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Effects of Smoking and Gender on Tetrahydroisoquinolines and Beta-Carbolines in a Healthy Population and During Alcohol Detoxification

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EFFECTS OF SMOKING AND GENDER ON TETRAHYDROISOQUINOLINES AND β–CARBOLINES IN A HEALTHY POPULATION AND DURING ALCOHOL DETOXIFICATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

by

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List of Abbreviations

5-HT	Serotonin
AAI	Annual Alcohol Intake
ABST SM	Abstaining smokers
ACAMP	Acamprosate
AcH	Acetaldehyde
ADM	Admission
ALT	Alanine aminotransferase
ANOVA	Analysis of Variance
ANCOVA	Analysis of Covariance
AP	Alcohol-Preferring
AST	Aspartate aminotransferase
AULOQ	Above upper limit of quantification
BC	β-carboline
BrAC	Breath alcohol
CDT	Carbohydrate-deficient transferrin
CI	Confidence interval
CIWA-AR	Clinical Institute Withdrawal Assessment-Alcohol Revised
CNS	Central nervous system
CO	Carbon monoxide
COMT	Catechol-*O*-methyl transferase

COV	Coefficient of Variation
CSF	Cerebrospinal fluid
CYP	Cytochrome P450
DA	Dopamine
DIPEA	N,N-diisopropylethylamine
DFN	Deviation from nominal
DOA	Drugs of Abuse screen
DSM-IV, SCID-I	Structured Clinical Interview for Diagnostics & Statistics Manual-IV
ECG	Electrocardiogram
EGTA	Ethylene glycol bis-2-aminoethyl ether-tetraacetic acid
ESI	Electrospray ionization
FD	Fluorescence detection
FTND	Fagerström Test for Nicotine Dependence
GABA	γ-amino butyric acid
GC	Gas chromatography
GEN	Gender
GGT	γ-glutamyl transferase
GTM	Grand Total Mean
HPLC	High-pressure liquid chromatography
HQC	High quality control
H	Harman

HS	Heavy-smoker
IQR	Inter-quartile range
IS	Internal standard
LLE	Liquid-liquid extraction
LLOQ	Lower limit of quantification
LOD	Limit of detection
LQC	Low quality control
LS	Light-smoker
MAO	Monoamine oxidase
MQC	Medium quality control
MP	Modified plasma
MS	Mass spectrometer
NE	Norepinephrine
NH	Norharman
NHP	Natural History Protocol
NMDA	N-methyl-D-aspartate
NS	Nonsmoker
PBA	Phenylboronic acid
PFBBr	Pentafluorobenzyl bromide
PP	Pooled plasma
Q-Q	Quantile-Quantile
REC SM	Recently smoked

Rs	Resolution
RSD	Relative standard deviation
SAL	Salsolinol
SPE	Solid phase extraction
SD	Standard deviation
SM	Surrogate matrix
SRM	Selective reaction monitoring
SS	Smoking status
TLFB	Timeline Follow Back
TIQ	Tetrahydroisoquinoline
ULOQ	Upper limit of quantification
YOH	Yohimbine

Abstract

EFFECTS OF SMOKING AND GENDER ON TETRAHYDROISOQUINOLINES AND β–CARBOLINES IN A HEALTHY POPULATION AND DURING ALCOHOL DETOXIFICATION

By Satjit Singh Brar, B.S.

A Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

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The purpose of this investigation was to evaluate the effects of smoking and gender on 1) tetrahydroisoquinolines (TIQs) and β-carbolines (BCs) in a population of healthy subjects and 2) TIQ's in an alcohol-dependent population undergoing in-patient detoxification. Comparison in plasma TIQ's between the populations was additionally conducted. To support the clinical investigations, a HPLC-FD method was developed and validated to assess plasma concentrations of BCs, harman and norharman, while a HPLC-ESI-MS/MS method was validated to quantify the TIQ's, R/S-salsolinol along

with dopamine. Forty-one young volunteers were recruited including 19 nonsmokers (NS), 11 light smokers (LS) and 11 heavy smokers (HS), stratified by their smoking history. Each group had, at least, 5 males and females. Plasma samples were obtained for analyte measurement within 30 minutes of smoking for LS and HS groups. Two–way ANCOVA was performed on the log-transformed concentrations. Significant differences were found between HS-NS and LS-NS in analyte concentrations. A comparison to eighteen subjects (6 NS, LS and HS) abstaining from smoking for 15 hours resulted in a difference only between NS and HS, suggesting that acute tobacco smoking has a major influence on circulating TIQs and BCs between smoking status groups. In a study involving thirty-five alcohol dependent subjects (12 NS, 11 LS, and 12 HS, balanced with gender), TIQ measurements were taken on day 1, 2, 3, 8 and 15 of inpatient detoxification. A significant effect of time was observed, with TIQ concentrations slightly increasing from admission to day 15. Both factors of smoking status and gender did not have a significant effect on plasma TIQ's at any of the time points evaluated. Although, measures of acute and chronic alcohol intake had no effect on TIQ levels, liver function showed moderate correlation with plasma TIQ's. Comparison of both populations showed that alcoholics had a lower average TIQ concentration than healthy subjects. The results indicate that smoking status 1) has an effect on plasma TIQs and BCs in healthy individuals and 2) does not have an effect in alcoholics during detoxification. The alcoholics possessed lower TIQ concentrations than the healthy subjects. No gender effect was observed in either study.

CHAPTER 1

INTRODUCTION AND BACKGROUND

1.1. Introduction

Alcohol and nicotine, the most frequently abused drugs, are likely also the most costly drugs in terms of health and societal costs. It is estimated that there are 15.1 million alcohol-abusing or alcohol-dependent individuals (approx 1 in 18 or 5.55%) in the United States (Allen et al., 2004). Alcoholism is a chronic, often progressive disease with symptoms that consist of a strong need to drink regardless of negative consequences. Like many other diseases, it has a generally predictable course, has recognized symptoms, and is influenced by both genetic and environmental factors that are being increasingly well defined. Although the prevalence is decreasing, approximately 20.8% of the US population aged 18 years or older, are current smokers. Tobacco use kills nearly half a million Americans each year, with one in every six U.S. deaths is the result of smoking (Volkow, 2006).

The co-occurrence of alcohol and nicotine dependence in people are common and well documented (Istvan and Matarazzo, 1984; Bien and Burge, 1990; Miller and Gold, 1998). Several studies have established that the vast majority (80-90%) of alcoholics smoke at a prevalence that is about three times higher than amongst the population as a whole (Dreher and Fraser, 1967; Crowley et al., 1974; Burling et al., 1982). Conversely, smokers consume twice as much alcohol as do non-smokers, and alcoholism has been estimated to be 10-14 times more familiar among smokers than nonsmokers (DiFranza and Guerrera, 1990). Furthermore, alcoholics who smoke use more cigarettes per day than do non-alcoholic smokers (Dawson, 2000). The close interrelationship between smoking and alcohol use is also exemplified by the observation that smoking cessation is more difficult to attain in previous or current alcohol abusers (Bobo et al., 1987) and that successful smoking or alcohol cessation improves the likelihood of alcohol intake reduction (Miller et al., 1983) or smoking cessation (Burling et al., 1982), respectively. Major reasons are that concurrent alcohol use, and/or prior alcohol exposure, may modify the reinforcing effects of nicotine, and vice-versa, and that each drug becomes a pharmacological cue for the expectation of the other. Results of the investigations on the relation of nicotine addiction and alcoholism suggest a synergism in the reinforcing properties of dependence.

1.2 Background

1.2.1 Current Biomarkers for Smoking Dependence and Alcoholism

In the clinical management of these problems, a critical necessity are effective and accurate biological markers that will enable clinicians to identify the extent of alcohol abuse and smoking dependence, as well as to monitor progress in treatment. Clinical laboratory procedures are commonly used to corroborate results of subjective patient interviews and clinical examinations when assessing drug dependence. In recent years, inherited components in the etiology of certain aspects of drug abuse are widely acknowledged and, as a consequence, the searches for biomarkers have gained importance. The markers of alcohol and smoking dependence may offer objective evidence of excessive dependence, especially in patients who deny their problems.

Abusers of alcohol and tobacco may exhibit several clinical and/or chemical changes. The more frequent the use of screening tests, combined with the use of new biochemical markers in patients who are suspected of alcohol abuse or tobacco use, will improve detection and permit intervention earlier in the course of illness. In the case of alcoholism, establishment of several biomarker candidates for excessive alcohol use has been attempted, including aspartate aminotransferase (AST), alanine aminotransferase (ALT), erythrocyte mean corpuscular volume (MCV), γ-glutamyl transferase (GGT), and Carbohydrate-deficient transferrin (CDT) (Yersin et al., 1995; Allen et al., 2004). Unfortunately, these current markers have variable sensitivity and/or low specificity (Moravcova et al., 2004). CDT has been reported to be the best laboratory marker of

the chronic alcohol abuse, but there are conflicting data on its accuracy and sensitivity (Stibler, 1991; Anton and Sillanaukee, 1996).

In the case of smoking, an array of biomarkers of tobacco smoke exposure have been proposed. Chemicals in tobacco smoke such as carbon monoxide or cyanide (the latter metabolized in the body to thiocyanate) can be measured in blood. However, the levels of these chemicals are nonspecific, i.e., there are significant sources of carbon monoxide and cyanide, including the body's own metabolism, other than tobacco smoke exposure (Benowitz, 1999). Thus, these markers are both nonspecific and insensitive markers of tobacco smoke exposure. Other markers that have been proposed to quantitate tobacco exposure consist of adducts of benzo[a]pyrene carcinogens to DNA (Binkova et al., 1995), 4-aminobiphenyl adducts to hemoglobin (Bartsch et al., 1990), adducts of polycyclic aromatic hydrocarbons (Crawford et al., 1994), urinary excretion of nicotine-derived nitrosoamines (Adikofer et al., 1984), and others. Specificity and sensitivity to these markers have yet to be acceptable for clinical use. The measurement of cotinine concentrations in biologic fluids has been used most widely by scientists to evaluate tobacco smoke exposure because cotinine reflects exposure to nicotine, which is almost specific to tobacco (Benowitz, 1996). The specificity of cotinine as a marker of tobacco exposure may come into question as food sources may contribute to overall exposure of cotinine. Moreover, cotinine is not a suitable marker in persons undergoing treatment with nicotine replacement therapy (e.g., gum, inhaler, transdermal patch). Therefore, substances present in tobacco or endogenously formed upon inhalation of tobacco, measurable in biological fluids, but not derived metabolically from nicotine

would be valuable for validating tobacco abstinence in persons undergoing nicotine replacement therapy. Additional benefit would come about if the biochemical measurement has implications in the mechanism by which smoking dependence and reinforcement transpires (i.e., causal repercussions).

Issues pertaining to alcohol and tobacco smoke exposure biomarkers primarily consist of a lack of sensitivity and specificity for the measurements. Importantly, the current candidates for biomarkers generally consist of molecules presenting as result of insult to the natural physiology. These types of markers may present false positive rates due to organ pathophysiology and may have a delayed quantifiable presentation in the body. Although some of the aforementioned markers attempt to relate to the acute and chronic exposure of alcohol and/or cigarette smoke, none of the markers show a mechanistic relationship to the reinforcing aspects of alcohol and nicotine. An understanding of the mechanism by which these drugs of abuse produce a reinforcing effect is essential to elucidate a biomarker with this type of characteristic.

1.2.2 The "Reward" pathway

During the past few decades, investigations on the molecular basis of alcohol and nicotine dependence and its etiology, *per se*, have concentrated on the discovery and validation of endogenous neurochemical factors. Drugs of abuse, such as ethanol (Thielen et al., 2004; Rodd et al., 2005) and nicotine (Balfour, 1989; Rausch et al., 1989; Crooks and Dwoskin, 1997; Staley et al., 2001) exert numerous pharmacological effects through their interactions with various neurotransmitter and neuromodulator

systems. Acute central nervous system (CNS) effects are mediated by different proteins and receptors, classically, the nicotinic acetylcholine receptors for nicotine and N-methyl-D-aspartate (NMDA) and γ-amino butyric acid (GABA) for ethanol (Gamberino and Gold, 1999). Tobacco smoke and alcohol intake are known to cause major acute and chronic neurochemical adaptations in the brain, including a profound enhancement of dopamine (DA) and serotonin (5-HT) transmission. The increased neurotransmission of these biogenic amines, particularly in the nucleus accumbens of the mesocorticolimbic system, is central to mechanisms regulating CNS effects of both nicotine and alcohol (Gamberino and Gold, 1999). Activation of DA and 5-HT transmission within the mesocorticolimbic pathways (dopaminergic system) has also been implicated in the reinforcing aspect of reward from natural stimuli (Groenewegen et al., 1991; Philips et al., 1991). It is suggested that compounds that interact with the nucleus accumbens and the dopaminergic neurons within these pathways plays a significant role in drug dependence and in drug-seeking behavior. In essence, this paradigm states that the pharmacological effect of the drug along with increased levels of DA and 5-HT within this dopaminergic 'reward' pathway, give way to the reinforcing properties of substances of abuse.

A primary means in which DA and 5-HT levels increase pertains to inhibition of monoamine oxidase (MAO) enzymes. As the actions of catecholamines are terminated via MAO-metabolic transformation, inhibition of MAO (e.g., pargyline, nialamide) can cause an increase in the concentration of norepinephrine (NE), DA, and 5-HT in the postsynaptic membrane of the brain and other tissues accompanied by a variety of

pharmacological effects (Nicotra et al., 2004). Both tobacco smokers and alcohol dependent subjects were reported to show a decrease in MAO activity in the brain and peripheral tissues. Several studies have illustrated that, in the alcoholic population, peripheral and central MAO-A and MAO-B activity is significantly decreased compared to control (Berggren et al., 2000; Coccini et al., 2002; Demir et al., 2002). In addition, researchers have found constituents in tobacco that inhibit both forms of monoamine oxidase, *in-vitro* (Berlin et al., 1995; Rose et al., 2001), and clinical studies have shown that *ex-vivo* MAO activity is lower in smokers than in nonsmokers (Norman et al., 1987; Ward et al., 1987; Berlin et al., 1995). Both ethanol and nicotine, the pharmacologically active constituents of alcohol and tobacco, and corresponding immediate metabolites of both drugs, are not inhibitors of either MAO isoenzymes.

Several studies exemplified that recently abstaining alcoholics produce higher circulating levels of acetaldehyde than control following ethanol administration (Collins, 1988). In addition, peripheral levels of acetaldehyde in smokers are known to be higher than non-smokers and depend on the number of cigarettes smoked (McLaughlin et al., 1990). Acetaldehyde is a highly reactive molecule that can react with many molecules via adduction, condensation and polymerization. The by-products of these reactions exert a wide variety of biological effects and neuropharmacological properties directly affecting psychological behavior. It is plausible that levels of stable adducts, consequent from acetaldehyde condensation with biogenic amines, also may be increased in alcoholics and smokers consuming ethanol and smoking, thus serving in body fluids as biochemical markers that are more

persistent that acetaldehyde itself. Moreover, these markers may provide a basis for the alcohol and nicotine seeking behavior seen in alcohol and smoking dependent individuals.

Of note, two classes of endogenously-formed compounds, the tetrahydroisoquinolines (TIQ's) and β-carbolines, have been indicted as chemicals that display MAO inhibition and are by-products associated with acetaldehyde from alcohol and tobacco smoke exposure. There is also evidence that these classes of compounds are potent inhibitors of 5-HT, DA, and NE reuptake mechanisms, *in-vitro* (Airaksinen et al., 1980; Komulainen et al., 1980; McNaught et al., 1996). Additional mechanisms that TIQ's and β-carbolines may be implicated in dopaminergic system modulation include dopamine receptor regulation, enzyme activity inhibition (e.g., catechol-O-methyl transferase), catecholamine biosynthesis (e.g., tyrosine hydroxylase) and mitochondrial metabolism (Bringmann et al., 2002; Toth et al., 2002) In essence, these compounds may be responsible for the increase in biogenic amine transmission within the dopaminergic 'reward' pathway. In addition, human exposure to TIQ's and β-carbolines via smoking and/or alcohol consumption may contribute to the pharmacological reinforcing effects.

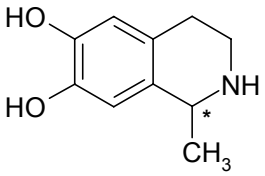
1.2.3 Tetrahydroisoquinolines (TIQ's: R/S-Salsolinol)

1.2.3a Chemistry and biosynthesis

TIQ's are a class of partially aromatic alkaloids that include R/S-salsolinol, 1-carboxysalsolinol, and tetrahydropapaveroline. TIQ's are compounds that are formed

as a result of a condensation reaction between DA and acetaldehyde or pyruvate (Rommelspacher et al., 1991a) and are natural metabolites of DA produced in the brain as well as other organs (Rommelspacher et al., 1995). Tetrahydropapaveroline (THP) is the dopamine - 3,4, -dihydroxyphenyl acetaldehyde condensation product whereas salsolinol is the dopamine-acetaldehyde condensation product (Duncan and Dietrich, 1980).

Salsolinol (SAL), 1-methyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline, is an isoquinoline analogue consisting of a catechol ring and a secondary amine that is able to be protonated at physiological pH (primarily ionized). Like many other TIQ's, SAL has an asymmetric center at the C-1 position of the heterocycle, thus leading to two stereo-isomeric forms (+)-(*R*)-SAL and (-)-(*S*)-SAL. The physicochemical properties of SAL have not been experimentally characterized. According to calculated results, the primary ionizable moiety is the secondary amine that possesses a pKa of ~ 9.4. SAL is very soluble in water possessing a log D of -1.68 (calculated from Advanced Chemistry Development, ACD/Labs, Software V 8.19 for Solaris © 1994-2008). The structure of SAL is shown in the figure below.



Chemical:	Salsolinol (SAL)
IUPAC name:	1-methyl-6,7-hydroxy-1,2,3,4-tetrahydroisoquinoline
Mol Weight (g/mol)	179.22
pKa	9.4 (secondary amine)
logD (calc)	-1.68
solubility	very soluble (1000g/L), slightly soluble in methanol

Figure 1-1: Structure and physicochemical characteristics of salsolinol. Chiral center denoted with asterisk.

SAL is present in various foods and beverages such as bananas, soy sauce, wine, and beer (Smythe and Duncan, 1985). The enantiomeric ratio within these food sources is close to 1, especially in dried banana, a food source rich in R- and S-SAL (Strolin-Benedetti et al., 1989), while the R-SAL enantiomer predominates in port wine (Dostert et al., 1991). The contribution of dietary SAL to the overall human exposure has not been extensively or well investigated. Attempts have been made to classify each enantiomer in terms of exogenous contribution or endogenous synthesis, but further studies are needed. In recent studies, it has been demonstrated that R-SAL predominates in human urine (Strolin-Benedetti et al., 1989). Results showed that the S-SAL enantiomer seemed to be formed in individuals who drink significant amounts of alcohol regularly. The differential enantiomer exposures are discussed to be affected by a genetic predisposition for an alcohol-induced SAL formation.

One biosynthetic pathway of SAL is the non-enzymatic condensation of dopamine and acetaldehyde to yield the racemic mixture of both enantiomers. The

Pictet-Spengler condensation reaction is known to form a 1:1 ratio of both R-SAL and S-SAL (Musshoff et al., 1999). Other pathways of biosynthesis of SAL have been proposed including the enzymatic formation by salsolinol synthase or the reaction of dopamine to pyruvic acid via intermediate formation of salsolinol-1-carboxylic acid (Naoi et al., 1996). Additional enzymes involved in this pathway have not been well characterized or further studied. Recently, the nature of R-SAL biosynthesis may be due to stereospecific enzymatic condensation of dopamine with pyruvic acid but substantiated evidence is still missing. A schematic depicting synthesis routes is shown in the figure below.

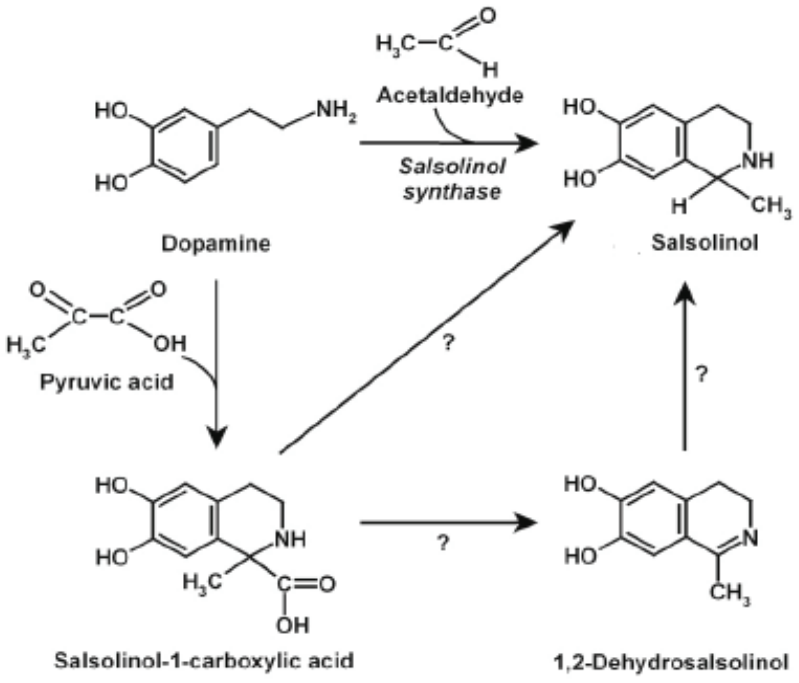


Figure 1-2: Biosynthetic pathway of Salsolinol. In addition to the condensation reaction product between dopamine and acetaldehyde, enzymatic formation has been purported (? = unknown enzyme). (adapted from Naoi et al, 2002).

Although the few reports describe the synthesis of the SAL isoquinoline, the metabolic disposition of SAL has been less extensively evaluated. It was thought that the metabolism of SAL yielded the neurotoxin 1,2-dimethyl-6,7-hydroxyisoquinolinium ion via N-methyl-transferase and amine oxidase (Naoi et al., 2002). Further studies need to be performed to evaluate the metabolic disposition of SAL and the stereoselective nature of the metabolism. Salsoline and isosalsoline are mono-O-methylated metabolites of SAL. Cateholamine-derived 6,7-dihydroxy-TIQs, such as SAL, serve as substrates for catechol-O-methyltransferase, in which SAL is O-methylated at the 7-position *in-vivo* (Collins and Origitano, 1983).

1.2.3b SAL *in-vitro* pharmacology

The neurotoxic properties of SAL have been extensively studied due to similar structural characteristics of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which is known to induce selective neuronal cell death of dopaminergic neurons (Martinez-Alvarado et al., 2001; Maruyama and Naoi, 2002). *In-vitro* studies have shown that incubation of dopaminergic neuroblastoma SH-SY5Y cells with N-methyl-salsolinol caused cell apoptosis (Storch et al., 2000). Other proposed catecholamine system dysfunctions invoked by SAL include autonomic dysfunction in Parkinson's Disease and production of positive ionotropic effect on guinea pig myocardium and isolated perfused rat heart (Chavez-Lara et al., 1989).

It has been suggested that SAL, the condensation product of the alcohol metabolite acetaldehyde and dopamine, may be involved in the balance of the reward

systems. It is well known that alcohol itself does not have any affinity for pre- or post-synaptic receptors in the neurons of the brain (Myers, 1989). The intra-cranial self-administration technique has shown that SAL induced reinforcement in the nucleus accumbens shell of rats occurs at concentrations that are pharmacologically possible. The mechanisms by which SAL induces this effect have been thoroughly evaluated. At relatively low concentrations (1 μmol/l), both enantiomers of SAL were able to antagonistically bind to dopamine receptors (D_2 and D_3), with the S-SAL form binding with higher affinity (Melzig et al., 1998). *In-vitro* data exemplified a substantial decrease in pro-opiomelanocortin gene expression caused by SAL, suggesting possible involvement of SAL in the role of opioid deficiency in alcoholism and the rewarding effect the SAL may have on mu-opioid receptors.

While R-SAL more specifically inhibits MAO-A (K_i = 31 μM) *in vitro*, 1,2,3,4-tetrahydroisoquinoline (TIQ) is more specific to MAO-B (K_i = 15 μM) (Bembenek et al., 1990; Naoi et al., 2004). S-SAL is an inhibitor of both MAO isoforms but less potent than that of the R-isomer (Naoi et al., 2004). It is speculated that these condensation products may also interfere with biogenic amine uptake and release. These compounds were found to inhibit the extraneuronal uptake of biogenic amines in the vas deferens, and SAL injections released DA and large amounts of 5-HT from the striatum, in rats (Duncan and Dietrich, 1980). In addition, SAL has shown to inhibit uptake and cause release of stored catecholamines via presynaptic α_2- and postsynaptic β-adrenergic receptor binding, to be an inhibitor of catechols-*O*-methyl transferase, and tyrosine and tryptophan hydroxylases, *in-vitro* (Haber et al., 1996). All sites of action

may lead to increased synaptic supply of dopamine within the dopaminergic "reward" pathway.

1.2.3c SAL *in-vivo* animal and human studies

In order to illustrate the drug reinforcing aspects of these compounds, several investigators showed that consumption of particular drugs of abuse (e.g. amphetamine, ethanol, cocaine) significantly increased upon exposure to TIQ's (Vetulani et al., 2001). Previous studies have found that chronic injection of TIQ causes an increase in alcohol intake in rats (Melchior and Myers, 1977; Myers and Oblinger, 1977; Duncan and Dietrich, 1980). Specifically, rats infused intraventricularly with 4.0 μg of salsolinol increased alcohol intake from 0.74 to 4.9 gm/kg/day. In another study, single infusions of racemic salsolinol in doses ranging from 0.1 - 1.0 μg increased alcohol consumption from 0.62 to 4.38 g/kg/day in the non-alcohol-preferring strain of Sprague-Dawley rats (Melchior and Myers, 1977). Animal studies have provided evidence that significant differences in SAL levels exist between alcohol-preferring (AP) and alcohol-non-preferring rats, with the AP rats showing significantly lower SAL content in the striatum and adrenal glands (Haber and Dumaual, 1999). A recent study also illustrated that SAL produces reinforcing effects when administered directly into the shell of the nucleus accumbens of AP rats (Rodd et al., 2003). Acute and chronic administration of selected TIQ's have been reported to alter alcohol consumption significantly.

In self-administration studies, it was exemplified that SAL exerts its reinforcing effect by stimulating receptors in the dopamine-rich nucleus accumbens (Rodd et al.,

2003). In conditioned fear stress tests, it was found that SAL induced a marked place preference in rats using doses ranging from 1-10 mg/kg i.p. (Matsuzawa et al., 2000). It was concluded that the potentiation and inhibition of this effect by morphine and β-funaltrexamine, respectively, indicate that SAL exerts its effect via the μ-opioid receptor.

A novel study utilizing a microdialysis-HPLC technique evaluated the action of R-SAL on 5-HT and DA metabolism in the brain (Naoi et al., 1996). It was concluded that R-SAL acts to stimulate a release of biogenic amines via inhibition of MAO and COMT. R-SAL was shown to be a more potent inducer of 5-HT and DA release from synapses than amphetamine itself. Additional animal studies have exemplified the *in vivo* formation of SAL upon high exposure of acetaldehyde (AcH), a primary metabolite of ethanol (Mostafa et al., 2003). Using a microdialysis-HPLC technique into the striatum, rats were treated with cyanamide, a potent inhibitor of aldehyde dehydrogenase, and 4-methylpyrazole (4-MP, a strong inhibitor of alcohol dehydrogenase), followed by ethanol (1 g/kg). High concentrations of SAL were detected in striatal dialysates and high AcH concentrations were found in the blood. The time course of changes in SAL concentrations correlated with blood AcH concentrations. In the other experimental groups, SAL in the dialysates and high AcH concentrations in the blood were not detected. It was concluded that high AcH concentrations induce the formation of SAL in the rat striatum. The delineation of individual SAL enantiomer exposure was not assessed.

Preclinical *in-vivo* pharmacological effects of SAL have been studied in rats. SAL has shown to have a modulatory role on cerebral benzodiazepine receptor (Kuriyama et al., 1987). The authors conclude that the decreased capacity of such a modulating mechanism may be involved in the exhibition of alcohol withdrawal syndrome, possibly by decreasing the function of endogenous ligands for benzodiazepine receptor in the brain.

Initial *in-vitro* and animal studies stimulated several researchers to evaluate SAL exposure in an alcoholic population. Of note, formal assessments of SAL effect on nicotine self-administration, or vice-versa, in rats have not been performed. SAL has been identified in urine, cerebrospinal fluid and the brain of humans (Melzig et al., 1998; Naoi et al., 2004). It was also found that blood acetaldehyde is positively correlated with urinary SAL ($r = 0.88$, $p < 0.001$), which supports the hypothesis that SAL is produced in-vivo from acetaldehyde condensation with dopamine (Adachi et al., 1986). Numerous human studies investigating plasma and urine concentrations of SAL have been published with regards to ethanol abuse (See Table 1-1). Baseline levels vary considerably within and between these studies. This may be due to methodological differences but some confounding factors have been identified, such as foodstuffs and tobacco smoke (Pais and Knize, 2000). The extent of these factors on SAL exposure levels has not been fully elucidated. Moreover, a majority of the studies were unbalanced with regards to group sample size and the effect of gender has not been explored. To date there are no clinical investigations that address the contribution of smoking to TIQ, and specifically SAL exposure, in the alcoholic and control

populations. SAL has not been reported as a cigarette smoke component. Moreover, dopamine is not present in tobacco and therefore tobacco smoke acetaldehyde can therefore only react with endogenous dopamine to contribute to circulating SAL concentrations.

Of equal importance, robust time-course measurements of SAL exposure have not been evaluated after acute and chronic ethanol exposure. Of the reports evaluated, sampling schedules only included a baseline measurements and, if assessed, one time point after ethanol exposure or after the start of detoxification, in the case for alcohol dependent subjects. The pharmacokinetic profiles of SAL have not been explored after acute ethanol exposure. Adequate sampling schedules after ethanol exposure would be needed in order to critically evaluate SAL concentrations between populations. A study performed by Rommelspacher et al, 1995 attempted to investigate the time course of SAL exposure in alcoholic subjects undergoing detoxification. Significant baseline differences were seen between alcoholic and control plasma SAL and declined over a three-month period, exemplifying the importance of the temporal effects of SAL exposure. The data suggests that alcohol dependent persons attempt to maintain particular physiological levels of SAL to possibly circumvent withdrawal effects or sustain a pleasurable "reward" feeling. Although the time-course of enantiomeric SAL plasma concentrations was assessed, sampling schedule was sparse and inadequate. Of important note, no formal preclinical or clinical pharmacokinetic investigations on the endogenous formation, exposure and elimination of R- and S-SAL have been performed to date.

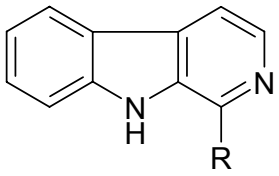
Table 1-1: Human studies evaluating Salsolinol (SAL), or the enantiomers, in alcoholic vs. control populations

Reference	Study	Results
(Haber et al., 1996)	N = 24 control subjects: SAL enantiomers quantified in plasma and urine before and after acute EtOH ingestion.	N =5 showed increase both isoforms in urine and plasma; 19 sub. decreased or unchanged levels. Subjects with low baseline tend to increase after EtOH; no change in enantiomeric composition.
(Haber et al., 1995a)	N= 27 (14 M, 13 F) control subjects: urinary excretion of SAL before and after EtOH ingestion.	Urinary SAL influenced by EtOH intake. 17 subjects showed increase while the others decreased. In subjects with low SAL conc., urinary SAL significantly increased after EtOH.
(Dostert et al., 1991)	N= 6 chronic alcoholics, deprivation of ethanol, N= 6 healthy control. Urinary R-and S- SAL.	Both populations possessed both SAL enantiomers in urine. Alcoholics abstained for 24 hrs, only (R)-SAL was detected.
(Collins et al., 1979)	N = 8 chronic alcoholics, N = 7 control; first 24-hours of urinary SAL and O-methyl-SAL.	SAL- Alcoholics: 28.8 ± 8.7 µg/d; NA 1.1 ± 0.3 µg/d; methyl-SAL Alcoholics: 111.4 ± 13.7 µg/d; NA 20.6 ± 7.1 µg/d (mean ± SD).
(Faraj et al., 1994)	N = 40 chronic alcoholics, N = 55 alcoholics w/cocaine dependence vs. control; plasma SAL and dopamine (DA) sulfate.	SAL- Alcoholics: 627 ± 195 pg/ml; w/cocaine dependence: 409 ± 76 pg/ml; control: 99.5 ± 7.5 pg/ml. Sig elevations of DA in both alcoholic groups compared to control. (mean ± SE).
(Faraj et al., 1989)	N = 18 chronic alcoholics in detox, N = 36 control; plasma SAL and DA-sulfate.	SAL- Alcoholics: 497 pg/ml (50 -1331 pg/ml); control: 93 pg/ml (0 –232 pg/ml). Sig elevations of DA in alcoholic groups vs. control. (mean and range)
(Rommelspacher et al., 1995)	N = 117 alcoholics (96 male) in detox, N= 20 control; plasma SAL-sulfate enantiomers and dopamine sulfate.	R-SAL- Alcoholics: 0.65 ± 0.82; control: 0.24 ± 0.07 ng/ml; S-SAL- Alcoholics: 0.35± 0.05; control: 0.08 ng/ml R- and S- SAL normalized after several months.
(Sjoquist et al., 1981)	Urine and CSF; SAL and O-methyl-SAL in control vs. alcoholics.	Alcoholics significantly higher concentration of SAL vs. healthy volunteers, single ethanol ingestion of 80g in control resulted in no effects.
(Sjoquist et al., 1982; Sjoquist et al., 1983)	Postmortem brain concentrations of SAL and O-methyl-SAL in alcoholics vs. control.	Alcoholics had sig higher concentrations of SAL (0.18 – 0.30 nmol SAL/g) than the healthy. (range)

1.2.4 β-carbolines (harman and norharman)

1.2.4a Chemistry and biosynthesis

β-carbolines, such as harman and norharman, are another class of aromatic compounds that are formed via the condensation of serotonin (5-HT) or other indolealkylamines with aldehydes (Airaksinen and Kari, 1981a). This class of compound possesses aromatic, planar, conjugated ring structures. The lipophilic nature of an array of β-carbolines has been studied, with harman (1-methyl-9H-pyrido-[3,4-*b*]indole) and norharman (9H-pyrido-[3,4-*b*]indole) resulting in relatively high logD values (Biagi et al., 1989). The pyridine nitrogen behaves as a base and is easily protonated, while the pyrrolic nitrogen is acidic and loses its proton in an alkaline environment, although outside its pH scale (pH>14).



Chemical:	Harman	Norharman
R:	CH_3	H
Mol Weight (g/mol)	182.2	168.2
pKa	7.37, 14.46	7.26, 14.23
logD (calc)	2.8	2.6
solubility	insol in H_2O, sol in dilute acids, methanol, and non-polar solvents	

Figure 1-3: Structure and physicochemical characteristics of the β-carbolines, harman and norharman.

There is substantial evidence suggesting that simple β-carbolines, may be biosynthesized from tryptophan, serotonin or its metabolites in animal cells, i.e., "mammalian alkaloids" (Melchior and Myers, 1977; Airaksinen and Kari, 1981a). The key biosynthetic pathway is shown below.

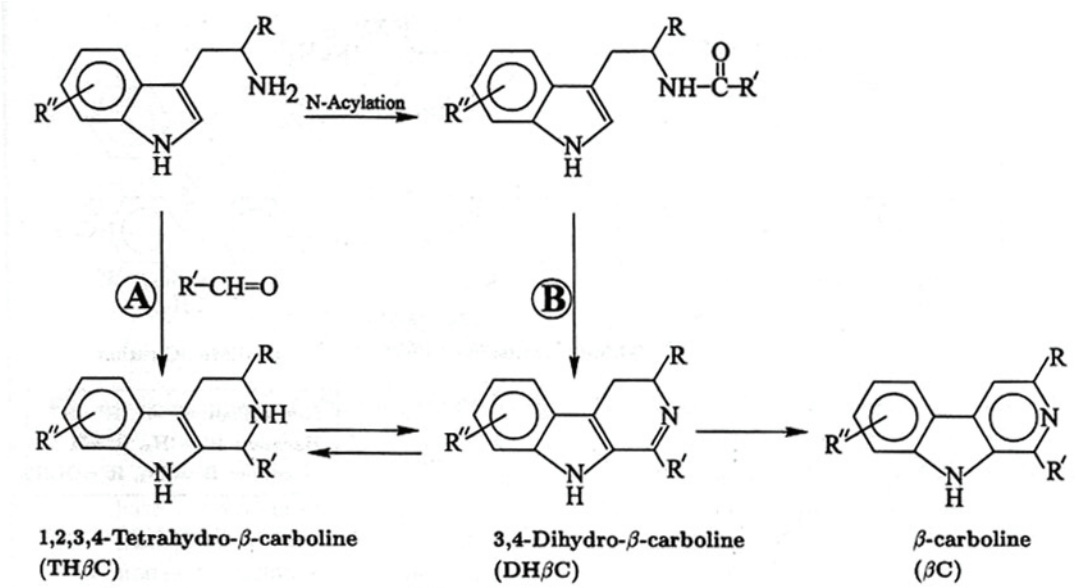


Figure 1-4: Reaction pathways for the formation of heteroaromatic β-carbolines (βC), 1,2,3,4 tetrahydro-β-carbolines (THβC) or 3,4 dihydro-β-carbolines (DHβC) via A) the Pictet-Spengler condensation or the B) N-acylation with cyclization. Adapted from (Collins and Neafsey, 1998).

Indolealkylamines like tryptophan or serotonin can non-enzymatically condense with acetaldehyde to form the tetrahydro-β-carbolines and spontaneously oxidize to the heteroaromatic harman or norharman (βC). Alternatively, biogenic amines may undergo enzymatic n-acylation and cyclization to yield dihydro-β-carbolines and to ultimately oxidize to form harman or norharman. Fekkes and co-workers reported that

the ingestion of tryptophan resulted in a low increase of the plasma concentration of norharman, but concluded that the primary norharman content in the body was from other sources (Fekkes et al., 2001). In general, the exact sequence of the specific steps in the pathways is yet to be established. Indeed, other, more complicated, biosynthetic pathways are also conceivable.

Few reports have evaluated the metabolism of the β-carbolines. The primary elimination route of norharman is the liver, where its half-life is approximately 20 minutes (Fekkes et al., 2001). Moreover, it has been reported that norharman binding in rat liver microsomes can be inhibited by CYP2E1 ligands and indole-3-carbinol (Stawowy et al., 1999), and is a known inhibitor of benzo[a]pyrene metabolism. Both β-carbolines are methylated by S-adenosyl-L-methionine N-methyltransferase on both the pyridyl and indole nitrogens (Matsubara et al., 1993). Driven by a combination of reported results, carboline based neurotoxic entities possibly involved in Parkinson's disease might be quarternary, cationic β-carbolines possessing methyl groups on both nitrogens (Matsubara et al., 1993). The charged molecules could form via sequential methyl transfer reactions within the brain from hydrophobic, blood-brain barrier permeable β-carbolines of environmental as well as endogenous biosynthetic origins.

Exogenous food sources such as cheese, charred beef and chicken contain variable amounts of β-carbolines (Pfau and Skog, 2004), but the post-prandial contribution of these foods to circulating β-carbolines have not been evaluated. Alcoholic beverages including wine and beer, contain substantial amounts of norharman and harman ranging from 0.3 – 22.7 ng/ml (Rommelspacher et al., 1996; Adachi et al.,

2000). Certain hallucinogenic plants can also biosynthesize β-carbolines and, as a consequence, have been purported to contribute to psychogenic pharmacological effects (Airaksinen and Kari, 1981b). Interestingly, the quantification of β-carbolines from tobacco smoke condensate and tobacco leaves have shown that harman and norharman are present in significant concentrations. The smoke of one cigarette contains 0.1-5.8 μg of harman and 1.3-6.2 μg of norharman (Poindexter and Carpenter, 1962; Herraiz and Chaparro, 2005). Of note, additional β-carboline may be formed endogenously from acetaldehyde inhaled from cigarette smoke and condensation with biogenic amines.

1.2.4b β-carbolines *in-vitro* pharmacology

Substantial data is available with regard to the *harmala* alkaloids and MAO inhibition, effects on membrane ion transport, blockade of the serotonin transporter, and antagonism of the GABA/benzodiazepine receptor complex (Collins and Neafsey, 1998). Speculations on the biological significance of harman and norharman consist of cytotoxic as well as neuroprotective properties. They have been proposed as endogenous ligands for benzodiazepine (Rommelspacher et al., 1980) and imidazoline (Hudson et al., 1999) receptors. Apart from the actions at these receptors, other effects of harman and norharman at the cellular level have been identified. These include activation of 5-HT_{2A} and 5-HT_{2C} receptors, potent inhibition of synaptosomal γ-hydroxybutyrate (McCormick and Tunnicliff, 1998) re-uptake and impairment of sodium-hydrogen exchange (Glennon et al., 2000).

Kinetic analysis revealed that β-carbolines from cigarette smoke were competitive, reversible, and potent inhibitors of MAO enzymes (Herraiz and Chaparro, 2005). It has been established that, *in vitro,* norharman and harman inhibit MAO B (K_i = 730 nM, brain tissue, rats) and MAO A (K_i = 220 nM), respectively (Rommelspacher et al., 2002). These results suggest that β-carboline alkaloids from cigarette smoke acting as potent reversible inhibitors of MAO enzymes may contribute to the MAO-reduced activity produced by tobacco smoke in smokers. Note that nicotine and corresponding metabolites (e.g., cotinine, thiocyanate) are not potent inhibitors of MAO, having inhibitory constants, (Ki's) 1000-fold or more higher than β-carbolines (Oreland et al., 1981). Moreover, the concentrations required to inhibit MAO by nicotine and its corresponding metabolites are not of physiological range (Volkow et al., 2005). *In-vitro* experimentation of harman and norharman effects on tyrosine hydroxylase and catechol-*O*-methyl transferase activity has not been explored.

1.2.4c β-carbolines *in-vivo* animal and human studies

Studies in animals have provided evidence of induction of ethanol intake by β-carbolines. When unanesthetized rats were infused with tryptoline, a β-carboline, the release of dopamine in the caudate nucleus and nucleus accumbens was significantly increased (Myers and Oblinger, 1977). In addition, it was shown that norharman plasma levels were significantly elevated in rats that ingested ethanol for two-weeks. Authors suggest elevated plasma norharman was due to binding to enzymes of the cytochrome P450 superfamily (Stawowy et al., 1999). A subsequent study involving

injections of a range of doses on norharman showed that administration of doses of 2.44 μmol/kg and 43.97 μmol/kg induced an increase of dopamine efflux by 70% and 160% (Baum et al., 1995). This was thought to indicate that norharman influences the mesolimbic dopaminergic neurons in a dose-response fashion, and the authors suggested that norharman is affecting the dopaminergic system via different receptors, namely MAO-A, MAO-B and non-MAO binding site.

Harman has been shown to induce preference for ethanol in rats (Rommelspacher et al., 1987). Increasing concentrations of ethanol were accessible to male Wistar rats for 21 days. Between day 8 and day 21, the animals were treated with several doses of harman and tetrahydronorharman (tetrahydro-beta-carboline) by means of continuous intraventricular infusion. Harman and tetrahydronorharman induced a significant preference for ethanol in a dose-dependent manner with harman being three times more potent than THN. The amount of ethanol consumed during the second and third weeks of the experimental period correlated with the harman concentration in the brain after the cessation of the treatment (p-value < 0.01). In an additional study, harman has been shown to induce volitional drinking of ethanol in the rat (Adell and Myers, 1994). The results demonstrated that the long-term exposure of hippocampal neurons to harman induces a preference for high concentrations of alcohol even in a line of rats lacking such a genetic predisposition. Harman administration increased the release of DA and 5-HT in the nucleus accumbens (Baum et al., 1996). These results have been corroborated by the results of harman administration enhanced the brain levels of 5-HT up to four times (Adell, 1996). No reports, to date, have investigated

the self administration of cigarette smoke and/or nicotine solution upon administration of harman or norharman to rats.

Interestingly, norharman has been shown to attenuate the withdrawal effects of alcohol in rats. Norharman injected intraperitoneally (6.3 mg/kg) attenuated the behavioral signs of alcohol withdrawal significantly in rats who were administered ethanol for 21 consecutive days and subsequently abstained (Fekkes et al., 2004). Conversely, Spies et al showed that norharman levels were significantly increased on days in patients who developed alcohol withdrawal syndrome compared with those who did not. An increase in norharman levels preceded hallucinations or delirium with a median period of approximately 3 days (Spies et al., 1996). The researchers suggested that norharman may be a possible substance that triggers convulsions and alcohol withdrawal syndrome.

In humans, these compounds are formed endogenously under normal conditions with the highest natural concentrations found in the substantia nigra (16 nmol/kg tissue)(Matsubara et al., 1993). Early investigations in humans exemplified that a high dose of ethanol (100g) resulted in an increase in urinary excretion of norharman. Moreover, it was shown that plasma levels of ethanol and acetaldehyde paralleled that of harman blood concentrations with a maximum concentration reached at ~1-2 hours after dosing (Rommelspacher et al., 1996). No harman was detectable in blood when no ethanol was given. Experimental studies showed that dosing with ethanol resulted in elevated harman levels while, conversely, elevated norharman levels were reported in alcoholics. In all studies reported (See table 1-2 below), considerable variability exist

in the results for both harman and norharman. In all studies involving alcoholics, the tobacco smoking of patients or control was not accounted for.

Recently, clinical studies have investigated the of role acute cigarette consumption on the plasma levels of harman and norharman. A clinical study determining the impact of smoking and drinking on norharman found that resumption of smoking after a period of abstinence generated elevated plasma levels of norharman among smokers (who did not drink alcohol excessively) as compared to the non-smoking, non-drinking (control) group (Breyer-Pfaff et al., 1996). It has been suggested that the acutely elevated plasma levels of β-carbolines in smokers may be due to recent cigarette consumption (Spijkerman et al., 2002). However, a clinical study investigating *ex-vivo* MAO-B platelet inhibition by harman and norharman demonstrated a baseline difference of β-carbolines in plasma and platelets between non-smokers (n=5) and smokers (n=19)(Rommelspacher et al., 2002). In plasma, baseline levels were two-fold higher in smokers than non-smokers (smokers: harman = 8.7 pg/ml, norharman = 19.2 pg/ml; nonsmokers: harman = 4.1 pg/ml, norharman = 9.5 pg/ml). After both groups consumed one and two cigarettes, harman and norharman levels were significantly higher in smokers than the nonsmokers at all time points.

Only few studies have evaluated the pharmacokinetics of harman or norharman in man. Oral administration of norharman was investigated in a small number of healthy subjects receiving doses of 7, 65, or 110 μg/kg body weight with considerable inter-individual differences in exposure (area under the curve)(Fekkes et al., 2001). Sublingual administration resulted in a maximum exposure after 5 minutes of

administration and was twenty-fold higher than oral administration. Elimination half-lives of 51 minutes (Breyer-Pfaff et al., 1996) and 25-30 minutes (Rommelspacher et al., 2002) for harman and norharman, respectively, have been estimated from human studies.

1.3 TIQ's and β-carbolines summary

As it has been noted in the literature, the modulation of drug abuse behavior and the consequent interaction of TIQ's and β-carbolines with the dopaminergic system demonstrate that these compounds may have a role in drug dependence. Among the brain neurotransmitters, dopamine is by far the one, if not the only to have been implicated in the behavioral stimulus effects of nicotine and alcohol. The dopaminergic system has been well established as the "reward system" in the brain. Therefore, compounds that interact with the nucleus accumbens and the dopaminergic neurons could have a significant role in drug dependence. TIQ's and β-carbolines possess various significant pharmacological properties to modulate dopamine transmission within this pathway, namely MAO inhibition. Furthermore, as the dopaminergic system has a significant role in the drug-seeking behavior, it is possible that other drugs of dependence such as nicotine or other ingredients of cigarette smoke may also interact with the TIQ's and β-carbolines. Additionally, these compounds may mediate smoking behavior.

Table 1-2: Human studies evaluating harman and norharman in alcoholic and control populations.

Reference	Study	Results
(Tsuchiya et al., 1996)	Urinary tetrahydro-β-carboline excretion in social drinkers before and after consumption.	In urine after drinking, 1-methyl-tetrahydro-β-carboline and tetrahydro-β-carboline excreted in significantly higher concentrations compared to sobriety.
(Rommelspacher et al., 1996)	Plasma H and NH in control population (N = 26), sober and after EtOH load vs. alcoholics (N = 138) undergoing detoxification.	On admission, H levels in alcoholics was two fold higher than control . Intoxicated nonalcoholics (after EtOH load) had lower levels of H and NH on day of admission than patients, irrespective of intoxication or not.
(Rommelspacher et al., 1991b)	Plasma H and NH in control population (N = 10) vs. alcoholic population (N=36 M, 7 F) under 21 days of detoxification.	Elevated H and NH levels seen throughout entire detoxification period At day 21, levels of NH in sober alcoholics: 99.5 ± 26.6 pg/ml vs. control: 26.9 ± 10.7 pg/ml . H in alcoholics: 41.6 ± 12.0 pg/ml vs. control 20.8 ± 8.3 pg/ml (mean ± SD).
(Wodarz et al., 1996)	24-hour urinary excretion of H and NH between alcoholics (N = 13) in a 42-day detoxification vs. control (N = 12).	Urinary H increased significantly (4.5 fold) compared to control during early detox; declined steadily throughout detox w/o reaching control levels. Urinary NH elevated 6 fold compared to non-alcoholics until day 42.
(Spies et al., 1996)	Plasma H and NH in chronic alcoholics (N = 35) and non-alcoholics (N= 25) admitted to an ICU following trauma.	NH significantly elevated in alcoholics vs. control on admission to hospital, and during first 2 weeks. NH increased in patients with alcohol withdrawal compared with other alcoholics.
(Spies et al., 1995)	Plasma H and NH in chronic alcoholics and nonalcoholics w/ carcinomas of upper digestive tract; evaluation during ICU.	Sober alcoholics (N = 6) and control not differ significantly with respect to H. HN levels were higher upon ICU admission (16.5 pg/ml, 2.7- 50.4 pg/ml) vs. control (N = 16) 3.2 pg/ml, 0.1 – 20.5 pg/ml. (median and range)
(Stohler et al., 1993)	Plasma H and NH in heroin dependent patients vs. control.	H and NH elevated in heroin dependent patients on admission to a detox program as compared to control.
(Rommelspacher et al., 2002)	Plasma and platelet H and NH in healthy smokers (N=19) and non-smokers (N=5), before and after smoking 2 cigarettes; platelet MAO-B activity.	Plasma baseline two-fold higher in smokers: (H = 8.7 pg/ml, NH = 19.2 pg/ml) than nonsmokers: H = 4.1pg/ml, NH = 9.5 pg/ml). Platelet levels differed 4-fold between non-smokers and smokers. After both groups smoked, H and NH levels significantly higher in smokers

A number of studies investigating the biological concentrations of the β-carbolines and TIQ's have been published. Considerable variability in results between and within studies has been reported in alcohol dependent patients compared to control. Several confounding issues are present that may contribute to the variability. For the most part, the majority of studies did not account for the effects of smoking on circulating β-carbolines and TIQ's. As the co-dependence of alcohol and smoking is prevalent, research involving the contribution of alcohol exposure on physiological concentrations of β-carbolines and TIQ's need to account for the exposure of tobacco smoke within the control and alcohol-dependent patients.

The information summarized above lead to the hypotheses that (1) TIQ's and β-carbolines may have implications in the etiology of smoking and alcohol dependence via the dopaminergic 'reward pathway', (2) TIQ's and β-carbolines are elevated in plasma and urinary levels differ between smoking and alcohol dependent populations compared to control, and (3) smoking and alcohol consumption differentially alters the blood levels of TIQ's and β-carbolines. All aforementioned points suggest that these compounds of interest possess tobacco-smoking and alcohol exposure biomarker characteristics. To date there are only a few studies that address the contribution of smoking to TIQ's and β-carbolines levels in the tobacco-smoking dependent population. In addition, the majority of the studies did not study the effect of gender.

CHAPTER 2

RESEARCH HYPOTHESES

2.1 Hypothesis

The hypotheses directing this research project are:

1) Within a healthy, non-alcoholic population, smokers will have higher concentrations of TIQ's and β-carbolines than non-smokers, in a dose-dependent manner (i.e., heavy-smokers > light-smokers > nonsmokers) (Figure 2-1). Concentrations in light- and heavy-smokers will be observed within thirty-minutes of smoking a cigarette.

2) The plasma concentrations of TIQ's will decline over a two-week period in a population of alcoholics during detoxification (Figure 2-2).

3) At all sampling times, smokers will have higher exposure of TIQ's compared to nonsmokers in both abstinent non-alcoholic and alcoholic populations. Moreover, heavy smokers are expected to have higher levels of TIQ's as compared to light-smokers (Figure 2-2).

4) The alcoholic population will have higher average exposure of TIQ's, and at admission and discharge of detoxification, compared to the control non-alcoholic population, regardless of smoking status.

5) A time-dependent withdrawal assessment, the CIWA-AR (Clinical Institute Withdrawal Assessment-Alcohol Revised), will correlate with the levels of TIQ's during detoxification of the alcoholic cohort.

6) A positive relationship between the degrees of smoking and/or alcohol dependence with TIQ's and/or β-carbolines will exist (e.g., the higher the degree of dependence, the higher the exposure).

This study was performed in two parts. Study I was a pilot study in forty-one male and female volunteers to study the effects of smoking on TIQ and β-carboline exposure. The outpatient study was non-interventional, designed to evaluate baseline measurements of plasma TIQ's and β-carbolines. Subjects were stratified according to smoking status of non-smokers (NS), light-smokers (LS) and heavy smokers (HS). Stratification was based on the number of cigarettes smoked per day and the Fagerström Test for Nicotine Dependence (FTND). Subject participation involved two morning outpatient visits in which a single blood sample was taken on each visit for the quantification of plasma TIQ's and β-carbolines. This study was designed to evaluate the effects of smoking and gender in baseline plasma TIQ's and β-carbolines in addition to the inter- and intra-individual variability that may be associated with the measurements.

A comparison was made to a second study involving an alcohol-dependent cohort undergoing detoxification treatment at the National Institutes of Health - National Institute on Alcohol Abuse and Alcoholism. This investigation proposed to evaluate detoxification-induced changes in plasma TIQ concentrations in n = 36 alcoholics undergoing a four-week, inpatient alcohol abstinence program. Subjects were stratified with respect to smoking status of NS, LS and HS and the analysis was balanced for gender. Plasma samples were collected during the first two weeks of detoxification: on admission, day 2, 3, 8, and 15 days after enrollment. A clinical endpoint, the Clinical Institute Withdrawal Assessment-Alcohol Revised (CIWA-AR), was used to assess a possible correlation of these levels to withdrawal symptoms. Plasma TIQ levels were assessed along with CIWA-AR, smoking history and exposure, and alcohol dependence measurements in order to assess their feasibility as a clinical biomarker for smoking and alcohol dependence. Importantly, the time-course of these compounds during early abstinence in an alcohol-dependent cohort was assessed. Evaluation of the contribution of smoking to levels of these compounds and, ultimately, the time-course was the primary objective of this study.

2.1.1 Study #1 Hypothesis

As nicotine is a drug of dependence interacting with the dopaminergic reward pathways in the brain, and cigarette smoking involves systemic exposure to aldehydes and/or TIQ and β-carbolines, we expect that smokers will have higher concentration as compared to control in a ‘dose-response’ fashion. In other words smoking status will

have an effect on plasma TIQ and β-carbolines with NS < LS < HS. A graphical representation of the research hypotheses are presented in the figure below.

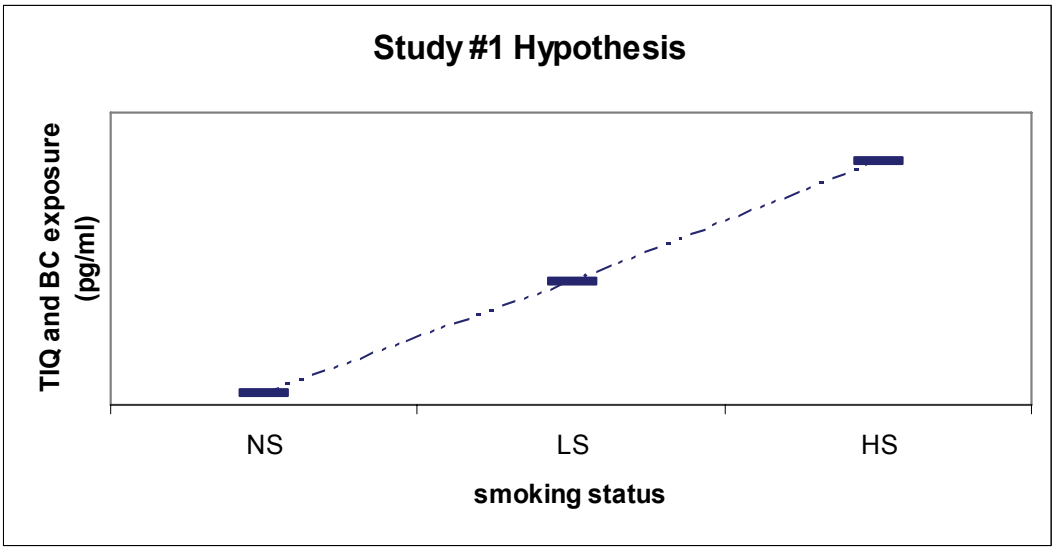


Figure 2-1: Proposed hypothesis of TIQ and β-carbolines relative average baseline plasma exposure in healthy nonsmoking (NS), light-smoking (LS), and heavy smoking (HS) populations.

Upon statistical evaluation, significant difference will be seen with the circulating TIQ's and β-carbolines when accounting for the factors of smoking and/or gender. A statistically significant positive correlation between the degree of dependence, as measured by the Fagerström Test for Nicotine Dependence (FTND) and the number of cigarettes smoked per day will be observed.

2.1.2 Study #2 Hypothesis

On the foundation that smoking will have a significant effect on plasma TIQ, it is expected that smoking tobacco should have an influence on the time-course of the

compounds in alcohol dependent patients. In these patients, the time course of the aldehydes adducts will differ at baseline and throughout the detoxification period, dependent on smoking status. The average aldehyde adduct concentrations, throughout the detoxification period, will be dependent on smoking status, with HS > LS > NS. Moreover, this relationship will be observed on day 15 of the detoxification period.

A withdrawal scale, the Clinical Institute Withdrawal Assessment (CIWA-AR) will decline during the initial stages of the detoxification period. Levels of circulating TIQ's and β-carbolines are expected to decline along with the CIWA-AR. A schematic representing the hypothesis for study #2 is shown below.

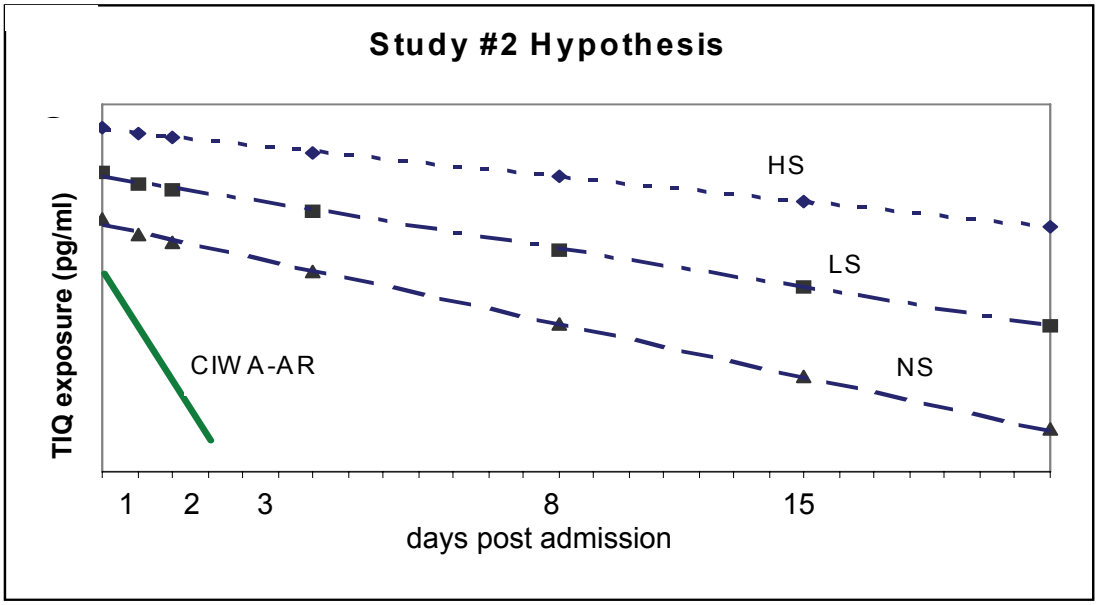


Figure 2-2: Proposed hypothesis of TIQ's relative average plasma exposure in alcohol dependent nonsmoking (NS), light-smoking (LS), and heavy smoking (HS) populations undergoing 3-weeks of detoxification. CIWA-AR profile denotes the withdrawal scale used within the first week of alcohol abstinence.

A statistically significant difference will be observed with regard to day 1, day 15 and average concentrations of plasma TIQ's and β-carbolines upon accounting for the two factors of smoking status and gender. A positive correlation between concentrations of TIQ's and FTND or number of cigarettes smoked per day. The CIWA-AR withdrawal scale will positively correlate with plasma TIQ's in a concentration dependent fashion. Overall a statistically significant difference will be seen between levels of smoking status and gender.

As the combination of alcohol drinking and cigarette smoking involves systemic exposure to aldehydes and/or TIQ and β-carbolines, we expect that smokers and alcohol dependent patients will have higher concentration as compared to control. Upon comparison between the studies, the alcohol dependent cohort will possess higher circulating levels of TIQ's with respect to the population observed in study #1, regardless of smoking status.

2.2 Rationale and Significance

The understanding of the neurochemical basis underlying the addictive properties of drugs of abuse is imperative for the rational development of new pharmacological treatments to reverse the addictive state, prevent relapse and/or reduce the intake of these drugs. The TIQ's and β-carbolines have demonstrated to have a variety of neuropharmacological effects that may be related to the reinforcing aspects of drug and alcohol abuse. However, sound evidence for the formation of these compounds and elevated concentrations after alcohol abuse and smoking is not yet

conclusive. Due to great inter-individual variations is plasma/urinary TIQ's and β-carbolines exposure levels and excretion rate, these compounds remain an insufficient marker to distinguish between alcoholics and non-alcoholics. The variability in the reported data might be a result of variables, including smoking status and gender, duration and amount of ethanol exposure, polymorphisms in metabolizing systems, and analytical problems associated with the quantification of the TIQ's and β-carbolines, all of which have not been thoroughly explored.

Currently, there are only a few studies reported in the literature that have studied the effect of alcohol and detoxification on exposure TIQ's and β-carbolines. Some of these investigations had questionable methodology, including not following the complete time-course of the levels, and not accounting for other variables influencing TIQ and β-carboline exposure, such as smoking. This research provides new, hitherto unknown information about the baseline levels of these compounds in regards to smoking status. In addition, this investigation assessed the effects of alcohol detoxification on the time-course of TIQ exposure in humans and attempted to establish a relationship to a clinical endpoint, the CIWA-AR.

These anticipated results will support the overall research hypothesis of activation of the central dopaminergic pathways as a consequence of smoking and alcohol dependence. Furthermore, if differences are found between NS, LS and HS, this would suggest that chronic exposure to TIQ's affects the withdrawal response, possibly as a result of chronic tolerance (e.g., CNS receptor down regulation). Measurement and examination of covariates, such as smoking and drinking history, will

allow comprehensive evaluation of TIQ and β-carboline levels in the alcohol and nicotine-dependent populations versus controls. Moreover, it will provide valuable information on its potential use as a state marker of alcohol and smoking dependence. Essentially, understanding TIQ and β-carboline exposure differences among individuals with smoking and/or alcohol dependence may provide clues about the dynamics of nicotine and alcohol seeking behavior and may provide a basis for enhanced treatment efficacy.

CHAPTER 3

BIOANALYTICAL ASSAY DEVELOPMENT FOR THE β-CARBOLINES, HARMAN AND NORHARMAN, IN HUMAN PLASMA

3.1 Introduction – Selection of Analytical method

In order to adequately evaluate physiological concentrations of β-carbolines in human plasma, a sensitive and specific analytical method is required. Several β-carboline alkaloids have been analyzed in different matrices including plant extracts, foods and beverages (Pfau and Skog, 2004). While the majority of these methods have been employed for quantification of the β-carbolines in foodstuffs (Pais and Knize, 2000), few analytical methods have been published for the support of the investigation of harman and norharman in human pharmacological studies. Applicability of transferring the assays for food sources to biological matrices have yet to be satisfactorily explored. Since the physiological concentrations of β-carbolines are reported to be in the low nanogram/ml to low picogram/ml range, and the available samples from humans are complex matrices, multi-step enhancement and preparation techniques are necessary for ultimate detection and quantification. In alcoholic patients

(Matsubara et al., 1986; Breyer-Pfaff et al., 1996; Rommelspacher et al., 1996; Tsuchiya et al., 1996; Wodarz et al., 1996) and patients undergoing elective tumor resection (Spies et al., 1995), the reported physiological concentrations ranged from ~5 pg/ml to 2 ng/ml of plasma and/or urine. In control subjects and smokers, similar plasma concentrations were seen immediately after smoking cigarettes (Breyer-Pfaff et al., 1996; Rommelspacher et al., 2002). Due to the highly lipophilic nature and the strong intrinsic fluorophore of the β-carboline alkaloids, bioanalytical methods used for quantification included primarily reversed-phase high-pressure liquid chromatography coupled with fluorescence detection (FD). The reported methodologies for quantification of harman and norharman in a biological matrix have been sensitive but several shortcomings exist for the published techniques. A list of published records of norharman and harman analysis in a biological matrix is presented in the table below.

Further critique of the reported bioanalytical assays is warranted, as the inadequacies in the chromatography, extraction, and validation of the β-carboline assay methods are apparent. Chromatographic baseline resolution for both harman and norharman has not been accomplished with majority of the assays presented, especially in reports that have supposed relatively low limits of quantification. Separation or resolution is an essential requirement in quantitative HPLC analysis and a baseline resolutions of $Rs > 1.5$ favors maximum precision and accurate quantification in reported results (Snyder et al., 1997).

Table 3-1: Reported methodologies for quantification of the ß-carbolines, harman and norharman in biofluids.

Author, year	Analytes	Matrix	Method	Extraction	Baseline resolution	LLOQ	Internal Standard	Calibration matrix
Moncrieff J, 1989	harman, harmine, harmaline	brain tissue, CSF, plasma	HPLC-FD	100 µl plasma, Protein precipitation	yes	1 ng/ml	sotalol	"blank" plasma
Pötsch L, Skopp G, 2002	norharman, harman	serum	HPLC-MS/MS	LLE, 1 ml serum	yes	50 pg/ml	harmine	serum
Bosin TR, Faull KF, 1988	norharman, harman, norharmol, and harmol	lung tissue	HPLC-FD GC-NICI-MS	PFBBr derivatization Protein precipitation	no (norharman and harman)	150 pg/ml	1-propyl-9H pyrido-indole (synthesized)	buffer
Schouten MJ, Bruinvels J, 1985	norharman, harman, tryptamine	platelets plasma	HPLC-FD	15 ml plasma SPE	no (norharman and harman)	not reported	none (external calibration)	buffer
Breyer-Pfaff U, Wiatr G, Stevens I, 1996	norharman, harman	plasma	HPLC-FD	1-2 ml plasma LLE	no	8 pg/ml	external standards no IS	buffer
Rommelspacher H, Schmidt LG, 1991	norharman, harman	plasma	HPLC-FD	4 ml plasma SPE	no	LOD 20 pg/sample	external standards no IS	buffer
Fekkes D, *et al*., 2001	norharman	plasma	HPLC-FD	2-5 ml plasma LLE	no	LOD 3-6 fmol	harman	buffer
Zheng W, *et al*., 2000	harman, harmine	blood	HPLC-FD	9-12 ml of blood, LLE	yes	210 pg/ml	no IS, (abs recovery ~60%)	mobile phase

With regard to quantification, several deficiencies are present in reported methods. Firstly, internal standards have not been used for the greater part of reported assays. Significant sample pretreatment and preparation steps utilized in these methods necessitate the use of an internal standard. A properly chosen internal standard can compensate for changes in sample size or concentration due to instrumental variations as well as variation in extraction recovery.

Requirements for a proper internal standard include, but are not limited to, well resolved from the analytes of interest, similar retention to the analyte, should not be present in the original sample, and stable and unreactive with sample or mobile phase (Snyder et al., 1997). For the assays in which an internal standard was employed, endogenous levels of internal standards are present which may compromise accuracy and precision estimates. For these methods, the concentrations of endogenous levels of internal standard in the plasma was not assessed or reported. In biological samples such as plasma or tissue where even lower levels are detected, the fluorescent 1-ethyl-9H-pyrido[3,4-b]indole or 1-propyl-9H-pyrido[3,4-b]indole have been used successfully as internal standards, but both require laborious organic synthesis (Bosin and Faull, 1988).

Secondly, appropriate bioanalytical validation metrics (i.e., accuracy and precision) have either not been presented or assessed. These aspects of method development are necessary to challenge the method and determine limits of allowed variability for the conditions needed to run the assay, both of which have significant bearing on suitable analyte quantitation in a biological matrix.

Most importantly, the calibration matrix that has been used for quantitation in published assays has been via external calibration in buffer or mobile phase. The premise behind the use of these matrices is the lack of a true blank matrix. Approaches used to minimize background noise and interference, via data processing and chromatographic separation, have not solved the seemingly insurmountable problem of a significant background signal when the analyte itself is present in the blank matrix used to prepare calibration standards (Li and Cohen, 2003). Calibration techniques used in reported β-carboline assays do not use the matrix the analyte(s) are intended to be measured in, but are quantitated via calibration curve in neat aqueous and/or organic solvents. This practice of calibration completely disregards sample extraction efficiency or matrix effects that may occur during the analysis or detection.

In summary, the reported bioanalytical methodologies for quantification of the β-carbolines, harman and norharman, in human plasma are not adequate for clinical study use due to poor resolution of analytes, lack of internal standard use in samples that have significant pretreatment steps, and, of utmost importance, insufficient and inappropriate validation of the bioanalytical assay. The current method developed for β-carboline quantification addresses the limitations associated with reported assay literature, whilst keeping the sensitivity needed for use in human pharmacology studies.

3.2 Physico-chemical Characteristics of Harman and Norharman

β-carbolines, such as harman (1-methyl-9H-pyrido-[3,4-*b*]indole) and norharman (9H-pyrido-[3,4-*b*]indole), are a class of aromatic compounds that are

formed via the condensation of indolealkylamines (e.g., tryptophan, serotonin) with aldehydes. Recall the structure and physicochemical characteristics from figure 1-3. The β-carboline class of molecules show commonality in possessing aromatic, planar, conjugated ring structures with extended π-electron systems. The lipophilic nature of an array of β-carbolines has been studied (Biagi et al., 1989), with harman and norharman resulting in relatively high logD values. The pyridine nitrogen behaves as a base and is easily protonated, therefore, all β-carboline derivatives studied possess pKa values that vary from 6.2-9.5 (Draxler and Lippitsch, 1995). Conversely, the pyrrolic nitrogen is acidic and loses its proton in alkaline environment, although outside the usual pH scale (pH>14). Due to their structural properties, norharman and harman exhibit a notable native fluorescence and atypical acid-base behavior in the ground and excited states (Pardo et al., 1992). Considering that β-carbolines are lipophilic and highly fluorescent, reversed-phase HPLC with fluorometric detection is a very useful technique to determine these compounds in a biological fluid. Therefore, the following experiments for assay development were designed to optimize fluorescence detection and chromatographic separation. The ultimate goal of the bioanalytical assay development was to quantify the β-carbolines, harman and norharman, in human plasma for the support of two clinical studies.

3.3 HPLC-Fluorescence Detection Method Development

3.3.1 Fluorescence Detection (FD) Experiments

Owing to their strong fluorescence characteristics, the β-carbolines have been investigated by numerous photophysical methods (Carmona et al., 2000). The fluorescent nature of these molecules may be due in part to their planar, conjugated ring structures, which possess extended π-electron systems. These compounds, in particular norharman, have been proposed as fluorescence standards due to their high quantum yields and their inability to be quenched by halide ions (Pardo et al., 1992). The pH-dependence of the absorption and fluorescence spectra of several naturally occurring β-carboline derivatives have been thoroughly investigated (Balon et al., 1993), (Wolfbeis et al., 1982). All reports infer that the polarity and acidity of the surrounding media greatly affect the fluorescence emission spectra, quantum yields and life-time of β-carboline derivatives. Unquestionably, much of the interesting photophysical properties of the β-carbolines arise from the polyfunctional hydrogen bonding nature of the β-carboline ring. Thus, the presence in this ring of the acidic pyrrolic and basic pyridinic acidic nitrogen atoms allows β-carbolines to act as hydrogen bond donor and acceptor molecules. As the fluorescence of an aromatic compound with acidic and/or basic ring substituents is usually pH-dependent (Skoog et al., 1998), spectral characteristics are likely to be different for the ionized and unionized forms of the molecule. The changes in the excitation and emission energy of ionizable species arise from the different number of resonance species that are associated with the acidic and basic forms of the

molecule. These observations suggest that analytical procedures based on fluorescence detection frequently require control of pH.

As protonation of the amine groups results in a loss of electron-donating characteristics, the pH dependence of the fluorescence behavior of these aromatic amines was studied. The aim of this investigation was to determine the most favorable $\lambda_{excitation}$ and $\lambda_{emission}$ for fluorescent detection analysis and to evaluate the effect that pH may have on the fluorescence excitation and emission of both β-carbolines, using standard applications for spectrofluorometric techniques.

Harman and norharman physiological concentrations are reported to be in the low picogram to nanogram per milliliter range, therefore optimization of fluorescence detection was based on:

1. evaluation of the dependency of signal intensity on pH for both analytes;
2. obtaining the most advantageous $\lambda_{excitation}$ and $\lambda_{emission}$ for fluorescent detection analysis, along with an adequate Stokes' shift for selective measurement; and
3. determination of any deviations from linearity of fluorescence signal in the expected physiological concentration range of analytes at optimal $\lambda_{excitation}$ and $\lambda_{emission}$.

The Stokes shift is fundamental to the sensitivity and selectivity of fluorescence techniques because it allows emission photons to be detected against a low background, isolated from excitation photons (Skoog et al., 1998). Optimal pH and $\lambda_{excitation}$ and $\lambda_{emission}$ results will aid in selecting an appropriate mobile phase for sensitive and specific detection of harman and norharman after chromatographic separation.

3.3.1a Materials and Reagents

All chemicals were or analytical grade quality and obtained from commercial sources. Solvents used for the spectrofluorometric measurements were of spectroscopic or HPLC grade and used without further purification.

1. Harman, purum ≥ 98% (Sigma-Aldrich Corp., St. Louis, MO)
2. Norharman (Sigma-Aldrich Corp., St. Louis, MO)
3. 0.05 M H_2SO_4, pH 1

   - H_2SO_4, double distilled (GFS Chemicals Columbia, OH), 18M
4. Potassium phosphate buffer (for buffers between pH 2-3 and pH 7.2)

   - monobasic potassium phosphate, KH_2PO_4 (Sigma-Aldrich Corp, St. Louis, MO)

   - dibasic potassium phosphate, K_2HPO_4 (Sigma-Aldrich Corp., St. Louis, MO)

   - phosphoric acid, H_3PO_4 (Sigma-Aldrich Corp., St. Louis, MO), 14.8M
5. Ammonium acetate buffer (for buffers pH 4 and 5.5)

   - ammonium acetate (Fisher Chem, Fairlawn, NJ)

   - glacial acetic acid (CMS Chempure Houston, TX)
6. Ammonium chloride buffer pH 9, 10, 12

   - ammonium chloride (Fisher Chem, Fairlawn, NJ)

   - ammonia, anhydrous (Sigma-Aldrich, St. Louis, MO)
7. 10 M NaOH (VWR, Westchester, PA)
8. Methanol, HPLC Grade (Burdick and Jackson, Morristown, NJ)
9. Acetonitrile (Burdick and Jackson, Morristown, NJ)
10. Milli-Q® water (Virginia Commonwealth University, Bioanalytical Laboratory)

3.3.1b Equipment

1. Instrumentation: Shimadzu RF-5301 Spectrofluorometer
2. Data acquisition: Shimadzu RF-5301PC Software
3. Cuvettes, Silica quartz 10.0 mm Shimadzu Scientific (Columbia, MD)
4. Corning pH meter, Model 240 (Corning, NY)
5. 10-µl, 100-µl, and 1000-µl VWR variable volume pipette and corresponding pipette tips.

3.3.1c Preparation of Solutions and Standards

1. 0.001 M Harman in methanol

   Approximately 10.3 mg of harman was weighed and placed in a volumetric flask of 50-ml methanol. For additional concentration levels, serial dilutions were performed using respective buffers.

2. 0.001 M Norharman in methanol

   Approximately 10.1 mg of norharman was weighed and placed in a volumetric flask of 50-ml methanol. For additional concentration levels, serial dilutions were performed using respective buffers.

3. 0.05 M H_2SO_4 (GFS Chemicals Columbia, OH), pH 1

   In a 100 ml volumetric flask, 270 µl of 18M H_2SO_4 was added to 100 ml Milli-Q® water.

4. Buffers - For each pH level, an appropriate amount of stock 0.5M base, stock 0.5M acid and Milli-Q® water was added to make a 100 ml 0.025M buffer at the respective pH.

a) Potassium phosphate buffers (for buffers between pH 2-3 and pH 7.2)

- 0.5 M monobasic potassium phosphate (KH_2PO_4)- 6.804 g added to 100 ml of Milli-Q® water in a 100 ml volumetric flask.

- 0.5M dibasic potassium phosphate (K_2HPO_4)- 8.708 g added to 100 ml of Milli- Q water in a 100 ml volumetric flask.

- 0.5M phosphoric acid (H_3PO_4) - 3.3784 ml was added to a 100 ml of Milli-Q® water.

b) Ammonium acetate buffers (for buffers pH 4 and 5.5)

- 0.5M ammonium acetate – 3.854 mg was added to 100 ml of Milli-Q® water.

- 0.5M acetic acid - 2.87 ml of glacial acetic acid, 17.4M was added to 100 ml of Milli-Q® water.

c) Ammonium chloride buffers (for pH 9, 10, and 12 buffers)

- 0.5M ammonium chloride – 2.67g of NH_4Cl was added to 100 ml of Milli-Q® water.

- 0.5M ammonia solution - 373 µl of anhydrous ammonia (13.4M) was added to 100 ml of Milli-Q® water.

5. 0.1 N NaOH – 5 ml of 10 M NaOH was added to 100ml of Milli-Q® water in a 100 ml volumetric flask.

3.3.1d Methods

Freshly prepared methanolic stock solutions of H and NH were prepared at a concentration of 0.001 M. Buffered solutions of various pH's were prepared at a concentration of ~ 0.025 M in order to maintain a maximum buffer capacity of at least 40%. Initially, the pH's (and respective buffers) that were evaluated for the optimization were the following: pH 1 (0.05 M H_2SO_4); pH 2 and 3 (KH_2PO_4/H_3PO_4 buffer); pH 4 and 5.5 ($NH_4C_2H_3O_2/CH_3COOH$ buffer); pH 7.2 (K_2HPO_4/KH_2PO_4 buffer); pH 9, 10 and 12 (NH_3/NH_4Cl buffer); and pH 13 (0.1 N NaOH). In order to determine the fluorescent properties of H and NH in common HPLC solvents, measurements were additionally made in 100% methanol and 100% acetonitrile.

Stationary excitation and emission spectra (uncorrected for instrument) were evaluated at each pH for the given concentration of H and NH. Blank buffer or solvent was additionally assessed for matrix evaluation. In order to obtain $\lambda^{max}_{excitation}$ and $\lambda^{max}_{emission}$ at each respective pH for both H and NH, fluorescence scanning experiments were performed. The excitation spectra were evaluated between the ranges of 220-400 nm, at a fixed $\lambda_{emission}$ of 500 nm. Upon appraisal of the spectra, the $\lambda^{max}_{excitation}$ was fixed, and the $\lambda_{emission}$ was scanned from between 250-550 nm. The excitation and emission slit width was set at 10 nm and 1.5 nm, respectively. All recorded spectra were taken at room temperature (22± 2 °C) at a scan speed at the medium setting with a response time of 0.1 seconds.

To make solutions for spectral pH optimization measurements, a predetermined amount of stock solution was added to a buffered solution to make a desired

concentration of 250 pg/ml (~1.4 x 10^{-6} M) and 220 pg/ml (~1.3 x 10^{-6} M) for H and NH, respectively. Dilution to this low of a concentration was necessary to avoid inner filter effects and reabsorption phenomena (Munoz et al., 2000). Final solvent for spectral measurement was 3:1 buffer to methanol mixture and was not degassed. At each pH excitation and emission spectra were scanned and recorded for both analytes (n = 3 for each measurement). Further pH optimization was performed at intervals of 0.1 pH units around the most favorable pH from the prior experiment.

For the linearity assessment of response the optimal $\lambda^{max}_{excitation}$ and $\lambda^{max}_{emission}$ with an optimal pH buffer was used. An approximately 1000-fold concentration range from ~5 pg/ml (~2.8 x 10^{-8} M) to 2.7 ng/ml (7.9 x 10^{-6}M) for both analytes was assessed and a calibration curve was plotted and evaluated for any deviations from linearity (10 concentrations in triplicate). The standards for the curve were prepared for the 0.001 M stock solution of H and NH and serial dilution was preformed to achieve the desired concentrations.

3.3.1e Results

For both β-carbolines, the spectral $\lambda_{excitation}$ and $\lambda_{emission}$ varied, reliant on the pH of the solvent environment. The $\lambda^{max}_{excitation}$ and $\lambda^{max}_{emission}$ for each analyte are presented in the table 3-2 below. Of note, all solvents were checked for background fluorescence that yielded a negligible fluorescence signal. Using the $\lambda^{max}_{excitation}$ for each solvent in the respective pH, the signal intensity at the $\lambda^{max}_{emission}$ was recorded. For situations where two or more $\lambda^{max}_{excitation}$ were observed, the longer wavelength

Table 3-2: Maximum excitation and emission λ (nm) for H and NH at varying pH's with speculated ionization state.

pH, buffer	Harman		Norharman		Major Ionization State
	Ex λ (nm)	Em λ^{max} (nm)	Ex λ (nm)	Em λ^{max} (nm)	
1 0.05M H_2SO_4	247, 297	432	248, 296	449	cationic
2 KH_2PO_4/H_3PO_4	246, 298	433	247, 297	448	cationic
3 KH_2PO_4/H_3PO_4	248, 298	437	247, 297	449	cationic
4 $NH_4C_2H_3O_2/CH_3COOH$	240, 303	430	244, 313	432	neutral
5.5 $NH_4C_2H_3O_2/CH_3COOH$	242, 305	428	242, 305	430	neutral
7.2 K_2HPO_4/KH_2PO_4	230, 278	360	247, 278	380	neutral
9 NH_3/NH_4Cl	241, 279	378	242, 285	382	anionic*
10 NH_3/NH_4Cl	238, 281	405	238, 281	400	anionic*
12 NH_3/NH_4Cl	232, 280	479	240, 282	480	anionic*
13 0.1 M NaOH	234, 283	479	230, 283	382, 485	anionic*
Methanol	247	360, 442	247	367, 440	neutral
Acetonitrile	233	355	230	360	neutral

*zwitterionic species may be present

(lower energy) was used for excitation to improve the selectivity of the detection. Fluorescence detection sensitivity can be severely compromised by background signals, which may originate from endogenous sample constituents. Minimization of the background noise can be performed by selecting excitation energies at longer wavelengths. Furthermore, at longer wavelengths, light scattering by dense media such as plasma is much reduced, resulting in greater penetration of the excitation light (Cullander, 1994). Moreover, the fluorescence emission spectrum is independent of the excitation wavelength, due to the partial dissipation of excitation energy during the excited-state lifetime (Lakowicz, 2007).

The pH dependency of signal intensity at $\lambda^{max}_{emission}$ is presented in figure 3-1 below for the pH range between pH 1 and 13. An important experimental design criterion for a reversed-phase HPLC pertains to the pH stability of the solid phase of the column. The majority of the manufactured HPLC columns are stable at a pH range between 2 and 9 with temperature control <50 ºC (Snyder et al., 1997). For this reason, further evaluation for the optimal fluorescence signal of H and NH was performed between the stable pH ranges of commercially available HPLC columns.

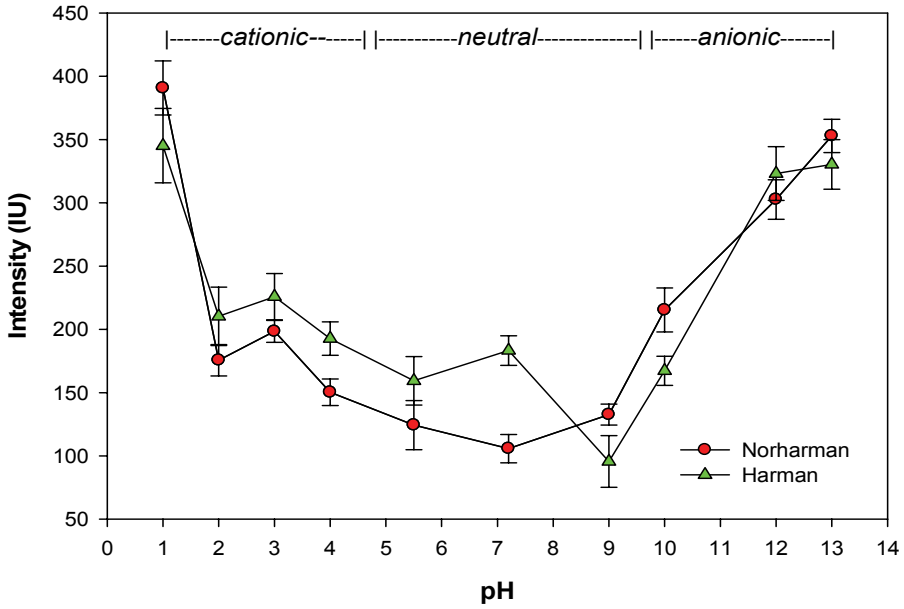


Figure 3-1: Harman and norharman fluorescence intensity and speculated ionization state as a function of pH at optimal excitation and emission wavelengths (mean ± SD, n=3).

Maximum fluorescent response was observed for H and NH at pH of ~3.0 in which H: $\lambda_{excitation}$ = 298 nm, $\lambda_{emission}$ = 437 nm and NH: $\lambda_{excitation}$ = 299 nm, $\lambda_{emission}$ = 440 nm. As the pH 3 solvent resulted in the most favorable fluorescence signal, supplementary experiments at 0.1 pH units below and above below pH 3 were appraised (range from pH 2.6-3.6). Using a 0.025 M buffer solution of KH_2PO_4/H_3PO_4, at the respective pH, fluorescent scanning experiments were performed (pH adjusted using 1M H_3PO_4). The spectral characteristics of the excitation and emission did not vary much over small pH range, therefore the signal intensities were evaluated at $\lambda^{max}_{excitation}$ = 298 nm and $\lambda^{max}_{emission}$ = 437 nm for H and $\lambda^{max}_{excitation}$ = 297 nm and $\lambda^{max}_{emission}$ = 449

nm for NH. The results of the pH dependency on fluorescence intensity are presented in figure 3-2.

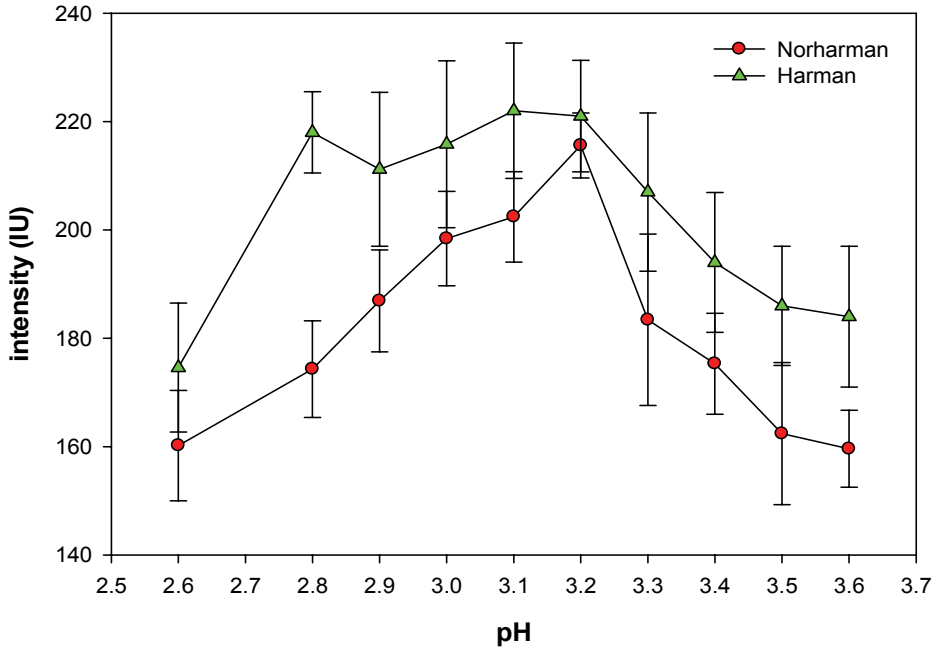


Figure 3-2: Harman and norharman fluorescence intensity between pH 2.6 and 3.6 (mean ± SD, n=3).

In the methanol and acetonitrile solutions, the neutral molecule's fluorescence is present within the UV range.

The additional pH experiments, at smaller pH intervals, resulted in an optimal fluorescence signal intensity at pH ~ 3.2, as deemed by the largest, most reproducible signal intensity for the given concentration of H and NH. From the pH optimization experiments, the resultant wavelengths were used for calibration curve experiments at pH 3.2; H: $\lambda^{max}_{excitation}$ = 298 nm, $\lambda^{max}_{emission}$ = 437 nm; NH: $\lambda^{max}_{excitation}$ = 297 nm, $\lambda^{max}_{emission}$ = 449 nm. The excitation and fluorescence spectra for H and NH are

exemplified in the figures below. For reference, the structure of the presumed ionic state is represented in the spectra.

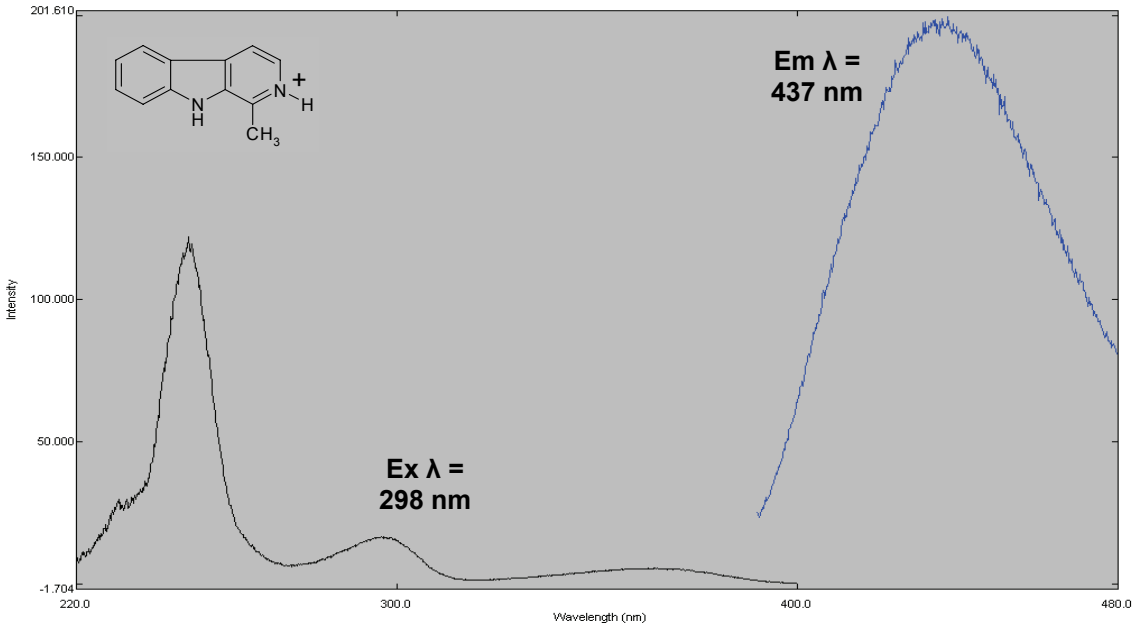


Figure 3-3: Excitation and emission fluorescence spectrum of harman in the cationic state, pH = 3.2.

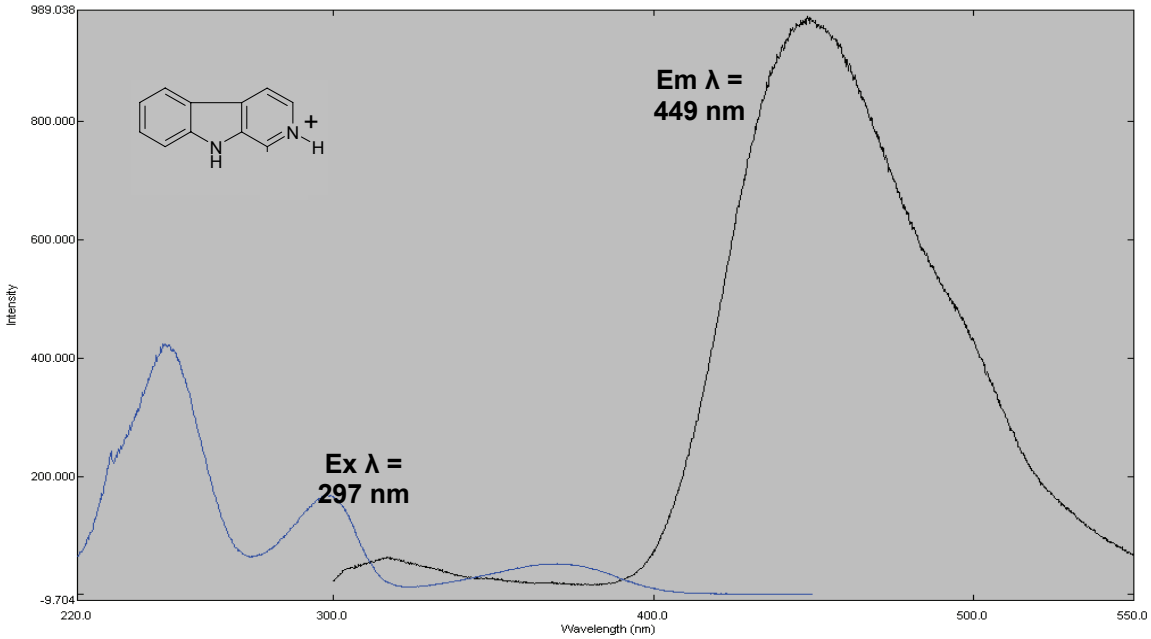


Figure 3-4: Excitation and emission fluorescence spectrum of norharman in the cationic state, pH = 3.2.

From this investigation, it was concluded that H and NH show good photophysical properties with strong fluorescence, large Stokes shifts (>90 nm) and relatively long excitation (>278 nm) and emission (>360 nm) wavelengths for both analytes throughout the pH range, all of which can minimize the effects of the background fluorescence. The difference in the wavelengths of maximum excitation and maximum emission is called the Stokes' shift ($\lambda^{max}_{emission} - \lambda^{max}_{excitation}$) (Gaigalas et al., 2001). Stokes' shifts that were observed as a function of pH are presented in the table below.

Table 3-3: Stokes shifts (nm) for harman and norharman at varying pH's

pH	Harman (nm)	Norharman (nm)	Speculated Ionization state
1	135	153	cationic
2	135	157	cationic
3	139	152	cationic
4	127	119	neutral
5.5	123	125	neutral
7.2	82	102	neutral
9	99	97	neutral
10	124	119	anionic*
12	199	198	anionic*
13	196	202	anionic*
MeOH	113	120	neutral
ACN	122	130	neutral

*zwitterionic species may be present

Larger stokes shifts were observed at pH's >10 (presumed anionic state). For practicality and utility of HPLC development, the acidic pH ranges between 1 and 3 yielded the larger Stoke's shifts. As the pH of approximately 3 resulted in the highest fluorescent intensity for a given concentration of H and NH, and the Stoke's shift at this pH was the largest, the pH 3.2, 0.025 M buffer solution of KH_2PO_4/H_3PO_4, was used to maximize sensitivity and selectivity for spectrofluorometric quantification experiments.

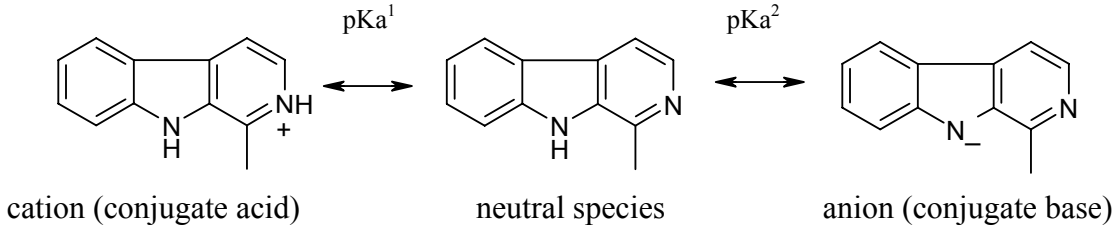
Calibration curve experiments resulted in linear response throughout the concentration range for both H ($R^2 > 0.998$) and NH ($R^2 > 0.997$). For each analyte, the inter-individual variability between slopes of the calibration curves was negligible and showed consistency for each analyte. Moreover, the y-intercepts obtained from each calibration regression were insignificant and statistically not different from zero. In this

spectrofluorometric system, the minimum concentration detected (LOD) for H and NH were 32 pg/ml and 25 pg/ml, respectively. This LOD calculation was based on the standard deviation of the noise and slope of the calibration curve (3 times the standard deviation of the noise divided by the slope of the calibration curve)(ICH, December 18, 1996). The standard deviation was estimated based on n = 3 blank sample signals or can be extracted from the calibration curve. Of note, the LOD is not a very stable characteristic because of its susceptibility to minor changes in the conditions of the analytical method, like temperature, purity of reagents, sample matrices, and instrumental system changes. This measurement was expected to change upon analysis of, H and NH in a biological matrix and upon transfer onto an HPLC-FD system. Additional experiments were conducted on additional, more concentrated solutions for testing fluorescence signal saturation. Non-linearity of the instrument response occurred at concentrations above 10 ng/ml, likely due to insolubility issues, and implying that dilution may be required of any samples above this concentration.

3.3.1f Discussion

The molecular resonance energy of a fluorophore in aqueous solutions are rather sensitive to the surrounding milieu that, in turn, tends to influence the energy of the electronic states. Thus, changes in solution properties, such as pH, usually lead to shifts in the wavelength of maximum emission and large changes in the quantum yield. As seen in experimentation and previously reported literature, the β-carbolines, H and NH, have been extensively evaluated for their spectral properties in different solvents.

The pH, and consequently ionization state, significantly alters the fluorometric measurement of H and NH. Owing to the acidic nitrogen moiety and the basic nitrogen atom in the pyridine nucleus, H and NH can exist in three differently charged ground state species. Their respective equilibria, shown in the scheme below are governed by two pKa's. The zwitterionic species may also be present, especially in the excitation spectra observed in the pH range of greater than neutral.



cation (conjugate acid) neutral species anion (conjugate base)

Figure 3-5: Structure and ionization states of the β-carbolines, governed by two pKa's

According to the proton transfer dynamics of the β-carbolines in differing pH's, the cationic form of H and NH yielded the maximal response for a given concentration (between pH 1-4), and the anionic and zwitterionic form (> pH 10). This is consistent with reported literature in which it was found that the fluorescence yield of the cationic species of norharman was the largest and found to be constant at pH's below 4. It has been observed that the molar extinction coefficients (log ε) for the cationic forms are 4.16 and 3.64 and the quantum yield (Φ_F) is 0.56 and 0.83 for NH and H, respectively (Balon et al., 1993). Within the 4-10 pH range the fluorescence signal, albeit reproducible, yielded the smallest response for the given concentration. Within this pH range, the neutral species for both H and NH are seemingly predominant.

For further HPLC method development, an initial pH of 3.2 was used for additional experimentation due to the following reasons: (1) at this pH, a relatively large fluorescence intensity was seen for a given concentration of H and NH; (2) the cationic form of H and NH predominates at this pH and, in concordance with previous literature, has the highest quantum yield; (3) for practical HPLC method development, the Stokes shift observed at this pH was relatively large, which was expected to improve selectivity and sensitivity of the method. Furthermore, there is small overlap between excitation and fluorescence spectra and the spectrum are independent of exciting wavelength, which are characteristic of superior fluorescence standards (Pardo et al., 1992). From these results, it was concluded that the 0.025 M buffer solution of KH_2PO_4/H_3PO_4 at pH = 3.2 was used for an initial mobile phase buffer for HPLC method optimization. At this pH, the resulting wavelengths were used were: $\lambda^{max}_{excitation}$ = 298 nm, $\lambda^{max}_{emission}$ = 437 nm for harman and $\lambda^{max}_{excitation}$ = 297 nm, $\lambda^{max}_{emission}$ = 449 nm for norharman.

3.3.2 Chromatographic Experiments and Optimization

The high-pressure liquid chromatography (HPLC) method was chosen due to the sensitivity of the method, its ready adaptability to accurate quantitative determinations, and its suitability for separating non-volatile species. Moreover, the physicochemical properties of the β-carbolines, H and NH, make the HPLC method conducive for separation and quantification. To address the concerns of previously reported assay methodologies, the following aims of chromatographic optimization were considered:

1. Baseline resolution of H and NH was paramount in order to ensure precision during quantification.
2. Retention time, peak shape (e.g., tailing factor and symmetry), resolution of peaks, selectivity and system suitability parameters (e.g., reproducibility of replicate injections) were to be optimized in order to ascertain the effectiveness of the final operating system.
3. In order to achieve adequate sensitivity to detect trace amount of H and NH in a minimal amount of human plasma, the chromatography was optimized to achieve the smallest amount of analyte(s) on column.
4. Exploration of a chromatographically suitable internal standard for further extraction method development.

As the analytes are considered to be a mixture of small molecules and are highly lipophilic, reversed-phase HPLC optimization was performed. Initial conditions of mobile phase pH were chosen based on the fluorescence detection optimization in which the cationic forms of H and NH yielded the optimal detection (in sensitivity and reproducibility). The preferred initial experimental conditions (Snyder et al., 1997) HPLC separation for ionized analytes were based on the conditions listed in table 3-4.

Specifically, the optimization of the chromatographic conditions was based on the USP XXIV 621 requirements for chromatographic separation and system suitability (USPharmacopoeia, 1999). Separation of the β-carbolines was based on the most favorable capacity factor, resolution of the peaks, and injection precision. Moreover,

peak shape for both H and NH were assessed for tailing factor, peak asymmetry, and number of theoretical plates.

Table 3-4: Initial Experimental Conditions for RP-HPLC Separation of Ionized H and NH (Snyder et al., 1997)

Separation variable	Initial Choice
Column	
Dimensions (length, ID)	15 x 0.46 cm
Particle Size	5 μm
Stationary phase	C_{18} and C_8
Mobile Phase	
Solvents A and B	Buffer and Methanol or Acetonitrile
% B	50%
Buffer (compound, pH, concentration)	25 mM potassium phosphate, pH 3
Flow rate	Isocratic, 1.0 ml/min
Temperature	Room temperature
Injection volume	50 μl

Capacity factor (k) describes the migration rates of the analytes on column and is related to retention time of the analyte:

$$k = \frac{(t_R - t_0)}{t_0} \qquad \text{(Equation 3.1)}$$

where t_R is the band retention time and t_0 is the column dead time. The goal of the solvent strength adjustment and buffer pH optimization was to position the bands within a k range of roughly 0.5 to 20. This range was based on the avoidance of initial

baseline disturbance overlapping the first band (early-eluting interferences) and to evade broadening of the last band that may be seen with long run times (Snyder et al., 1997).

An inadequacy apparent from reported assays for H and NH quantification biological matrices is resolution, R_s, between β-carboline analytes. With baseline resolution, the HPLC data system is able to draw an accurate baseline under each band-peak, thereby increasing the accuracy of the band-area measurements and resultant calculation of sample concentrations (Snyder et al., 1997). The measurement of efficiency of the separation of H and NH in a mixture was determined by the equation:

$$R_s = \frac{2(t_2 - t_1)}{W_2 - W_1} \qquad \text{(Equation 3.2)}$$

whereby t_2 and t_1 are the retention times for the two components, H and NH, and W_2 and W_1 are the corresponding widths of the bases of the peaks (bandwidth), obtained by extrapolating the relatively straight sides of the peaks to the baseline. The goal baseline resolution that corresponds to $R_s > 1.5$ will allow for adjacent bands of dissimilar bands and account for the usual deterioration of an HPLC method from day-to-day use (Snyder et al., 1997).

Another useful parameter is the reproducibility of replicate injections of the analytical solution of H and NH. The reproducibility of the replicate injection is best expressed by the injection precision, or percent relative standard deviation, %RSD. The calculation is expressed by the equation:

$$\%RSD = \frac{100}{\bar{x}}\left[\sqrt{\frac{\sum_{i=1}^{n}(x_i - \bar{x})^2}{n-1}}\right] \qquad \text{(Equation 3.3)}$$

in which, %RSD is the relative standard deviation in percentage, $\bar{x}$ is the mean of a set of n measurements and x_i is an individual measurement. The x term refers to the peak area response corresponding to the reference standard. The injection precision suggested limit is a %RSD < 3% for n ≥ 5 chromatographic measurements (USPharmacopoeia, 1999).

Additional information such as peak shape (i.e., tailing factor, peak asymmetry) and number of theoretical plates should also be assessed to evaluate the performance of the HPLC method. More specifically, column performance can be defined in these terms for a test substance run under "optimal" conditions. The column plate number, N, is an important characteristic of the column and is defined as the ability of the column to produce sharp narrow peaks for achieving good resolution between band pairs (Snyder et al., 1997). A relationship used to measure plate number is:

$$N = 2\pi\left(\frac{t_R h'}{A}\right)^2 \qquad \text{(Equation 3.4)}$$

where t_R is the band retention time, and h' and A are the peak height and area, respectively. Equation 3.4 is often used in HPLC data systems to determine the value of N and is used for measuring column performance (Snyder et al., 1997). Representative values of an ideal N value for columns of differing lengths and particle sizes vary and are generally reported under 'ideal conditions'. For the purposes of

initial HPLC method development, an acceptance limit of $N > 2000$ was proposed as a criterion. The plate number was evaluated primarily for the evaluation of column efficiency and evaluation of column lifetime throughout the HPLC method development.

While the column plate number is a useful measure of column quality and efficiency, the peak shape and tailing assessment is also important during development (Snyder et al., 1997). Lack of symmetry in peak shape, A_s (exactly symmetrical peaks have a value of 1.0), can result in issues in imprecise quantitation, poor retention reproducibility, and degraded resolution (Snyder et al., 1997). The peak symmetry is calculated at 10% of full peak height and samples of interest generally should have A_s values of <1.5 (Snyder et al., 1997).

Chromatographic optimization for H and NH separation in neat solution was based on the initial criteria and limits aforementioned. These suggested limits were used as a reference to set up initial system suitability criteria during the early HPLC method development process. The goal of this chromatographic investigation was to obtain a range of HPLC system parameters (i.e., %B, pH of mobile phase, flow rate) from neat solution in order to further optimize the conditions from those of extracted human plasma matrix. This safeguard provided initial HPLC parameters for further method chromatographic exploration from extracted plasma, especially in the case of selectivity issues that may arise. The results of the optimization were carried further in subsequent validation and analysis with human plasma matrix.

3.3.2a Materials and Reagents

All chemicals were or analytical grade quality and obtained from commercial sources. Solvents used for the chromatographic optimization measurements were of spectroscopic or HPLC grade and used after filtration with a 0.2 μm porosity filter (Corning, Corning, NY).

1. Harman, purum ≥ 98% (Sigma-Aldrich Corp., St. Louis, MO)
2. Norharman (Sigma-Aldrich Corp., St. Louis, MO)
3. Potassium phosphate buffer (for buffers between pH 2-5 and pH 7.2)
   - monobasic potassium phosphate - KH_2PO_4 (Sigma-Aldrich Corp, St. Louis, MO)
   - dibasic potassium phosphate, K_2HPO_4 (Sigma-Aldrich Corp., St. Louis, MO)
   - phosphoric acid, H_3PO_4 (Sigma-Aldrich Corp., St. Louis, MO), 14.8M
4. Methanol, HPLC Grade (Burdick and Jackson, Morristown, NJ)
5. Acetonitrile (Burdick and Jackson, Morristown, NJ)
6. Milli-Q® water (Virginia Commonwealth University, Bioanalytical Laboratory)

3.3.2b Equipment

1. Instrumentation: Waters HPLC, 600 Controller, 717+ Autosampler, 2475 Fluorescence Detector, with In-line Degasser.
2. Data acquisition: Waters Empower® Software
3. Corning pH meter, Model 240 (Corning, NY)
4. 10-μl, 100-μl, and 1000-μl VWR variable volume pipette and corresponding pipette tips.

5. Columns (all end-capped):

   - Waters Symmetry®, C_{18}, 3.5 μm (150 x 4.6mm)

   - Waters Symmetry®, C_{18}, 5 μm (75 x 4.6mm)

   - Agilent Zorbax Eclipse XDB®, C_8, 5 μm (150 x 4.6mm)

6. Vials/Caps/Septa: Waters®, 1ml glass shell with polypropylene caps

3.3.2c Preparation of Solutions and Standards

1. 0.001 M Harman in methanol

   Approximately 10.2 mg of harman was weighed and placed in a volumetric flask of 50-ml methanol. For additional concentration levels, serial dilutions were performed using methanol.

2. 0.001 M Norharman in methanol

   Approximately 10.0 mg of norharman was weighed and placed in a volumetric flask of 50-ml methanol. For additional concentration levels, serial dilutions were performed using methanol.

3. Buffers - For each pH level, an appropriate amount of stock 0.5M base, stock 0.5M acid and Milli-Q® water was added to make a 100 ml 0.025M buffer at the respective pH. For the potassium phosphate buffers (for buffers between pH 2-5 and pH 7.2):

   - 0.5 M monobasic potassium phosphate (KH_2PO_4)- 6.804 g added to 100 ml of Milli-Q® water in a 100 ml volumetric flask.

   - 0.5M dibasic potassium phosphate (K_2HPO_4)- 8.708 g added to 100 ml of Milli- Q water in a 100 ml volumetric flask.

- 0.5M phosphoric acid (H_3PO_4) - 3.3784 ml was added to a 100 ml of Milli-Q® water.

3.3.2d Methods

The steps taken during the optimization of chromatographic separation were based on current HPLC method development practice (Snyder et al., 1997). Initial specific conditions were described previously, and the results of which were used in subsequent experiments. Moreover, optimal analyte solvent (pH ~3) along with excitation and emission wavelengths at the most favorable pH were utilized for mobile phase pH and detection, respectively. A systematic (along with a trial-and-error) process was continued until successful separation was achieved. Based on the physicochemical characteristics of H and NH, the reversed phase separation conditions and parameters that were explored were column chemistry (packing) and configuration, mobile phase strength (%B), pH and concentration of buffer, flow rate, temperature of column, injection volume and sample plug solvent composition. For all experiments, the conditions were evaluated via univariate experimental design.

Specifically, the types of columns that were evaluated ranged from a single C_8 column to two different C_{18} columns with differing column configurations (in length and particle size, see section 3.3.2.2 under "Columns"). A C_8 or C_{18} column made from specially purified, less acidic silica and designed specifically for the separation for basic compounds is generally suitable for all samples and is strongly recommended (Snyder et al., 1997). An optimal column was chosen on the basis of the ability for H

and NH to be retained and eluted from the column, the retention time, and the resolution and peak shape of H and NH bands. Isocratic methods were explored initially for method development simplicity.

With respect to the mobile phase composition, optimization experiments were performed with the following prioritization of conditions (most important to less important): (1) the percent organic, %B, (2) flow rate of mobile phase, (3) pH of buffer, and (4) concentration of buffer. In particular, the %B was varied from 5% to 50% in increments of 5%. Both methanol and acetonitrile, or a combination of both, were utilized to determine best retention and separation conditions. The initial experiments used a H_3PO_4/KH_2PO_4 buffered mobile phase at ~ pH 3 at a concentration of 50 mM. These initial pH conditions were used to reflect the results from the spectrofluorometric experiments described earlier. The initial concentration of the buffer was used to maintain adequate buffer capacity and, at the same time, to avoid salt precipitation upon introduction of larger %B. The flow rate of the mobile phase was subsequently explored from between 0.5 ml/min to 1.5 ml/min. Along with chromatographic evaluation, the pressure was evaluated for consistency within the chromatographic run and between injections.

As H and NH are expected to be in its cationic state at the initial pH conditions (pH ~ 3), the buffered aqueous composition was subsequently optimized. Besides the notion that this pH yielded the largest signal intensity for a given concentration of H and NH, a low pH protonates column silanols and reduces their chromatographic activity (Snyder et al., 1997). Secondly, the low pH is far enough from the pKa values

for the basic functional groups of H and NH to maintain its ionization. Therefore, at the initial low pH conditions it was hypothesized that the retention of H and NH will not be affected by the small changes in pH and the reversed-phase HPLC method will be more rugged. Optimization at pH 3.0, 3.2, 3.5, 4.0, 4.5, 5.0 and 7.2 was explored to evaluate effects of pH conditions on H and NH chromatography. pH values lower than 3.0 were not evaluated as the solid phase stability of the columns used may be compromised. In conjunction with pH assessment, the concentration of the buffer was explored to maintain the pH. This was performed to ensure the pH reproducibility and buffering capacity of the final mobile phase upon dilution. The buffer concentrations that were explored were between 10 mM and 50 mM at increments of 10 mM. Higher buffer concentrations, provide an increased buffer capacity but may not show favorable solubility in mobile phases with a high organic content (%B). As the initial conditions for the HPLC separation were for the ionized form of H and NH, consideration of buffers with a marginal buffer capacity (i.e., low concentration) were avoided to circumvent less reproducible separations for the ionized H and NH. A H_3PO_4/KH_2PO_4 buffered mobile phase was adjusted accordingly with acid or base to achieve a desired pH and concentration.

Along with the column configurations and mobile phase compositions, the sample injection volume and composition of the sample solution (i.e., injected solution) was also evaluated. The volume of sample introduced into the HPLC was constrained by the injection loop size (100 µl for the Waters HPLC system used). Injection volumes < 10 µl were not used due to irreproducibility that may occur with sample

introduction from the sample loop and needle. Sample volume was assessed upon chromatographic evaluation using the system suitability and peak shape requirements aforementioned. The goal of the sample volume investigation was to avoid an undesirable change in separation due to a sample size that is too large (e.g., column overload) and to increase detection sensitivity for the trace analysis of H and NH by using the largest possible sample size. The composition of the sample solvent was kept close (±5%) to the composition of the mobile phase to evade extra-column effects associated with the HPLC (e.g., band broadening or peak asymmetry). It has been exemplified that injecting the sample in a solvent that is stronger than the mobile phase usually results in early bands that are distorted and tailing (Snyder et al., 1997).

The initial solution concentrations used for the chromatographic optimization were 406 pg/ml and 452 pg/ml for NH and H, respectively. Injections for each optimization step (i.e., when a chromatographic condition was changed) were performed in triplicate. Univariate optimization was performed in a step-wise fashion. This sequential single-factor approach requires all factors but one to be held constant while the univariate search was carried out on the factor of interest (Massart et al., 1988). After optimization of the conditions, three levels of concentrations for both analytes were investigated for precision, system suitability requirements, and peak shape reproducibility. The concentrations were 202 pg/ml and 260 pg/ml for the low level, 406 pg/ml and 452 pg/ml for the medium level, and 1.12 and 1.25 ng/ml for the high level for H and NH, respectively. A total of n=6 injections were performed for each level using the optimized chromatographic conditions.

For the calibration and sensitivity analyses for both analytes, a range of reported physiological concentrations for both analytes were used to determine the linearity of the response and to determine the lowest amount of analyte on column. The concentrations (n=16) that were employed for both analytes ranged from 5 ng/ml down to 5 pg/ml for both β-carbolines. Areas under the peak for both analytes were used for calibration quantitation. Once the calibration range was established and assessed via linear regression, injections were run in triplicate for those injections giving an adequate signal-to-noise ratio for detection (S/N > 3). The lowest detectable amount on column was defined as a S/N > 3 with %RSD < 2% for n=6 repeated injections.

3.3.2e Results

Using a neat solution, the chromatography was optimized with respect to the column chemistry and configuration, the percent of organic modifier, pH and concentration of the buffer, flow rate and injection volume. Chromatographic retention of both H and NH was seen for all columns investigated. Although they were not baseline resolved, peak(s) were observed within one minute for each column when 100% methanol or 100% acetonitrile was used for the mobile phase. For these extreme conditions, the capacity factor (k) was < 0.2, resulting in the need to use a weaker, more polar mobile phase. Adequate retention for the analytes, as defined by $0.5 < k < 20$, was seen for a %B < 50%. Although resolution of the analytes was not observed at various solvent strengths for both configurations of the C_{18} columns, the C_8 column employed for investigation resulted in a sufficient capacity factor and resolution. The

results of solvent strength (%B is 50:50 methanol:acetonitrile) on the capacity factor for both H and NH are exemplified in the figure below.

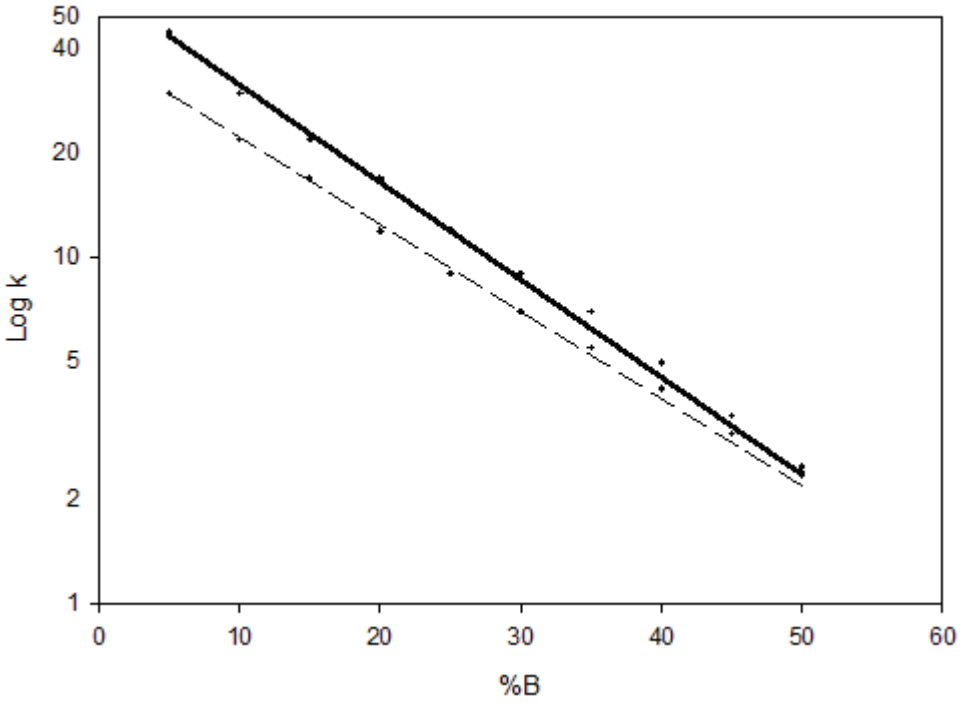


Figure 3-6: Capacity factor (k), on logarithmic scale, as a function of organic strength of mobile phase (%) for H (solid line) and NH (dashed line) on the C_8 column. Other HPLC parameters: flow rate 1 ml/min, %A is pH = 3 H_3PO_4/KH_2PO_4 buffer, 50 mM, and injection volume = 20 µl, column temp = RT. (mean response on n=3 injections, %RSD < 2% for all)

Under these conditions, it can be seen that the organic strength of the mobile phase has a significant influence on the retention and the capacity factor for both H and NH. As NH is slightly less lipophilic, the capacity factor is less than that of H at all %B. Moreover, the separation (and resolution) between the analytes appear to increase with a decrease in %B. At first approximation, the retention behavior of H and NH

decreases logarithmically with respect to mobile phase strength. The dependence of RP-HPLC retention on %B has been studied exhaustively, and the retention behavior of H and NH is in agreement with other highly lipophilic compounds (Snyder et al., 1997). The successive reductions in %B from 50% to approximately 15% yielded results in the desired capacity factor range ($0.5 < k < 20$). When the mobile phase strength was much weaker (%B <15%) the retention for both H and NH were unacceptably long. Moreover, the peak shape, albeit symmetrical, decreased in sharpness and, in turn, the S/N ratio. Superior resolution was obtained with the lower strengths of %B at the cost of a longer run time. From the optimization results of organic strength, a range that was most favorable was between 15% - 35%. Within this range of organic composition, the analytes showed baseline separation with the resolution increasing with a decrease in %B. Moreover, at this concentration a sufficient S/N ratio was observed for both analytes. This range was kept in mind in order to evaluate selectivity of the assay during method development in the biological matrix.

In the initial organic strength experimentation, the optimization was based on the separation of the cationic form of both H and NH (pH~3 buffered aqueous phase). For ionic compounds, a change in pH can result in a 10-fold or greater range in capacity factor (Snyder et al., 1997), but also have a profound effect on the resolution of the analytes. Therefore, based on the initial isocratic conditions and using a %B of 30% methanol, the pH was adjusted to evaluate the effects on retention and resolution for H and NH. As seen in figure 3-7, a systematic increase in the capacity factor is observed with increases in pH of the mobile phase. Between the low pH range of 3.0 to 4.0, the

capacity factor is relatively consistent with sufficient resolution between H and NH. As the pH approaches the pKa of H and NH (i.e., ~7.3) the capacity factor increases and less resolution is observed between analytes, due to a shorter retention (see figure below).

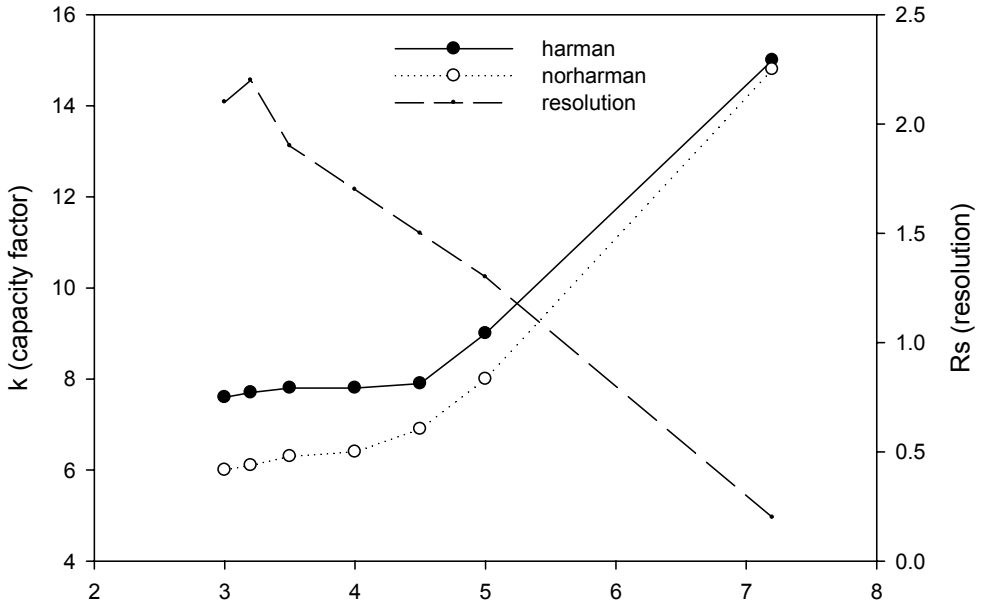


Figure 3-7: Capacity factor (k) and resolution (Rs) on Cartesian scale, as a function mobile phase pH (H_3PO_4/KH_2PO_4 buffer, 50 mM) for H (●) and NH (○) on the C_8 column. Other HPLC parameters: flow rate 1 ml/min, %B is 30% methanol, 50 mM, and injection volume = 20 µl, column temp = RT. (mean response on n=3 injections, %RSD < 3% for all)

At all pH measurements, H possessed a larger capacity factor than NH and the retention behavior became similar upon increasing pH towards the pKa.

Peak shape, asymmetry and peak tailing was evaluated at each pH. Peak asymmetry was close to unity (As~1.4) for the mobile phase pH between 3.0-3.5. An

increase to more neutral pH yielded less symmetric peaks, significant tailing, and less resolution. For these experimental conditions, the peak shape of both H and NH deteriorates progressively as the pH is increased above 4, presumably due to a decreased buffer capacity of the phosphate buffer at this pH. The buffer capacity at each pH was further explored by changing the concentration between 10-50 mM at pH values below 4.0. Influence of the concentration of phosphate buffer (20 - 50 mM) on the peak separation and shape was negligible at pH values below 4.0. Although separation with the 10 mM buffer concentration at low pH yielded adequate separation, reproducibility was slightly compromised, presumably due to a lower buffering capacity.

Under the conditions with 30% methanol as the organic modifier, at low pH (3.0-3.5) an acceptable retention factor for both H and NH was seen with satisfactory baseline resolution and symmetrical peak shape. A buffer concentration of 20 mM was used for further method development as this concentration would less likely precipitate with the organic solvents used and would maintain sufficient buffer capacity.

Flow rates between 0.8 – 1.2 ml/min were explored, ensuring that the pressure requirements for the columns utilized were met (<1800 psi). For the C_8 column used (5-µm particle size, 15 x 0.46 cm) the run times and resolution varied, with no intended change in selectivity. The majority of the optimization was performed at controlled room temperature (22ºC) with successive increases in column temperature yielding shorter run times, sharper peaks, and slightly decreased resolution. Temperature optimization was performed in the later stages of HPLC method development using the

biological matrix. Injection volumes, at the concentration used for optimization, did not show peak distortion or irreproducibility between 20 and 80 µl, suggesting that sensitivity optimization may be explored between these injection volumes at the later stages. The sample solvent composition was kept similar to that of the mobile phase composition. Deviations of ±15% organic composition for the solvent, relative to the mobile phase, resulted in peak distortion and double-peaking. The final optimized conditions, for the neat solution, that were used for system suitability, peak shape assessment, and calibration assessment are presented in the table below.

Table 3-5: Final Experimental Conditions for RP-HPLC Separation of Ionized H and NH in neat solution (mobile phase).

Separation variable	Optimized condition
Column	Agilent® Zorbax Eclipse XDB, C_8 15 x 0.46 cm, 5 µm, endcapped
Solvents A and B	
% B	methanol, 30%
Buffer (compound, pH, concentration)	KH_2PO_4/H_3PO_4 buffer (pH=3.2, 25mM)
Flow rate	Isocratic, 0.9 ml/min
Column temperature	22ºC (room temperature)
Injection volume	80 µl
Detection	$\lambda_{excitation}$ = 300 nm, $\lambda_{emission}$ = 433 nm

Under these chromatographic conditions, separation of H and NH was optimized (figure 3-8). With this separating system, good resolution, plate numbers and band symmetries were found for H and NH and separated rapidly at a mobile phase flow rate of 0.9 ml/min.

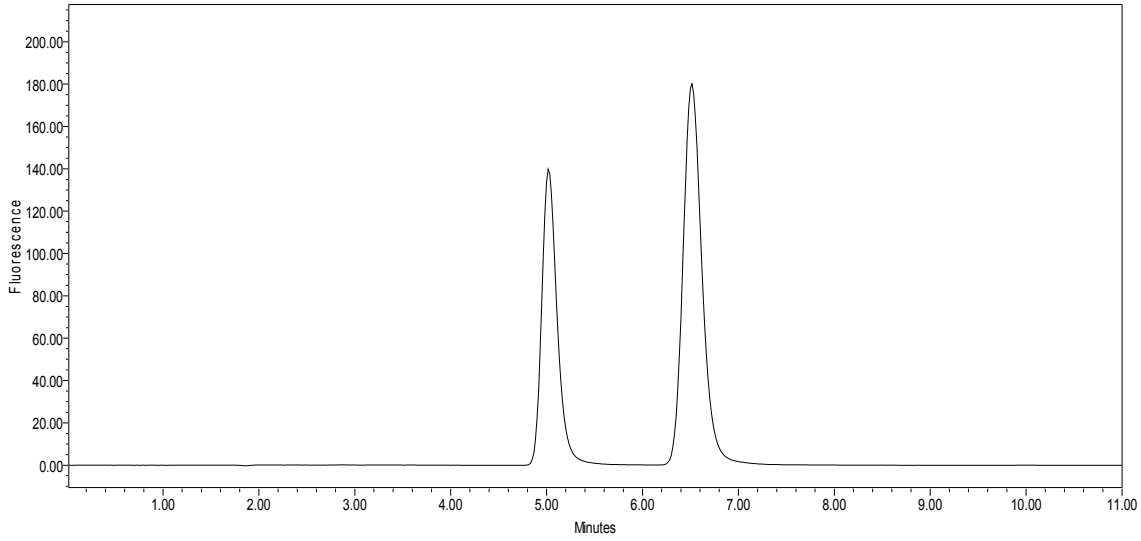


Figure 3-8: Chromatogram of norharman (406 pg/ml) and harman (452 pg/ml) in a *neat* solution of MeOH: KH_2PO_4/H_3PO_4 buffer (pH 3.2) (25:75). Baseline resolved retention times are at 5.02 min and 6.51 min for norharman and harman, respectively.

Along with run time, capacity factor and retention time, the peak shape and system suitability measurements were assessed to finalize the chromatographic conditions. The system suitability parameters are presented in the table below.

Table 3-6: System suitability measurements for the final RP-HPLC Separation Conditions for H and NH in neat solution (mobile phase). Peak shape measurements are based on the medium concentration used for both analytes (chromatogram in Figure 3-8).

Suitability parameter	Norharman	Harman
Resolution, *Rs*	2.99	
Retention time (min)	5.02	6.51
Number of theoretical plates, *N*	4641	5104
Peak tailing factor, *T*	1.21	1.05
Peak asymmetry factor, *As*	1.41	1.10
%RSD (n = 6) of Area: Low	2.6 (202 pg/ml)	2.3 (260 pg/ml)
Med	1.3 (406 pg/ml)	2.4 (452 pg/ml)
High	0.9 (1.12 ng/ml)	1.3 (1.25 ng/ml)

The optimized RP-HPLC conditions were consequently used to assess linearity of the response via calibration curves. Linear regression parameters and statistical assessment resulted in linearity throughout the concentration range for both H and NH, with adequate precision and accuracy for all concentrations. The regression was performed on the relationship between analyte concentration (pg/ml) and peak area response with a $1/x^2$ weighting factor. The calibration curve results are presented in the table below.

Table 3-7: Calibration curve measurements (n=6 injections) for final RP-HPLC separation conditions for H and NH in neat solution.

Calibration curve parameter	Norharman	Harman
Slope ± SD	4315 ± 82	5121 ± 128
Intercept ± SD	23 ± 43	52 ± 67
% RSD (all concentrations)	< 3.2	< 2.7
% DFN (all concentrations)	< 6.7	< 9.2
R^2 (range)	0.9982 (0.9811 – 0.9994)	0.9965 (0.9872 – 0.9991)
Linear range	20 pg/ml – 2524 pg/ml	26 pg/ml – 2653 pg/ml
Lowest amount on column, LOD (pg)	1.6 pg	2.1 pg

The calibration results showed linearity throughout the specified range, deemed by the values of $R^2 > 0.99$ for both analytes. For concentrations tested above the range, signal saturation was apparent, suggesting that samples with concentrations above the linear range may require dilution. The slopes for both H and NH yielded reproducible results upon replication, with H possessing larger response factor sensitivity. In other words, a given change in concentration of H produced a larger response than that of NH. The accuracy assessment as defined by %DFN (%deviation from nominal), showed that a linear regression model was appropriate for calibration purposes and interpolation between concentrations may be performed for unknown concentration evaluation. The method exemplified precision with superior relative standard deviations (%RSD) in comparison to the limits aforementioned.

3.3.2f Discussion

The RP-HPLC method for the quantitative analysis of a neat solution of H and NH has been optimized for further bioanalytical method development. The most favorable fluorescence detection environment (i.e., pH) and initial RP-HPLC conditions used for highly lipophilic compounds were used as a springboard for further modifications. The initial pH of ~3 resulted in the most sensitive signal per concentration of analyte for spectrofluorometric results. The major species of H and NH at this pH was in its cationic form, classifying this sample as ionic. In turn, both organic modifier (%B) and pH were optimized, as both are recognized to have a profound effect on retention behavior of ionized samples.

For the cationic forms of H and NH, the C_8 column configuration employed confirmed satisfactory baseline resolution and retention, as deemed by the capacity factor. As expected, the less lipophilic NH eluted before H in all chromatographic runs and selectivity was maintained. A range of organic content for the mobile phase was determined to be between 15-35%. Differing strengths of organic solvents (i.e., methanol and acetonitrile) can be used for H and NH separation and was considered for selectivity and interference chromatographic optimization in the biological matrix. In comparison to previously published methods, improvement in the resolution of H and NH was sought, with this range demonstrating adequate and reproducible resolution. This range %B was to be further investigated using the biological matrix, in the case of interfering endogenous analytes in the chromatography. In the case of pH adjustment, a range between pH 3.0-3.5 provided favorable retention and resolution, giving room

for selectivity investigation in the biological matrix. The concentration of phosphate buffer in this pH range provided significant buffering capacity to maintain reproducibility without compromising the chromatographic endpoints required.

The concentration range used for calibration assessment was in physiological range. Using a neat solution of H and NH, concentrations of up to ~ 2.5 ng/ml (200 pg on column) may be detected using the calibration regression; concentrations above may require dilution. A low picogram level can be detected on column, suggesting that this method may be sensitive enough to detect reported amounts in human plasma. A formal assessment of sensitivity for the method (lower limit of quantification) was not assessed during the current point in the method development and was postponed to the validation in the actual biological matrix.

Upon assessment of the system suitability requirements, reproducible and symmetrical bands for both H and NH were seen under the ideal chromatographic parameters. The chromatographic conditions from these results were used for subsequent experimentation in the biological matrix. Moreover, favorable ranges for organic content and pH of the mobile phase were carried forth for further optimization in plasma. From the detection and chromatographic investigations, the experimental conditions that were to be used in the human plasma matrix are included in the table below.

Table 3-8: Experimental conditions, with ranges, to be used for RP-HPLC Separation of Ionized H and NH in human plasma matrix.

Separation variable	Optimized condition
Column	Agilent® Zorbax Eclipse XDB, C_8 15 x 0.46 cm, 5 μm, endcapped
Solvents A and B	
% B	methanol and/or acetonitrile, 15-35%
Buffer (compound, pH, concentration)	KH_2PO_4/H_3PO_4 buffer (pH=3.0-3.5, 25mM)
Flow rate	Isocratic, 0.8 – 1.2 ml/min
Column temperature	22 - 40ºC
Injection volume	20-80 μl
Detection for H and NH	$\lambda_{excitation}$ = 300 nm, $\lambda_{emission}$ = 433 nm

3.3.3 Extraction Optimization and Internal Standard Investigation

As an essential part of the bioanalytical method development, the sample preparation is intended to provide a reproducible and homogeneous solution that is suitable for injection into the HPLC system. The goal of the sample preparation is to produce a sample aliquot that is relatively free of interferences, will not damage the HPLC column/system and is compatible with the intended HPLC method. Furthermore, the careful method development of the sample extraction method deserves scrutiny because the method precision and accuracy are frequently determined by the sample pretreatment procedure (van der Wal and Snyder, 1981).

Norharman and harman have been analyzed in different matrices including fried food, plant extracts, beverages and biological matrices such as urine, blood plasma and

organs. The majority of the extraction techniques have been in part to the evaluation of heterocyclic amines (HCA) in food sources. These methods include liquid-liquid extraction with lipophilic solvent at alkaline pH (Airaksinen and Kari, 1981a) or solid-phase extraction using one ion-exchange column (Herraiz, 2000) or extraction with phthalocyanin-based blue cotton, blue rayon or blue chitin (Hayatsu et al., 1991; Bang et al., 2002). Although these methods proved quite successful in the quantification of the β-carbolines in food sources, their applicability in human plasma has yet to be determined.

Since the amounts of norharman and harman in the human plasma matrix are low, and plasma is considered a complex matrix, multi-step enrichment and preparation techniques are necessary. A liquid-liquid extraction method involving alkalinization and tert-butyl-methyl-ether extraction (Breyer-Pfaff et al., 1996) was developed to consolidate norharman and harman from 2-ml of human plasma. Although the method proved sensitive enough to detect physiological concentrations, an internal standard was not used and method ruggedness and reproducibility were not reported. Solid-phase extraction (SPE) procedures have also been performed for extraction of H and NH from plasma using C_{18} (Spijkerman et al., 2002) and phenyl (Rommelspacher et al., 1991b) SPE sorbent beds, but the methods have inadequacies such as poor sensitivity and the lack of an internal standard, respectively.

In order to improve upon the inadequacies of reported methods, the following extraction investigations were designed to provide a reproducible quantitative recovery of both H and NH analytes from human plasma. The goals for optimization of the

extraction was to (1) obtain the highest recovery of each analyte to enhance sensitivity and assay precision, (2) to attain a reproducible extraction method, and (3) to minimize the sample-pretreatment steps in order to decrease the opportunity for errors. Moreover, the investigation involved an exploration of a suitable internal standard for calibration purposes and, in the case of extensive sample preparation, correct for sample losses due to the extraction.

With respect to the exploration of the extraction process, SPE techniques were chosen over the LLE (liquid-liquid extraction) *a priori*. SPE was chosen due in part to its advantages over LLE methods including a more complete extraction of the analyte(s), a more efficient separation of interferences, easier collection of the total analyte fraction, and more efficient separation process that LLE methods (Snyder et al., 1997). Practically, SPE is effective in removing interferences and "column killers" and this extraction technique permits the trace enrichment or concentration of the analyte, especially in the case for the low reported concentrations of H and NH. A primary disadvantage of SPE is the variability associated between extraction columns of similar column chemistry and configuration (Snyder et al., 1997). For this reason, employment of an internal standard may be necessary to account for differences in the efficiency of analyte removal from the sample.

Although SPE methods provide fast and efficient pre-analysis sample clean-up and concentration, the development requires attention to four related factors: (1) proper physical and chemical characterization of the analyte and the sample is required. Factors such as the analytes' polarity relative to the sample matrix, the presence of

ionizable functional groups, solubility and molecular weight determine how strongly the analyte is retained by the packing bed; (2) A proper retention strategy needs to be explored. Generally, the analyte is retained on the packing bed while the interferences are unretained or washed off the packing bed prior to eluting the analyte; (3) A suitable packing type and bed size should be investigated. Different packing types offer different selectivities that should be exploited to maximize the structural differences between the analyte and sample interferences. The goal is to obtain the cleanest extract with the highest recovery. (4) Suitable conditioning, wash and elution solvents should be explored for maximal and reproducible recoveries. Attention should be given to the solvent strength relative to the packing material. The final conditioning solvent should be relatively weak which will not act as an elution solvent. Buffers should be used in the case of ionizable species. The wash solvents should remove the weakly retained interferences but should not be strong enough to elute the analyte. Finally, the elution solvents should be strong enough to completely elute the analyte in a small volume. Post-column concentration may be employed to improve the sensitivity of the method. Each of the aforementioned factors should be considered and optimized to result in a robust and sensitive SPE method.

The physicochemical characteristics of H and NH have to be considered when developing a SPE method for human plasma. As the human plasma matrix is quite complex and may possess many interfering substances, the lipophilicity, pKa, and chemical structure plays an important role in the isolation of the analytes of interest from the matrix. Successful isolation of the analytes from the biological matrix

depends on the relative affinities of the analyte between the biological matrix and the adsorbent and relative ease of eluting the compound for subsequent analysis (McDowall, 1989). Both β-carbolines are regarded as hydrophobic molecules that are strongly to moderately non-polar. Moreover, the structure owns to a functional group that is basic in nature, the ionizable secondary amine. The imidazoline nitrogen is suspected to play a minor role in the retention characteristics on an SPE column, as the pKa is present in an extreme range (pKa > 14). Due to these characteristics, typical SPE phases that may be used for the extraction of H and NH consist of reversed phase nonpolar bonded phases that are strong to intermediate in hydrophobicity. Some examples of the typical solid phases that exemplify these characteristics include octadecylsilane (C_{18}), octylsiloxane (C_8), cyclohexyl and phenyl. Due to the ionizable secondary nitrogen, strong cation exchange columns may be used for the SPE method. The physicochemical characteristics of H and NH render retention mechanisms comprising of hydrophobic van der Waals forces and π-π interactions.

Since several sample preparation steps are required, especially in the case of SPE, quantification requires the application of an internal standard. Deuterated norharman or harman would be most suitable internal standards for mass spectrometry analysis but are not commercially available. In biological samples such as plasma or tissue where lower levels (pg/ml) are detected, the fluorescent synthetic 1-ethyl-9H-pyrido[3,4-b]indole or 1-propyl-9H-pyrido[3,4-b]indole have been used successfully as internal standards in HPLC analyses with fluorescence detection (Pfau and Skog, 2004). The synthesis of these compounds is quite laborious and requires extensive spectral and

structural confirmation prior to appropriate use. Therefore, an appropriate internal standard was explored for the use in H and NH quantification in plasma.

For HPLC-FD the internal standard is generally a different compound from the analyte, but is well resolved in the HPLC separation (Snyder et al., 1997). The main purpose of the internal standard is to compensate for changes in sample size and recovery from SPE. When added prior to sample preparation, a properly chosen internal standard can be used to correct for sample losses. These sample losses may be due to variability associated between extraction columns of similar column chemistry and configuration. The internal standard should be chosen, in part, to mimic the physicochemical characteristics of the analyte in the pretreatment steps (Snyder et al., 1997). Therefore, requirements for the choice of an appropriate internal standard include: (1) adequate resolution in the HPLC separation from the other analytes, whilst maintaining a similar retention (capacity factor) behavior; (2) should not be in the original sample or be a precursor of the endogenous analytes; (3) should mimic the analyte in any of the sample pretreatment steps; (4) should be commercially available in high purity; and (5) should be stable and unreactive with components of the matrix or mobile phase. These necessities were evaluated in conjunction with the calibration. The calibration plot incorporates the ratio of the peak area of the analyte (H or NH) to the internal standard peak area plotted vs. the concentration of H or NH. Calibration plots are generally assessed for reproducibility and its capacity to be characterized by a simple regression model.

The following experiments were performed to appraise an appropriate SPE method for the extraction of H and NH from human plasma. Moreover, the exploration of a suitable internal standard for the extraction method was performed. The methodology for assessment of the appropriateness of an SPE method and internal standard included the following:

1. An internal standard that possessed similar physicochemical characteristics to H and NH was sought through reported literature. The physicochemical characteristics evaluated included lipophilicity, solubility, and the ability to give an adequate fluorescence signal.
2. Chromatographic resolution of both H and NH along with the internal standard was explored by using the aforementioned HPLC parameters (see table 3-8). The criteria for the acceptance of chromatographic parameters included resolution of H and NH along with the internal standard, peak shape and favorable capacity factors for all compounds.
3. SPE extraction was initially evaluated for the H and NH analytes in a neat buffered solution in order to obtain optimal conditioning, loading, washing and eluting conditions for the highest and most reproducible absolute recoveries.
4. SPE conditions were further evaluated using the internal standard along with both analytes to obtain optimal absolute and relative recoveries and reproducibility.
5. The SPE methodology obtained from neat buffered solution was subsequently used for the extraction method from human plasma. Further exploration of additional

extraction steps (i.e., protein precipitation) were evaluated in the event that further sample clean up was required.

6. Calibration curves were constructed in neat solution and plasma to evaluate the amount of plasma required to quantify a low amount (~5 pg/ml), concentration dependency of the extraction recovery, and to assess the concentration range to be used for calibration curves.

The optimized SPE extraction method was to be carried forth for method validation purposes.

3.3.3a Materials and Reagents

All chemicals were or analytical grade quality and obtained from commercial sources. Solvents used for the chromatographic optimization measurements were of spectroscopic or HPLC grade and used after filtration with a 0.2 μm porosity filter (Corning, Corning, NY). In addition to all reagents used for the aforementioned optimized investigations, the extraction experiments also incorporated the following materials and reagents.

1. Yohimbine HCl, 98% (Sigma-Aldrich Corp., St. Louis, MO)
2. Potassium phosphate buffer (for buffers at pH 7.0)

   - monobasic potassium phosphate, KH_2PO_4 (Sigma-Aldrich Corp, St. Louis, MO)

   - dibasic potassium phosphate, K_2HPO_4 (Sigma-Aldrich Corp., St. Louis, MO)
3. Borate buffer (for buffers pH 8 and 9)

- Sodium tetraborate decahydrate, $Na_2B_4O_7$*10 H_2O (Sigma-Aldrich Corp., St. Louis, MO)

- Boric acid, H_3BO_4 (Sigma-Aldrich Corp., St. Louis, MO)

4. Acetonitrile, HPLC grade (VWR, West Chester, PA)
5. Methanol, HPLC grade (VWR, West Chester, PA)
6. Perchloric acid, 70% $HClO_4$, double distilled (GFS Chemicals, Columbus, OH)

3.3.3b Equipment

1. Solid Phase Extraction (SPE) cartridges - Mixed mode (C_{18}-cation exchange) and lipophilic sorbent beds (C_2, C_8, C_{18} and phenyl), 100 mg bed mass, 1.5 ml volume (Alltech, Deerfield, IL)
2. Centrifuge – Eppendorf 5804R, Benchtop Temperature Controlled (Westbury, NY)
3. SPE manifold – 24-port, with stopcocks (Alltech, Deerfield, IL)
4. Vacuum for SPE manifold (KMF Neuberger Vacuums, Trenton, NJ)
5. Borosilicate glass test tubes, 15mm x 125mm, 16 ml volume (VWR, West Chester, PA)
6. Polypropylene tubes, 15 ml volume (VWR, West Chester, PA)

3.3.3c Preparation of Solutions and Standards

1. Harman and Norharman in methanol – combination standards were made for both analytes. A stock solution of 1 µg/µl (1 mg/ml) was made for H and NH. Approximately 3 mg for both H and NH was added to a silanized, 4-ml amber vial in

which an appropriate volume (~3 ml) was added to dilute to a desired concentration, ensuring to account for salt weight and purity. Serial dilutions of 50 ng/µl and 500 pg/µl were made from the stock solution to create smaller "stock solution" concentrations. All concentrations used for the experiments were diluted, with methanol from these three stock solutions.

2. Yohimbine in methanol - A stock solution of 1 µg/µl (1 mg/ml) was made. Approximately 2 mg for both H and NH was added to a silanized, 4-ml amber vial in which an appropriate volume (~2 ml) was added to dilute to a desired concentration, ensuring to account for salt weight and purity. Serial dilutions of 100 ng/µl and 1 ng/µl were made from the stock solution to create smaller "stock solution" concentrations. All concentrations used for the experiments were diluted, with methanol from these three stock solutions.

3. Protein precipitation solutions – all subsequently kept at 4˚C until use.

a) Acetonitrile:Methanol – approximately a 1:1 ratio of both organic solvents

b) 1 M $HClO_4$ – for 100 ml, 8.54 ml of a 70% $HClO_4$ solution (11.6M) was added to a volumetric flask and qs to 100 ml with Milli-Q® water.

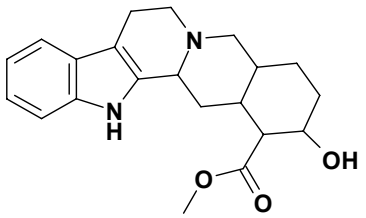
c) 1 M $HClO_4$: Acetonitrile – approximately a 75:25 ratio of acid to organic solvent.

3.3.3d Methods

A literature review was conducted in order to find a suitable internal standard for H and NH quantification. Desired characteristics for the internal standard included

similar physicochemical properties, structural similarity, and solubility as compared to H and NH. Although an internal standard need not be structurally similar to the analytes, the structural attributes of the internal standard was important for the fluorescence detection and chromatographic behavior seen with H and NH. Internal standards for use in the extraction of β-carbolines from food sources included sotalol, ibogaine, harmaline, and harmine. With the exception of sotalol, the reported internal standards possess a similar planar β-carboline scaffold. Harmaline and harmine were avoided as internal standards as they may be present in human plasma and/or may serve as precursors of the H and NH analytes. The fluorescence qualities of ibogaine is not well understood but is suspected to be sufficient in its quantum yield. However, ibogaine could not be purchased for the experimentation due to its classification as a Schedule I hallucinogen (Sigma-Aldrich, 1997).

Yohimbine (17-hydroxyyohimban-16-carboxylic acid methyl ester) was chosen as an initial internal standard as the physicochemical and structural characteristics are similar to those of the β-carboline analogues. Yohimbine has been successfully quantified in human plasma via HPLC with fluorescence detection (Owen et al., 1985) in sub-nanogram levels. Most importantly, this compound is not found endogenously in humans and is not suspected to be a metabolic precursor of the analytes of interest. The strong fluorescent characteristics are similar to those of H and NH, with yohimbine possessing a relatively large quantum yield (Gurkan, 1974). The structure and physicochemical characteristics of yohimbine are shown in the figure below.



MW* (g/mol)	390.9
pKa	8.44, 14.39
Log D	2.4
Solubility*	slightly soluble in H_2O (10 mg/ml), sparingly soluble in dilute acids, soluble in ethanol, methanol

Figure 3-9: Structure and physicochemical characteristics of yohimbine (calculated using Advanced Chemistry Development (ACD/Labs) Software V 8.14 for Solaris, © 1994-2007, ACD/Labs; *(Sigma-Aldrich, 2002)

Similar to the H and NH compounds, yohimbine has two ionizable nitrogen functional groups and a planar, conjugated ring structure. Both of which can prove beneficial for the optimization of chromatographic and SPE experiments.

Chromatographic experiments were performed to obtain most favorable resolution of the analytes and the proposed internal standard yohimbine (YOH). Optimization for H, NH and the YOH separation were made in neat solution, prior to SPE exploration. Initial chromatographic conditions were based on those obtained from prior optimization of H and NH separation (see table 3-8). The percent of organic phase was varied from 15-35% methanol or acetonitrile. If needed, pH of the aqueous phase was altered between 3.0-3.5 to optimize the peak shape of all analytes and YOH. Within this pH range, YOH is suspected to be ionized at the pyridine nitrogen site of the structure, similar to the other β-carboline analogues. The influence of mobile phase flow rate was investigated to obtain ideal capacity factors and

resolution. The fluorescence detection parameters for YOH was taken from reported results: $\lambda_{excitation}$= 280 nm, $\lambda_{emission}$ = 360 nm (Owen et al., 1985). Of note, YOH could not be detected using the excitation and emission wavelengths needed for H and NH detection.

In the case of H and NH, a range of expected physiological concentrations were tested for quantification and linearity in the presence of the internal standard. Calibration curves were constructed within expected physiological ranges (5 pg/ml – 2.0 ng/ml for both H and NH) to determine linearity and the least quantifiable amount on column. To evaluate the suitability of YOH as an appropriate IS, similar concentrations were used for assessment of linearity and to determine an ideal concentration needed for the internal standard.

Initial exploration of a potential SPE method was performed in water at a concentration of 1 ng/ml for both analytes and IS. Mixed mode (C_{18}-cation exchange) and lipophilic sorbent beds (C_2, C_8, C_{18} and phenyl), with 100 mg bed mass and 1.5 ml volume (Alltech ®) were assessed for retention capacity of H, NH and YOH. Loading, washing, and elution solvents, with pH adjustments were investigated for adequate and reproducible recovery.

For the lipophilic sorbent beds, loading solvents included unbuffered and buffered aqueous solutions at least 2 pH units above the pKa of H and NH. At pH > 7 both analytes are expected to be in its neutral state, thereby increasing the retention onto the lipophilic sorbent bed. Buffering solutions included pH 7 phosphate buffer (20 mM) and pH 8 and 9 $H_3BO_4/Na_2B_4O_7$ buffer (20 mM). Analyte retention onto the SPE

column was assessed by evaluation of breakthrough upon loading. Ideal conditions were regarded as possessing minimal to no analyte breakthrough with maximum retention of the analytes. Washing solvents included water, buffered solutions (same as above), and a range of aqueous mixtures with organic solvent (5-30% methanol). The washing solvent with the highest percent organic, with minimal analyte breakthrough, was subsequently employed for the plasma analysis for the eluting of potential interfering substances. Elution solvents included 100% organic solutions of methanol and/or acetonitrile that was or was not acidified with 0.5% acetic acid. The pH adjustment of the elution solvent was explored to ensure the ionization of the analytes to, in turn, release the analytes from the SPE sorbent bed. Elution volumes ranging between 1 and 3-ml were explored with the acceptance criteria being the lowest volume of elution solvent that yielded the highest recovery. If needed, concentration of the elution solvent was performed via N_2 in order to achieve an adequate signal for HPLC analysis. Upon dry-down, the residue was dissolved in approximately 100 µl of mobile phase. This step required the use of a control to assess for sample loss due to the N_2 (dried down and reconstituted vs. neat solution in reconstitution solvent). For each extraction condition, experiments were performed in triplicate. The SPE vacuum flow was not to exceed 1 ml/min to minimize analyte breakthrough.

Absolute recovery (extracted vs. unextracted area under the peak) was evaluated for each H and NH at 50 pg/ml, 500 pg/ml, 1.0 ng/ml and 2.0 ng/ml. Moreover, similar concentrations were used to assess absolute recovery for YOH. Ideal conditions yielded maximum and most reproducible recovery for all analytes and internal standard. In

addition, relative recovery (analyte to internal standard peak area ratio for extracted vs. unextracted) was assessed to compare the relative retention behavior of H and NH to YOH. The SPE conditions that yielded the most reproducible results were used for assessment of H, NH, and YOH extraction recovery from pooled plasma. Recovery experiments were performed in triplicate.

The ideal chromatographic parameters and SPE conditions were consequently used for evaluation of H and NH extraction from plasma. Pooled human plasma (EDTA anticoagulant, non-smoking, drug-free, pooled from n = 20 males and females) was utilized as the matrix for SPE recovery exploration. Optimization was performed using a 1.0 ng/ml concentration of both H and NH with 1 ng of YOH internal standard. The concentrations used for H and NH recovery assessment included 10 pg/ml, 50 pg/ml, 500 pg/ml, 1.0 ng/ml and 2.0 ng/ml. Moreover, "blank" plasma (unspiked) and a "zero" concentration level (spiked with 1 ng YOH internal standard) to evaluate the chromatographic interference of the internal standard and assess if an endogenous H and NH signal is present, respectively. No more than 100 µl of standard solution was spiked into the plasma, ensuring that > 90% of the total volume was plasma matrix. The amount of pooled plasma that was used for the assessment was 1-ml. In the case that the required sensitivity (~5 pg/ml) was not obtained larger volumes were used (up to 3-ml). Larger volumes of plasma were not used due to the clinical sampling volume constraint and practicality of using a 100 mg sorbent bed for SPE (volumes greater than 3-ml may cause decreased capacity of retention for the SPE sorbent bed).

In the event that further sample clean-up be required, protein precipitation techniques were evaluated as a pretreatment step prior to the optimized SPE method. The viscosity and sample size of the plasma was thought to have a functional hindrance on the loading of the sample, with the viscous sample not being able to flow through the SPE tube at a given flow rate of 1 ml/min. For 1-ml of pooled plasma, protein precipitation solutions included 1-ml cold acetonitrile, 1-ml cold acetonitrile:methanol (50:50), 1-ml cold $HClO_4$ and 1-ml cold $HClO_4$:acetonitirile (75:25); each of which have shown to be effective in the extraction of highly lipophilic compounds (Souverain et al., 2004). All solutions were kept in 0 ºC prior to use. For each experiment, pooled plasma spiked with 1.0 ng of each analyte and internal standard was subjected to protein precipitation, with a 1:1 mixture of plasma to protein precipitation solution. The mixture was vortexed for 2 minutes and refrigerated between 10 ºC for 10 minutes. The sample was later centrifuged at 2300 rpm for 10 minutes at 4 °C. The supernatant was pH adjusted to pH = 9 with 100 µl of 1 N NaOH and $H_3BO_4/Na_2B_4O_7$ buffer (pH = 9.0, 20 mM) and subjected to the SPE cartridge.

A comparison was made to the plasma sample with and without protein precipitation. The plasma samples evaluated without protein precipitation were treated with a buffer dilution (4:1 buffer to plasma) to ensure proper flow rates and minimize viscosity effects through the SPE column. The final SPE and protein precipitation extraction method was to be used for subsequent method validation experiments.

3.3.3e Results

The proposed YOH internal standard was chromatographically resolved from the H and NH analytes using the following conditions:

Table 3-9: Experimental conditions for RP-HPLC Separation of Ionized H, NH and the internal standard, YOH, in neat solution.

Separation variable	Optimized condition
Column	Agilent® Zorbax Eclipse XDB, C_8 15 x 0.46 cm, 5 μm, endcapped
Solvents A and B	
% B	35% methanol
Buffer (compound, pH, concentration)	KH_2PO_4/H_3PO_4 buffer (pH=3.2, 25mM)
Flow rate	Isocratic, 0.95 ml/min
Column temperature	40ºC
Injection volume	50 μl
Run Time	11 minutes
Detection for H and NH	$\lambda_{excitation}$ = 300 nm, $\lambda_{emission}$ = 433 nm
YOH	$\lambda_{excitation}$ = 280 nm, $\lambda_{emission}$ = 360 nm

Slight modification of the flow rate and % organic composition from the initial RP-HPLC conditions yielded adequate resolution and peak shape of both H and NH (Rs >1.9) and the internal standard YOH. The capacity factor ($3 < k < 9$) and number of theoretical plates (> 4000) for all compounds were in the desired range. Peak shape (including asymmetry and tailing factor) was slightly compromised, but yielded sufficient results for quantification purposes. The HPLC chromatogram for the

separation of NH, H and the proposed internal standard, YOH is shown in the figure below.

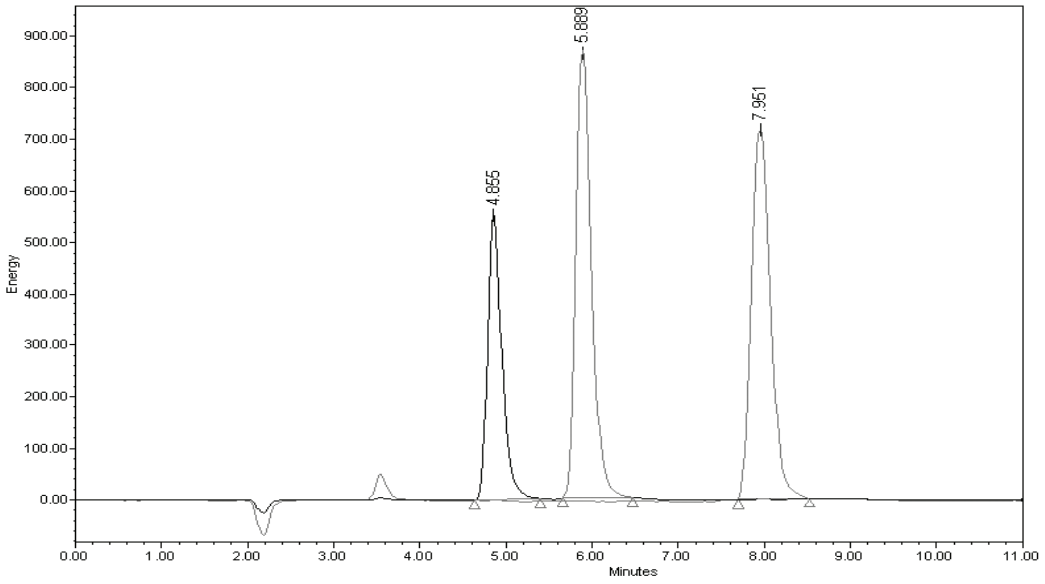


Figure 3-10: Sample chromatogram of spiked NH and H (500 pg/ml each), with YOH as internal standard (1 ng/ml) in neat solution. Retention times for NH, H, and YOH were at 4.86, 5.89, and 7.95 minutes, respectively.

Chromatographic response was linear throughout the concentration range through ~2.5 ng/ml for H ($R^2 > 0.996$), NH ($R^2 > 0.997$) and YOH $R^2 > 0.998$). The minimum amount quantified on column (as deemed by the limit of detection) for H, NH and YOH was 1.6 pg, 2.1 pg, and 53 pg, respectively. The optimized RP-HPLC conditions were used for the evaluation of a suitable SPE extraction methodology.

Lipophilic sorbent beds including C_8, C_{18}, and phenyl, yielded high (>80%) and reproducible (%COV< 6%) absolute recoveries in buffered water matrix for all analytes and internal standard. The phenyl sorbent bed was initially chosen for subsequent

plasma analysis to increase the selectivity of the extraction. All solutions used for SPE from a neat matrix required optimization. The loading conditioning solution required buffering to maintain significant retention of all the compounds. In the case of the loading solution, 97±3% of H and NH was retained while 92±4% of YOH was retained upon loading of a pH = 9, $H_3BO_4/Na_2B_4O_7$ buffer (pH = 9.0, 20 mM) adjusted solution (n=3 extractions). The systematic decrease in pH of loading and conditioning solvents (from pH 8 to 7), decreased the retention of the compounds slightly but caused significant amount of variability in the retention capacity (%COV > 9% for all compounds).

With the specified pH for conditioning, the washing solvents included water and the $H_3BO_4/Na_2B_4O_7$ buffer (pH = 9). The amount of organic solvent for the washing step could not exceed 10% methanol without significant analyte breakthrough. A water washing step was employed prior to elution in order to minimize salt transfer into the elution solvent step. The water wash step, although not buffered, did not yield any significant analyte breakthrough. At least 2-ml of elution solvent was required to elute H, NH and YOH from the phenyl sorbent bed. The organic solutions that were capable of complete elution included 100% methanol and 100% acetonitrile. Acidification of the elution solvent was not necessary to elute the compounds from the sorbent bed. As consequence of the elution volume, a dry-down procedure was required to concentrate the analyte mixture prior to introduction to the HPLC. Concentration of the sample via N_2 did not lead to significant sample loss. Silanization of all glassware used in the extraction procedure was used as precautionary measures to ensure maximum and

recproducible recoveries. For a neat solution of H, NH and the internal standard YOH, the SPE method in depicted in figure 3-11 was employed.

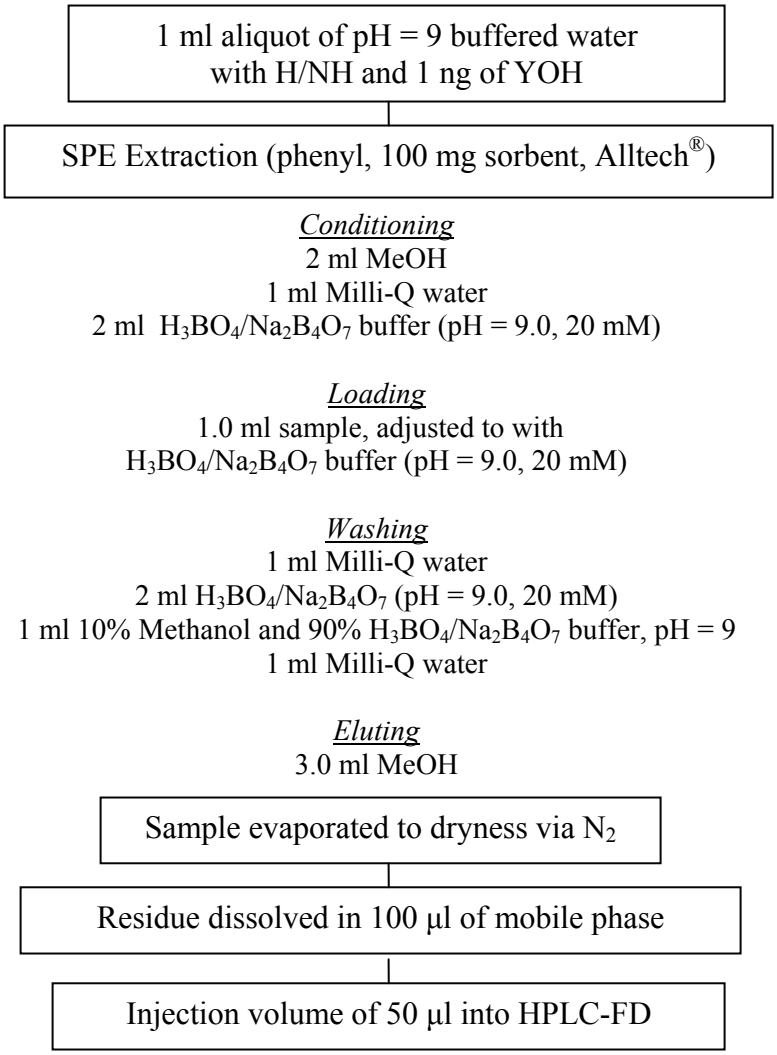


Figure 3-11: Optimized SPE method in a 1-ml neat buffered solution for H, NH, and the internal standard YOH.

The concentration-dependency for the extraction of H, NH, and YOH from a neat matrix was evaluated. Absolute recovery of both analytes and YOH at

concentrations of 50, 500, 1000, and 2000 pg/ml was > 94% with %COV < 4.3 % for each analyte, across the concentration range evaluated. The relative recovery for both H and NH at all concentrations was > 90% with %COV < 2.9 %.

Table 3-10: Absolute recovery from buffered water of norharman, harman and yohimbine following SPE (n=3 for each concentration). (% mean ± SD).

	50 pg	500 pg	1000 pg	2000 pg
Harman	95.6 ± 2.1	97.1 ± 0.5	96.4 ± 1.9	98.7 ± 5.2
Norharman	97.6 ± 4.3	98.6 ± 4.1	95.8 ± 1.2	93.4 ± 4.7
Yohimbine (IS)	94.4 ± 5.2	95.9 ± 1.2	95.2 ± 2.3	94.5 ± 3.6

The recovery experiments performed in the neat buffered matrix were also performed in the pooled plasma matrix. For plasma experiments, further sample clean up was needed for elution through the SPE cartridge. Protein precipitation was employed to ease the loading of the plasma sample and to remove potential additional interferences. Moreover, the use of this additional cleanup step would potential extend the lifetime of the HPLC column used for the analysis. The efficacy of the various precipitants in removing the protein from plasma sample and extracting the H and NH is shown in table 3-11. Using 1 ng/ml level of analyte concentration the protein precipitation optimization yielded highest and most consistent relative recovery using 1-ml cold $HClO_4$:acetonitirile (75:25). Moreover, this particular protein precipitation treatment yielded statistically similar results to the samples not subjected to protein

precipitation (harman: *p*-value = 0.2701 and norharman: *p*-value = 0.1685, via unpaired Student's t-test).

Table 3-11: Relative recovery from pooled plasma of norharman and harman (1 ng each) using different protein precipitation methods prior to SPE (n=3 for each concentration, % mean ± SD).

	None*	ACN	ACN:MeOH	$HClO_4$	$HClO_4$:ACN*
Harman	85.3 ± 2.1	73.6 ± 14.1	79.1 ± 9.8	80.3 ± 5.9	89.7 ± 3.2
Norharman	83.2 ± 1.9	8.2 ± 11.9	77.2 ± 8.1	5.8 ± 7.9	91.9 ± 2.7

(All solutions were used in a 1:1 ratio of protein precipitation solution to plasma. ACN: acetonitrile; MeOH: methanol, $HClO_4$: perchloric acid) *statistically similar via Student's t-test, *p*-value > 0.05

The concentration dependency of the extraction procedure was evaluated at five different concentrations (see table 3-12). The "blank" plasma (no spiked H, NH, or YOH) yielded a significant chromatographic signal for H and NH but not YOH, suggesting that there are no interferences in the pooled plasma for the YOH signal. The "zero" concentration level (spiked with only 1 ng YOH internal standard) resulted in a significant signal for H and NH, which is suspected that these are constitutive of the pooled plasma. Chromatograms for the "blank" and "zero" level are present in the validation section for this chapter.

Table 3-12: Concentration-dependency of relative recovery of harman and norharman from 2-ml of pooled plasma after protein precipitation and SPE (n=3 for each concentration, % mean ± SD).

Concentration (pg/ml)	Harman	Norharman
10	81.6 ± 2.3	80.9 ± 1.3
50	85.1 ± 2.5	82.5 ± 1.6
500	88.2 ± 3.9	91.4 ± 5.3
1000	89.1 ± 4.2	91.3 ± 3.1
2000	91.7 ± 6.9	93.6 ± 7.4

Throughout the concentration range, the relative recovery was > 80% suggesting that the method of extraction resulted in a relatively high recovery from pooled plasma. A slight concentration dependency on the extraction was observed with larger concentrations yielding higher recoveries. The extraction method throughout the concentration range was reproducible with % COV < 6% for both analytes across the concentrations. In is expected that the constitutive contribution of H and NH in pooled plasma would increase the relative recovery from samples without endogenous analyte.

The amount of plasma needed to attain an adequate lower limit of quantification was initially explored. For the experiments three volumes of "zero" concentration were evaluated, 1-ml, 2-ml, and 3-ml of pooled plasma. The 2-ml plasma volume yielded statistically higher signals (peak area) compared to that of the 1-ml volume (*p*-value > 0.182 for both analytes, via unpaired Student's t-test). In comparison with the 3-ml volume, peak areas of H and NH resulted in a less that proportional increase in signal, suggesting that these larger volumes affects the retention capacity of the SPE cartridge.

Moreover the variability in the extraction increased with the increase to the 3-ml volume. This evaluation of volume suggests that 2-ml of pooled plasma was optimal is obtaining an adequate signal from presumably, drug-free, tobacco smoke-free plasma. Further evaluation of the accuracy, precision, recovery and endogenous signals of H and NH will be presented in the following section on validation of the method.

3.3.3f Discussion

Prior to developing the extraction method and choosing an appropriate internal standard, proper characterization of the physical and chemical characteristics of H and NH was necessary. Factors such as the β-carbolines' polarity relative to the matrix, the presence of charged functional groups, and solubility, etc. was considered to assess how strongly the analyte would be retained by the packing bed. For the extraction of H and NH from human pooled plasma, YOH was successfully used as an internal standard. Possessing similar physicochemical characteristics (i.e., logD and pKa), a strong fluorophore, and similar retention characteristics on the lipophilic SPE sorbent beds, YOH proved to be suitable for H and NH extraction conditions. The chromatographic baseline resolution between H and NH was maintained with the addition of the well resolved YOH, maintaining a relatively short chromatographic run time (~11 minutes). From the original chromatographic conditions, the addition of YOH required only negligible changes in chromatographic parameters. The signal response for both H and NH analytes was not compromised, maintaining peak shape, reproducibility and

sensitivity, while yielding a linear response for the YOH internal standard throughout the concentration range evaluated.

The primary approach used for the extraction involved retaining H and NH on the packing bed while interferences were unretained or washed off of the sorbent bed prior to elution of the analytes. Packing beds of different selectivities were exploited to explore to maximize structural differences between the analytes and the sample interferences. The phenyl sorbent bed was chosen, in part, due to the high and reproducible recoveries seen in the neat buffered matrix. Moreover, the retention chemistry of the phenyl ring of the sorbent is hypothesized to interact with the phenyl rings associated with the H and NH analytes and the proposed internal standard, improving selectivity of the extraction method. In essence, the selectivity of the phenyl sorbent bed was expected to provide a unique, non-polar retention mechanism via π-π and hydrophobic interactions with the phenyl rings of the analytes and internal standard. This phase has been successfully utilized in the extraction of other aromatic hydrophobic compounds from biological fluids.

In addition to the SPE column configuration, thorough experimentation of suitable conditioning, wash and elution solvent was performed. For each step, attention was given to the solvent strength and pH relative to the packing material. A pH = 9 buffered conditioning step ($H_3BO_4/Na_2B_4O_7$ buffer: pH = 9.0, 20 mM) minimized the variability associated with the absolute and relative recovery of H and NH to YOH. The pKa's of all compounds tested for the extraction dictated the need for a buffered conditioning and loading step in order to control the ionization of the potentially

charged compounds. The wash solvents were used effectively and removed weakly retained interferences, but were not strong enough to elute the analytes and internal standard. A 10% methanol buffered solution proved successful in the removal of interferences while maintaining a high and reproducible recovery. The methanol elution solvent was strong enough to completely elute the analytes in a relatively small volume. Using the optimized extraction procedure provided high and reproducible absolute recoveries from the neat matrix, prompting its use in the plasma matrix.

A 1-ml plasma sample was insufficient to obtain a significant endogenous peak of both H and NH from pooled plasma (healthy, not drug-dependent subjects). Therefore, a 2-ml plasma sample was necessary to detect a significant chromatographic response. Extraction of plasma samples greater than 2-ml resulted in the functional issues of irreproducible SPE solvent flow and pressure fluctuations of the vacuum manifold. Dilution of the sample, albeit a solution, required relatively large volumes to be passed through the SPE cartridge, increasing the chances of analyte breakthrough and prolonging the time for analysis. The effects of the sample volume on the SPE extraction required an additional protein precipitation step to the SPE procedure in order to provide a cleaner extract and to minimize SPE time. A 1-ml cold $HClO_4$:acetonitirile (75:25) treatment provided a clean extract and reproducible recovery for subsequent HPLC analysis. The 1 M $HClO_4$ solution alone provided a superiorly clean extract but the addition of acetonitrile provided additional extraction efficiency. As all compounds of interest are highly lipophilic, the addition of a strong solvent like acetonitrile, would increase the extraction yield more so than the use of the aqueous $HClO_4$ solution.

Upon investigation of the concentration dependency of the extraction of H and NH from 2-ml of plasma, recoveries varied only slightly but were all above 80%. The endogenous contribution, in addition to the spiked H and NH, are expected to cause an increased absolute and relative recovery from sample not containing constitutive H and NH. Therefore, the relative recoveries obtained in the concentration dependency investigation are thought to be a product of endogenous and spiked H and NH. On a side note, the artifactual formation of the β-carbolines upon subjecting the biological matrix to an acidic environment may cause circulating precursors to condense with aldehydes (Tsuchiya et al., 1999). The evaluation of artifactual formation of H and NH from the extraction process will be assessed in the next section. For the most part, the recoveries obtained from the final extraction procedure are relatively high (>80%), and more importantly reproducible.

Extraction from the pooled plasma matrix resulted in no interfering substances for the internal standard, as deemed by a lack of a substantial signal, at the expected YOH retention time, from the blank sample (no H, NH or YOH spiked). Moreover, the addition of YOH to pooled plasma did not affect the signal for either H or NH, suggesting that YOH is not a precursor of the analytes of interest.

In summary, a combination protein precipitation-SPE extraction procedure was developed for the analysis of H and NH in 2-ml of human pooled plasma. An internal standard, YOH, was successfully employed for the extraction process. The optimized procedure was subsequently used for the validation of the complete method. The final extraction method is summarized in the figure below.

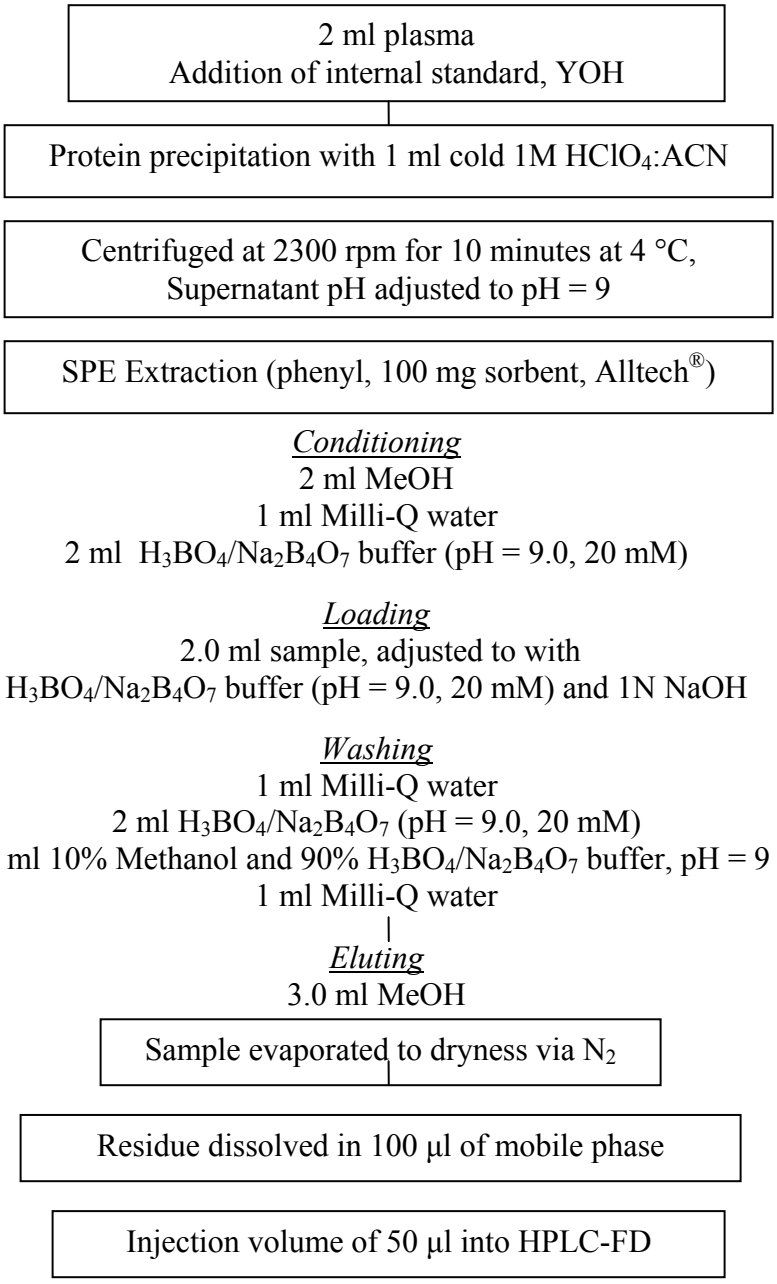


Figure 3-12: Optimized protein-precipitation/SPE method using 2-ml human plasma and YOH for H and NH quantification.

3.3.4 Validation of Assay Method

The general issue of analytical method validation has been discussed and documented in great detail (Karnes et al., 1991; FDA, 2001; Bansal and DeStefano, 2007; Viswanathan et al., 2007), with many official groups establishing guidelines and recommendations. However, these guidelines are generally not specific or only apply to certain applications. Preferred approaches for each phase of an assay validation includes the assessment of selectivity, sensitivity, accuracy, precision, reproducibility and precision (FDA, 2001). Other parameters of interests include those of extraction efficiency, calibration range, matrix effects, a dilution integrity, and response function (e.g., nonlinear or linear)(Bansal and DeStefano, 2007). As for any bioanalytical method, the extent to which an assay should be validated depends on the intended application of the method and, in turn, different validation parameters require different levels of scrutiny. Along with testing the acceptability of a method, the validation process challenges the bioanalytical method and determines allowed variability for the conditions needed to run the method.

Special consideration is required for the validation of assays quantifying constitutive components of a biological matrix. In the case for putative biomarkers such as H and NH, appropriate clinical and assay controls should be incorporated to produce unbiased clinical results. As most biomarkers are endogenous compounds with quantifiable baseline levels in the biological matrix of interest, the nature of biomarkers posts a challenge to find analyte-free biological matrix to prepare calibrator standards. As calibrators used for drug compound analysis, the biomarker assays require the

similar parameters (e.g., accuracy and precision) for validation of the assay method. One primary difference between biomarker assays and that of drug compounds is the preparation of calibrators not in the intended sample matrix, but in a "surrogate matrix". As a consequence of this divergence, validation is required to demonstrate that the analytical concentration-responses relationships are similar in the sample matrix and the surrogate matrix.

Of the reported methods used for the quantification of H and NH in biological matrices, the majority of the validation was performed in a neat buffered matrix, without the formal assessment of a matrix effect. The buffered solutions utilized for calibration curves are most likely not representative of calibrations used in biological matrix. It has been recommended that validation be performed and calibration curves/control samples be prepared in a matrix same as those being analyzed (Findlay and Das, 1998; Shah et al., 2000). As the background signal of constitutive H and NH poses an issue with calibration curve construction, a strategy limiting or completely eliminating the background needs to be investigated. There is no limitation to what can be an appropriate substitute matrix for standard preparation. Removal of the endogenous analyte or alterations of the biological matrix have proven successful form quantification purposes, but this results in a matrix that it is no longer representative of the test sample matrix. Therefore, if modification of the matrix is necessary, behavior (i.e., slope) of the calibration curves between the unadulterated and the altered matrix needs to be statistically similar to deem matrix surrogacy. A "matrix effect" should not

be present upon testing the calibration between the two matrices. Only when surrogacy is proven, quantification using the modified matrix may be performed.

Considering the issues of endogenous H and NH background and the lack of a true blank matrix, the following strategy was employed for the H and NH assay bioanalytical validation in human plasma:

1. A pooled plasma matrix was used for the initial exploration of validation, with the parameters such as precision, linear range, and selectivity being assessed. In this investigation the goal was to determine, in an unadulterated matrix, the precision of the bioanalytical assay throughout a given concentration range. Accuracy was expected to be confounded by the constitutive concentrations of H and NH and was not scrutinized during the pooled plasma analysis. Moreover, an exploration of a suitable concentration of internal standard was evaluated at this step. Short-term and long-term stability studies were conducted in the unadulterated pooled plasma.
2. Exploration of a suitable surrogate matrix included dilution of pooled plasma, to decrease the signal for the endogenous H and NH background signal. The pooled plasma matrix was modified until devoid of a significant H and NH chromatographic signal (<20% of LLOQ), while minimizing the difference of composition in the true matrix. Therefore, the goal was to obtain a minimal dilution factor, attempting to maintain a comparable matrix to the unadulterated plasma matrix. Subsequently, H and NH were spiked into the modified matrix to assess the validation parameters aforementioned. Full calibration curves (n = 6) and

quality control samples were used to evaluate the linear range, accuracy and precision. In addition, an AULOQ (Above Upper Limit of Quantification) dilutional control was evaluated for precision and accuracy. Of importance, the Lower Limit of Quantification (LLOQ) was determined during this step using n = 6 replicates. Sensitivity of the method was enhanced by increasing the sample volume, if needed. Recovery assessment was evaluated using this matrix to assess the relative recovery and, more importantly, the precision.

3. Upon calibration curve evaluation of both pooled plasma and modified plasma matrix, n = 6 individual, unmodified plasma sources were subsequently spiked with H and NH standards. Owing to the different constitutive concentrations of H and NH in the individual plasma sources, it was expected that the intercepts for each calibration regression would differ. Therefore, precision was the primary assessment in this portion of the investigation.
4. To appraise surrogacy of the modified plasma matrix, parallelism studies were performed. The slopes of all calibration curves from pooled plasma matrix, modified matrix and the n = 5 different sources were to be statistically similar (parallel) in order to deem the modified matrix and appropriate "surrogate" matrix. If statistically different, the method was considered to have a significant matrix effect and issues of selectivity. In the case that selectivity was of concern, chromatographic or extraction re-optimization experiments were conducted to rid the interference.

5. For all successive experiments, including ultimate sample analysis, the calibration curves and quality control samples were to be constructed using the "surrogate" plasma matrix.

To account for matrix effects associated with quantification, studies for the small molecule validation in a surrogate matrix requires the implementation of "parallelism" studies. In essence, the surrogate matrix should behave similarly to the sample matrix for intended use with regard to calibration. Parallelism (of concentration–response curves) is defined as the concentration– response curves of the test (surrogate) and standard (unmodified) being identical in shape and differ only in a constant horizontal difference (Singer et al., 2006). If the two curves are shown to be sufficiently similar (equivalent) in shape by its linearity and slope, and tested statistically for similarity, "surrogacy" of the modified matrix is verified. The parallelism studies were based on reported strategies for large-molecule validation (Miller et al., 2001). The design of the method validation procedures were based on previously published reports (Karnes et al., 1991; FDA, 2001; Lee et al., 2006)

3.3.4a Materials and Reagents

All chemicals were or analytical grade quality and obtained from commercial sources. All solvents used for the H and NH validation analysis were of spectroscopic or HPLC grade and used after filtration with a 0.2 μm porosity filter (Corning, Corning, NY). The validation required the following materials and reagents, in addition to those aforementioned:

1. Perchloric acid, 70% $HClO_4$, double distilled (GFS Chemicals, Columbus, OH)
2. Sodium Hydroxide, 10 N (VWR, Westchester, PA)
3. Ascorbic Acid, reagent grade crystalline, $C_6H_8O_6$, (Fluka, Buchs, Switzerland)
4. Semicarbazide HCl, purum >99% (Aldrich, St.Louis, MO)
5. Pooled human plasma, n = 20 donors – Healthy, fasting, drug-free, non-smoker, collected in EDTA (Biochemed Services, Winchester, VA)
6. Individual human plasma – six different donors, healthy, drug-free, non-smokers or smokers, collected in EDTA (from clinical study)

3.3.4b Equipment

The same equipment used for extraction and optimization procedures were used for subsequent validation.

3.3.4c Preparation of Solutions and Standards

1. Harman and Norharman in methanol – combination standards were made for both analytes. A stock solution of 1 µg/µl (1 mg/ml) was made for H and NH. Serial dilutions of 50 ng/µl and 500 pg/µl were made from the stock solution to create smaller "stock solution" concentrations. All concentrations used for the experiments were diluted, with methanol from these three stock solutions. In order to minimize error of volumetric transfer of the H and NH standard solution to the plasma matrices, stock calibrators were made to ensure that an appropriate mass of each concentration level was transferred with 10 µl of volume. All standards were made fresh and checked via

HPLC-FD prior to each and any experiment to confirm concentrations did not deviate from batch to batch.

Approximately 3 mg of H and NH were weighed and subsequently placed in a 4 ml silanized amber vial. The appropriate volume of methanol was used as the solvent to ensure a 1 mg/ml (µg/µl) concentration of solution was made (e.g., 3.2 mg in 3.2 ml of methanol), accounting for salt and impurity of the powder form. The primary standard concentrations were 1 µg/µl, 50 ng/µl, and 500 pg/µl.

Table 3-12: Preparation of Primary H and NH standards at 1 µg/µl, 50 ng/µl, and 500 pg/µl levels.

Final STD conc	amount of STD	methanol
1 µg/µl H and NH	~3 mg	~ 3 ml
50 ng/µl H/NH	100 µl of H and NH (1 µg/µl)	1.8 ml
500 pg/µl H/NH	30 µl of H/NH mix (50 ng/µl)	2.97 ml

From the primary stock solutions, 1-ml volumes of calibrators and quality control standards were created to ensure that the mass required for each level was transferred into plasma matrix via a 10 µl volume. The preparation of all the concentration levels is presented in the table below.

Table 3-13: Preparation of calibrator and quality control standards. Standards were prepared at concentrations to ensure that a 10 µl volume was transferred into the matrix of interest.

STD	Final STD conc	amount of Primary STD	methanol
2.5 ng	250 pg/µl	5 µl (50 ng/µl)	995 µl
1.0 ng	100 pg/µl	200 µl (500 pg/µl)	800 µl
800 pg	80.0 pg/µl	160 µl (500 pg/µl)	840 µl
625 pg	62.5 pg/µl	125 µl (500 pg/µl)	775 µl
500 pg	50.0 pg/µl	100 µl (500 pg/µl)	900 µl
400 pg	40.0 pg/µl	80 µl (500 pg/µl)	920 µl
250 pg	25.0 pg/µl	50 µl (500 pg/µl)	950 µl
100 pg	10.0 pg/µl	20 µl (500 pg/µl)	980 µl
50 pg	5.0 pg/µl	10 µl (500 pg/µl)	990 µl
25 pg	2.5 pg/µl	5 µl (500 pg/µl)	995 µl
12.5 pg	1.25 pg/µl	2.5 µl (500 pg/µl)	997.5 µl
6.25 pg (2ml)	0.625 pg/µl	2.5 µl (500 pg/µl)	1997.5 µl
3.12 pg	0.321 pg/µl	1.0 ml (0.625 pg/µl)	1000.0 µl

All standards were checked chromatographically, with triplicate injection, to ensure stability of stocks and reproducibility of construction. Solution stability, of all compounds was assessed at room temperature and storage temperature (-20˚C). H and NH solutions were stable for > 24 hours at room temp and > 1 month in storage temperature.

2. Yohimbine in methanol - A stock solution of 1 µg/µl (1 mg/ml) was made for YOH. Approximately 2 mg for both H and NH was added to a silanized, 4-ml amber vial in which an appropriate volume (~2 ml) was added to dilute to a desired concentration,

ensuring to account for salt weight and purity. Serial dilutions of 100 ng/µl and 1 ng/µl were made from the stock solution to create smaller "stock solution" concentrations. All concentrations used for the experiments were diluted, with methanol from these three stock solutions. YOH solution was stable for > 24 hours at room temp and > 1 month in storage temperature (-20˚C).

3. Protein precipitation/antioxidant solution – A 1 M $HClO_4$: acetonitrile solution (75:25) was made by making a 1 M $HClO_4$ solution and adding appropriate volume of acetonitrile to make the appropriate ratio. For the protein precipitation solution, an antioxidant (ascorbic acid) and aldehyde trapping agent (semicarbazide) was added. Per 750 ml of $HClO_4$ in the protein precipitation solution, 112.5 mg of ascorbic acid and 45 mg of semicarbazide was added. The final solution was subsequently kept at 4˚C until use.

4. Mobile phase (aqueous) – For 4 L of buffer a 90% K_2HPO_4/H_3PO_4: 10% methanol solution was made. Methanol was added to the aqueous phase to hinder bacterial growth in the buffer. Monobasic K_2HPO_4 (11.02 g) was added to 3.6 L of Milli-Q® water along with 621 µl of H_3PO_4 as the acidic component to make a pH=3.2, 25 mM solution. The pH was recorded to ensure the required buffering before and after adding 400 ml of methanol.

5. Extraction buffer – A 1.0 L solution of $H_3BO_4/Na_2B_4O_7$ buffer (pH = 9, 10 mM) was made for the pH adjustment, conditioning and was solvent. Approximately 0.316 g of H_3BO_4 and 1.854 g of $Na_2B_4O_7$*10 H_2O was added to 1.0 L of Milli-Q® water and

subsequently measured for pH. A 500 ml wash solution of 75% $H_3BO_4/Na_2B_4O_7$ buffer 25% methanol was made from this solution.

3.3.4d Methods

Validation of the bioanalytical assay was performed using optimized ideal extraction conditions (see figure 3-12). The HPLC-FD parameters were modified slightly because of selectivity issues approached in the later validation stages of the assay. Therefore, all subsequent experiments and results were obtained with parameters denoted in table 3-14.

Table 3-14: Experimental conditions used for validation of the RP-HPLC bioanalytical assay for H and NH in a human plasma matrix.

Separation variable	Optimized condition
Column w/guard column	Agilent® Zorbax Eclipse XDB, C_8 15 x 0.46 cm, 5 μm, endcapped
Solvents A,B, and C	
%A	80% KH_2PO_4/H_3PO_4 (pH=3.2, 25mM)
%B	15% acetonitrile
%C	5% methanol
Flow rate	Isocratic, 0.9 ml/min
Column temperature	40ºC
Sample temperature	4ºC
Injection volume	75 μl
Detection for H and NH	$\lambda_{excitation}$ = 300 nm, $\lambda_{emission}$ = 433 nm
for YOH	$\lambda_{excitation}$ = 280 nm, $\lambda_{emission}$ = 360 nm

Another major distinction from the previous experimentation incorporated the use of an antioxidant/aldehyde trapping agent. Several investigators noted a major concern in the quantitative analysis of β-carboline alkaloids is the suppression of artifactual formation during sample preparation (Allen and Holmstedt, 1980; Bosin and Faull, 1988; Tsuchiya et al., 1999). The successful use of semicarbazide as an aldehyde trapping reagent and ascorbic acid as an antioxidant in decreasing artifactual formation has been exemplified (Rommelspacher et al., 1984; Adell and Myers, 1994; Fekkes et al., 2004). Therefore, a solution of semicarbazide (4.5 mmol), ascorbic acid (0.5 nmol), and EDTA (0.05 nmol) per 1.0 ml of 1M $HC1O_4$ was implemented in the extraction of H and NH from human plasma to circumvent any issues pertaining to artifactual formation.

Before formal matrix experiments, stock solution stability for H, NH and YOH, post preparative (extracted samples/autosampler tray) and benchtop stability experiments were performed. Stock solution and benchtop stability for both analytes and internal standard at two different concentrations was assessed for 6 hours at room temperature.

3.3.4d-1 Pooled plasma experiments

Using pooled plasma (from presumably healthy, fasting, drug-free, non-smokers, collected in EDTA) initial partial validation experiments were explored to evaluate the linear concentration range for H and NH, a concentration of the internal standard, YOH, needed to produce a representative response for calibration purposes,

precision of the method and stability. Parameters such as accuracy and LLOQ were not scrutinized during this initial exploration due to the probable significant confounding effects of endogenous H and NH in the pooled plasma.

The linearity of the developed method was evaluated by preparing n=2 standard curves for the two analytes in duplicate (2 x 1 day) with varying (n = 5) concentrations of YOH. The concentrations of both H and NH used for a nine-point standard curve were 3.2, 6.3, 12.5, 25, 50, 100, 250, 500, 1000, and 2500 pg/ml. YOH internal standard amounts evaluated included: 100 pg, 300 pg, 500 pg, 750 pg and 1000 pg, for each calibration curve. Moreover, along with at least 9 non-zero standards, a blank sample (matrix processed without internal standard) and a zero sample (matrix processed with internal standard) were processed. A 10 µl volume of standard calibrator solutions were used at each concentration level to ensure that the volume of the stock solution added was <10% of the total matrix volume. Initial experiments were performed using 1-ml of human pooled plasma. In the case that adequate sensitivity was not obtained, 2-ml of pooled plasma was used. Plots of peak area ratio (H or NH / YOH) against analyte concentration were constructed. Plots of the residual against concentration assessed the behavior of the response variance across the calibration range. If the residuals for the linear regression analysis were heteroschedastic in nature, an appropriate weighting factor was investigated ($1/x$ and $1/x^2$).

Initially, the linear range was approximately based on an observable chromatographic response (peak) for both H and NH between the concentration ranges, until formally tested. In the case that higher concentrations distorted the peak signal,

the upper limit was deemed to be the highest standard that did not distort the peak shape, in order to have a reliable estimate of the peak area response. In the case that the upper limit was lower than that reported in physiological levels, an AULOQ (above upper limit of quantification) dilutional control was used in subsequent calibration curves. The calibration function (e.g., linear or weighted-linear) was established through observation of reverse calculated standard concentrations of which were reverse predicted from the curve. The appropriateness of the concentration of YOH was based on the peak height of the YOH being approximately at the mean of H and NH response of the concentration range tested. Moreover, the analyte to internal standard ratio at the upper and lower limits were not to exceed 10 or be less than 0.1, respectively, in order to minimize error associated at the extremes of the linear calibration curve.

The optimized internal standard and linear range were subsequently tested in the pooled plasma with n = 6 replicates (2 calibration curves x 3 days) to assess precision of the method. Moreover, quality control samples were created at the following levels:

- Low QC is ≤ 3 X LLOQ
- Medium QC approximately the geometric mean between low and high QC.
- High QC between 75% to 90% of highest calibrator.
- AULQC (above upper limit quality control 2.5 x highest calibrator and diluted.

The LQC, MQC, and HQC concentrations were used to assess relative recovery from the pooled plasma. The comparison of an extracted to unextracted analyte to

internal standard ratio, at each concentration, was used to calculate the relative recovery. Experiments were performed in triplicate.

It was expected that a systematic bias (positive y-intercept) would be present in the calibration curve because of the presence of endogenous H and NH. Therefore, accuracy and precision calculations were performed on the analyte to internal standard ratio, across the concentration range. For accuracy assessment, the predicted area ratio was based on the linear regression parameters, where the predicted area ratio is:

Predicted area ratio = (slope of calibration curve x conc.) + y-intercept

Accuracy and precision, for each analyte to internal standard ratio, were calculated as follows:

Accuracy (%DFN – deviation from nominal)

%DFN = {observed ratio – predicted ratio} / predicted ratio x 100%

Precision (%COV – coefficient of variation)

%COV = {standard deviation of the observed ratio / average ratio} x 100%

Within-run and between-run precision and practical LLOQ was assessed in the later stages of validation. The basis for accepting the precision was that >75% of all standards must be within ±15 % COV, with the exception of the LLOQ where a ±20 % COV, would be permitted.

For long-term stability, standard solutions using a high, low, and zero (unspiked) control concentrations in biological matrix were portioned and stored under the conditions of study sample storage (-80˚C). Measurements were taken in triplicate over

a six-month period on four separate occasions. For each sample, the ratio of the analyte to internal standard was recorded and concentration back-calculated and compared between measurements and across occasions for statistical deviations (accuracy and precision within ±15 % nominal value). Freeze-thaw stability was assessed over three cycles, in which the initial freeze was 24 hours and subsequent cycles was held in -80˚C for 12 hours. A low and high concentration control (n=3) was used to assess deviations from expected (evaluation of precision).

Of primary importance, the linear regression parameters for the calibration curves, including slope and y-intercept, for the pooled plasma validation were assessed upon evaluating the estimates and corresponding 95% confidence interval (CI) along with the precision of the slope and y-intercepts (% COV). In the case that a significant peak was observed in the zero standard (with only IS spiked), the back calculated concentration from the regression slope with a fixed intercept of zero (corrected baseline) was compared to the positive y-intercept value obtained from the calibration regression (non-corrected). This was to determine if the calibration curve was able to reliably quantify the endogenous H and NH.

3.3.4d-2 Pooled plasma results

Initial exploration of a calibration range and an appropriate concentration of the internal standard were performed in pooled plasma. Using 1-ml of plasma, a linear concentration range between 12.5 pg/ml and 1.0 ng/ml was seen for both H and NH. The calibration levels detected less than 12.5 pg/ml, although detectable, did not show

adequate reproducibility in measurement (%COV> 17% for both H and NH). At the concentration of 2.5 ng/ml, chromatographic peak distortion was seen for both analytes (saturation of signal). Sensitivity for the calibration was subsequently improved using 2-ml of plasma. A mass of 300 pg of YOH yielded an analyte to internal standard ratio at the upper (1.0 ng/ml) and lower limit (12.5 pg/ml) of approximately < 11.5 and > 0.04, respectively for both H and NH. These ratios loosely met the criteria for minimizing error associated with the extremes of the linear calibration curve. Larger concentrations (>300 pg/ml) of the YOH internal standard yielded results that were not representative of the linear calibration range for both H and NH. According to the "blank" sample (no IS) measurements, there were no significant peaks associated with the retention time of the YOH, exemplifying selectivity of the YOH chromatography. Significant peaks at the representative H and NH retention times were present (data not shown). Using the optimal concentration range of H and NH (6.3, 25, 50, 100, 250, 500, 1000 pg per 2 ml plasma) and 300 pg of internal standard, n = 6 calibration curves were constructed in pooled plasma with quality control samples of 12.5 pg (LQC), 400 pg (MQC) and 750 pg (HQC), and 2.5 ng (AULQC, dilutional control). Area ratios of the calibration points and quality control samples with precision estimates (%COV) are presented in the following tables for NH (Table 3-15) and H (Table 3-16). Moreover, the individual linear regression parameters for each calibration curve for both NH and H are presented with a goodness of fit (R^2) metric for linearity. An average calibration curve is presented for both NH (Figure 3-13) and H (Figure 3-14). Of note, the calibration curve for both H and NH was constructed upon calculation of an average

response (across n=6 calibration levels). A linear regression, utilizing a $1/x^2$ weighting factor, was performed on the average response for each concentration level. The calibration levels all showed acceptable precision for NH and H with %COV less than 13.5% and 11.3%, respectively. The precision of the quality control samples ranged from 5.3% to 12.11% for NH while the H quality control precision resulted in a %COV ranged 3.5% to 9.9%. The dilutional control (2.5 ng/2ml) precision was acceptable for both analytes.

Table 3-15: Peak area ratio for norharman in pooled plasma (n=6), with precision assessment (%COV) and calibration curve parameters ($1/x^2$ weighting).

pg NH	calib #1	calib #2	calib #3	calib #4	calib #5	calib #6	average	sd	%COV
6.25	0.148	0.142	0.144	0.140	0.141	0.136	0.142	0.004	2.93
25	0.254	0.268	0.270	0.273	0.256	0.244	0.261	0.011	4.38
50	0.381	0.398	0.382	0.398	0.342	0.410	0.385	0.024	6.16
100	0.793	0.752	0.722	0.761	0.639	0.711	0.730	0.053	7.28
250	1.923	1.653	1.782	1.423	1.673	1.513	1.661	0.180	10.83
500	3.669	3.069	2.826	3.023	3.262	2.862	3.118	0.312	10.00
1000	5.946	7.446	6.562	5.562	5.110	6.610	6.206	0.838	13.50
12.5 (LQC)	0.177	0.196	0.186	0.176	0.177	0.169	0.180	0.010	5.28
400 (MQC)	2.421	2.525	2.112	2.852	2.110	2.228	2.375	0.287	12.11
800 (HQC)	5.532	4.913	5.022	4.571	4.639	4.433	4.852	0.399	8.22
2500 (AUL QC)	4.402	3.902	3.424	3.553	3.616	3.323	3.703	0.395	10.67
zero	0.078	0.071	0.059	0.082	0.071	0.065	0.071	0.008	11.76
slope	0.0068	0.0072	0.0065	0.0065	0.0068	0.0064	0.0067	0.0003	
y-int	0.0670	0.0713	0.0660	0.0780	0.0810	0.0691	0.0721	0.0061	
R2	0.9874	0.9910	0.9933	0.9982	0.9832	0.9971	0.9917	0.0057	

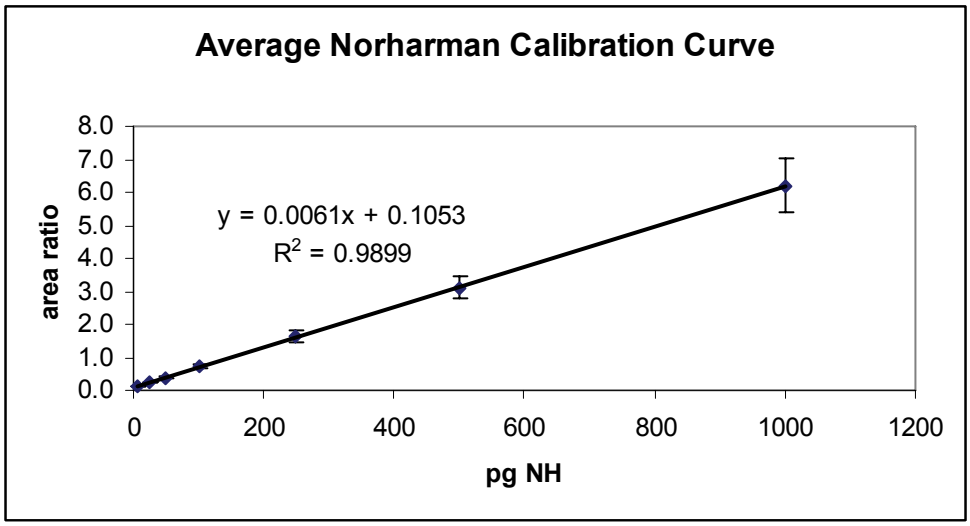


Figure 3-13: Average calibration curve (n=6) for Norharman in pooled plasma. Linear regression performed on the average of the response across concentrations. Each point represents the average response ± SD.

Table 3-16: Peak area ratio for harman in pooled plasma (n=6), with precision assessment (%COV) and calibration curve parameters ($1/x^2$ weighting).

pg H	calib #1	calib #2	calib #3	calib #4	calib #5	calib #6	average	sd	%COV
6.25	0.173	0.165	0.152	0.171	0.156	0.168	0.164	0.008	5.16
25	0.363	0.373	0.331	0.387	0.346	0.323	0.354	0.025	7.09
50	0.636	0.587	0.590	0.587	0.527	0.600	0.588	0.035	5.96
100	0.976	1.093	1.193	1.217	1.088	1.046	1.102	0.090	8.20
250	2.364	2.873	2.339	2.609	2.440	2.668	2.549	0.206	8.09
500	4.390	5.573	5.573	5.193	5.185	5.003	5.153	0.438	8.51
1000	9.620	11.371	10.939	12.098	9.628	9.092	10.458	1.184	11.32
12.5 (LQC)	0.228	0.236	0.223	0.245	0.226	0.230	0.231	0.008	3.45
400 (MQC)	3.539	3.833	3.863	4.032	4.154	4.377	3.966	0.290	7.30
800 (HQC)	7.688	8.532	8.213	9.328	7.102	7.541	8.067	0.797	9.88
2500 (AUL QC)	6.491	7.424	7.153	6.373	6.093	6.543	6.680	0.504	7.55
zero	0.065	0.061	0.059	0.078	0.071	0.065	0.067	0.007	10.60
slope	0.0094	0.0113	0.0109	0.0118	0.0096	0.0091	0.0104	0.0011	
y-int	0.0580	0.0524	0.0642	0.0688	0.0721	0.0830	0.0664	0.0108	
R2	0.9963	0.9877	0.9980	0.9938	0.9897	0.9974	0.9938	0.0043	

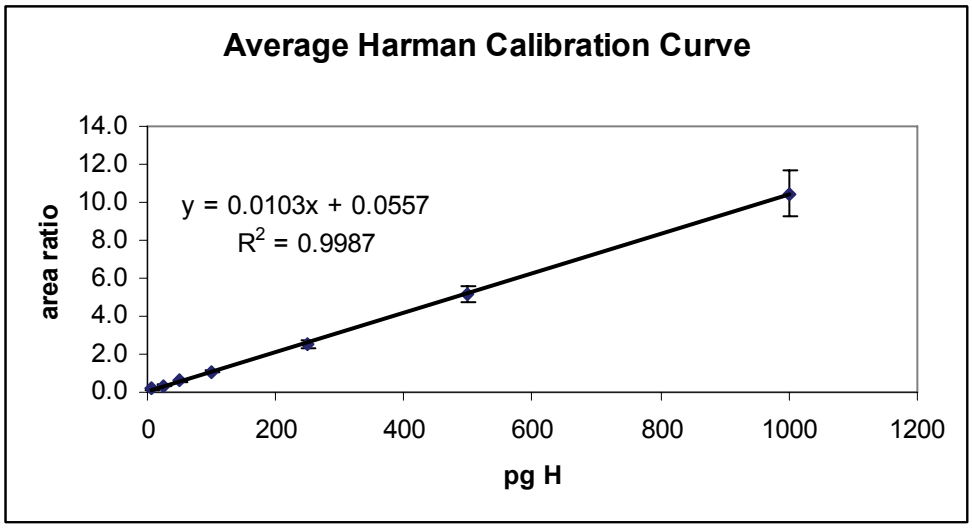


Figure 3-14: Average calibration curve (n=6) for Harman in pooled plasma. Linear regression performed on the average of the response across concentrations. Each point represents the average response ± SD.

The "zero" level, consisting of 2-ml plasma with only internal standard, yielded a significant response for both H and NH, suggestive of the significant constitutive nature of these analytes in the matrix. The peak area ratios for both analytes were reproducible, yielding %COV of 11.8% and 12.2% and for NH and H, respectively. With respect to accuracy of the measurements within the calibration range, back-calculated concentrations were compared to the linear-regression, corrected for the baseline. Throughout the concentration range, %DFN were less than 13.6% and 14.8% (absolute value) for NH and H, respectively.

Regarding the linear regression of the calibration curves, a weighting factor of $1/x^2$ was required to rid the unequal variance associated with the residuals (data not shown). Therefore, a weighted linear regression was performed on each individual

calibration curve for both analytes. Linearity of the calibration curves, as assessed by the coefficient of determination (R^2), was acceptable for both analytes with NH possessing R^2 ranging between 0.983 - 0.998 and H having a range between 0.987 - 0.998. For both analytes, the 95% CI for each individual calibration slopes included the estimate of the other individual calibration slopes, signifying similarity between the slope calibration parameter of each curve. Of major note, each calibration curve yielded a significant y-intercept for both analytes, indicating a significant background. The y-intercepts were significantly different from zero with NH having an intercept (mean ± SD) of 0.072 ± 0.006 (p-value > 0.34) and H having an intercept of 0.066 ± 0.011 (p-value > 0.17). Moreover, the y-intercepts obtained from the linear regressions for both analytes were reproducible.

In order to assess the precision and accuracy of the endogenous measured amount of H and NH in pooled plasma, a comparison of the "blank" sample was made to the y-intercept of the regression of its respective calibration construction. In addition, a concentration of the endogenous measurement of NH and H was calculated from the respective calibration curve, corrected for the significant y-intercept. Accuracy (%DFN) was calculated by evaluation of the 100% x (observed concentration – predicted concentration) / predicted concentration. In this investigation, the observed concentration for the "zero sample" was obtained from the respective calibration curve, correcting for the y-intercept (equaling zero). The predicted concentration was obtained from evaluation of the y-intercept of the calibration curve and back-calculating the concentration using a baseline-corrected calibration curve. A comparison was

made to assess if a difference in the values was seen and if the calibration curve was able to accurately quantify the endogenous H and NH within pooled plasma. The results from each calibration curve for each analyte is presented below.

Table 3-17: Accuracy and precision assessment for "zero" calibration level for norharman.

		predicted		*observed*		
calibration	slope	y-intercept	corrected conc	zero	corrected conc	%DFN
1	0.0068	0.067	9.9	0.078	11.5	16.4
2	0.0072	0.071	9.9	0.071	9.9	-0.4
3	0.0065	0.066	10.2	0.059	9.1	-10.6
4	0.0065	0.078	12.0	0.082	12.6	5.1
5	0.0068	0.081	11.9	0.071	10.4	-12.3
6	0.0064	0.069	10.8	0.065	10.2	-5.9
	avg		10.8		10.6	
	stdev		1.0		1.3	
	%COV		9.1		11.9	

Table 3-18: Accuracy and precision assessment for "zero" calibration level for harman.

		predicted		*observed*		
calibration	slope	y-intercept	corrected conc	zero	corrected conc	%DFN
1	0.0094	0.058	6.2	0.065	6.9	12.1
2	0.0113	0.052	4.6	0.061	5.4	16.4
3	0.0109	0.064	5.9	0.059	5.4	-8.1
4	0.0118	0.069	5.8	0.078	6.6	13.4
5	0.0096	0.072	7.5	0.071	7.4	-1.5
6	0.0091	0.083	9.1	0.065	7.1	-21.7
	avg		6.5		6.5	
	stdev		1.6		0.9	
	%COV		24.0		13.4	

This evaluation was to deem the calibration curve as useful for the quantification of H and NH in pooled plasma. According to the corrected calibration curves, the accuracy for the measurement was in a range of -12.3 and 16.4% for NH and 12.1 and -21.7% for H. The precision for the NH and H measurement was 11.9 and 13.4%, respectively. Upon statistical comparison (unpaired t-test, two-tailed assuming unequal variance), the concentration calculated from the y-intercept and the observed response ratio (from the "zero" calibration sample), was similar. No statistically significant difference was detected (p-values < 0.05) for both NH and H. According to the analysis, and using the baseline corrected calibration curves, the pooled plasma contained 10.6 pg of NH and 6.5 pg of H per 2-ml, which is within reported physiological range.

Long-term stability, in pooled plasma was evaluated at three different levels of High (500 pg/2ml), Low (25 pg/2ml) and zero (unspiked H and NH) over a six month period. Of note, the theoretical concentrations for the High and Low controls are those of spiked and constitutive, additive. The concentrations were measured, using baseline corrected calibration curves, at 15 days, 1, 3 and 6 months after standard pooled plasma preparation. At each time-point the analysis was performed in triplicate. Over a six-month period, there was a negligible variation in concentration for both H and NH, with most of the variability seen at the zero concentration level (unspiked H and NH; see table 3-19).

Table 3-19: Long-term stability assessment for "zero", Low and High level of Harman and Norharman in 2 ml pooled plasma over a 6-month period. Each value presented as mean of n=3 back calculated concentrations (pg/2ml)

Norharman	zero control	low control	high control
day 0	12.5	37.3	520.1
day 15	14.1	32.1	515.8
month 1	13.2	33.8	518.3
month 3	10.7	34.1	531.4
month 6	9.1	33.1	522.6
concentration (pg/2ml)	10.6	35.6	510.6
mean	11.9	34.1	521.6
stdev	2.0	2.0	6.0
%COV	16.9	5.7	1.1
%DFN	12.5	-4.3	2.2

Harman	zero control	low control	high control
day 0	7.2	31.2	518.1
day 15	5.2	35.4	522.1
month 1	6.4	32.1	537.5
month 3	7.7	32.5	518.4
month 6	7.1	35.6	527.9
concentration (pg/2ml)	6.5	31.5	506.5
mean	6.7	33.4	524.8
stdev	1.0	2.0	8.1
%COV	14.4	6.0	1.5
%DFN	3.4	5.9	3.6

Freeze-thaw stability assessment did not yield any significant changes in H or NH at all concentration levels evaluated through three-cycles, with %DFN and %COV not exceeding 6.7% for both analytes at all concentration levels.

The relative recovery, across the quality control concentrations, did not show much variation within and across concentrations (see table 3-20). Of note, the analyte to internal standard ratio obtained from the extraction procedure includes constitutive H and NH, contributing to the relative recovery. Therefore, the recoveries obtained from

the analysis are expected to be larger than if the endogenous analyte was not present in the matrix.

Table 3-20: Relative recovery from 2 ml pooled plasma of norharman and harman (n=3 for each concentration). (% mean ± SD).

	12.5 pg	400 pg	800 pg
Harman	87.2 ± 4.1	90.9 ± 1.4	91.6 ± 2.3
Norharman	85.2 ± 3.9	89.2 ± 3.5	90.3 ± 2.8

The information obtained from the pooled plasma analysis was used to design a more formal validation using a modified matrix for assessing surrogacy. Using 2-ml of matrix, the calibration range between 6.3 and 1000 pg of both H and NH with 300 pg of YOH internal standard was used for validation purposes. Quality control samples used for subsequent tests included 12.5 pg (LQC), 400 pg (MQC) and 750 pg (HQC), and 2500 pg (AULQC, dilutional control). The results of minimal deviation for long-term and freeze-thaw stability tests proved the chemical robustness of analyzing H and NH in the plasma matrix.

3.3.4d-3 Surrogate matrix experiments

Similar experiments to the aforementioned were performed using a modified pooled plasma matrix, with an emphasis on both accuracy and precision assessment. The modification employed the dilution, with pH = 9 buffer, of plasma until chromatogram for H and NH was devoid of a significant peak area response (unable to

integrate under the peak). Moreover, the goal was to minimize the dilution factor in order to maintain unmodified plasma composition as close possible. Dilution factors (buffer to plasma) such as 0.5:1, 1:1, 2:1, 4:1, 5:1 and 10:1 were evaluated to decrease the signal. Experiments in triplicate were evaluated for each dilution factor under two conditions: plasma spiked with 300 pg internal standard only and "blank" plasma. Devoid of peak areas for both H and NH were assessed.

Upon appropriate dilution of the modified matrix, full calibration curves (n = 6) and quality control samples were used to evaluate the linear range, accuracy and precision. The concentrations evaluated in 2-ml of modified matrix included a seven-point standard curve including 6.3, 25, 50, 100, 250, 500, 1000 pg. Quality controls (QC) for the low, medium and high were 12.5, 400, and 750 pg, respectively. In addition, an AUL (Above Upper Limit) dilutional control of and 2500 pg was evaluated for precision and accuracy. Of importance, the Lower Limit of Quantification (LLOQ) was determined during this step using n = 6 replicates, separate from the calibrators. At this point, recovery assessment was evaluated using this matrix to assess the relative recovery and, more importantly, the precision or the recovery. The room temperature stability measurements, as mentioned before, was also performed in this in modified, pH = 9 buffer matrix.

A more formal evaluation for precision and accuracy was conducted by evaluation of n = 6 each of the LLOQ, low, medium, high, and AUL control sample concentrations in one day for within-run assessment. Between run-precision and accuracy was assessed in triplicate over three days for the same controls. The accuracy

(%DFN) and the precision (%COV) between and within-runs were found acceptable at a level of within ±15%. All quality control samples, including those that failed with no assignable cause, were used for the final calculation.

Using the modified matrix, extraction efficiency (recovery) was assessed at three quality control levels in triplicate of 12.5 pg, 400 pg and 750 pg (LQC, MQC, and HQC). The normalized extraction efficiency was evaluated by reviewing the H or NH / YOH ratio from an extracted sample to the peak area response ratio obtained from the unextracted sample. In addition to the relative recovery, the precision of the extraction method was evaluated at this step.

As the case for the calibration curves obtained for the pooled plasma, the linear regression parameters for the calibration curves, including slope and y-intercept, for the modified plasma validation were assessed. The estimates and corresponding 95% confidence interval along with the precision of the slope and y-intercepts (% COV) were evaluated. For approval of the matrix modification via dilution factor, the mean of the y-intercepts should not be statistically different from zero (as deemed by a one-sided unpaired Student's t-test). The individual confidence intervals of the y-intercept estimates should include zero for each individual calibration curve.

The slope of the linear regressions within all calibration curves should not be statistically different from one another (as deemed by a 95% CI). Moreover, the mean of all calibration slopes between the pooled plasma and the modified plasma matrix were evaluated for "parallelism" (i.e., the average slopes obtained between each matrix

should be statistically similar). Lack of similarity was evaluated by a two-sided unpaired Student's t-test.

3.3.4d-4 Surrogate matrix results

A modified matrix, using pH = 9 buffer used for dilution, was constructed from pooled plasma until chromatogram for H and NH was devoid of a significant peak area response. The minimum dilution factor required to rid significant chromatographic signal was the 4:1 dilution (buffer: plasma). Dilutions less than this resulted in significant chromatographic peaks for both H and NH. From previous experiments in pooled plasma, it was expected that at least a 3:1 fold dilution would be required to significantly decrease the detectable signal. Although the analysis of larger dilution factors (5:1 and 10:1) resulted in peaks that were not able to be integrated, the 4:1 dilution was used for validation purposes to keep the matrix as similar to the original sample as possible. Full calibration curves (n=6) were constructed using 2-ml of modified matrix along with corresponding quality control samples. For NH, throughout the concentration range the precision (%COV) was less than 12.1% and the accuracy (%DFN, absolute value) was less than 14.9% (see table 3-21). In addition, the analysis of H yielded acceptable results with a precision < 9.7% and an accuracy of < 15.3% (table 3-22). Of note, the analysis met the criteria in which >75% of all standards must be within ±15 % COV, with the exception of the LLOQ where a ±20 % COV.

Further evaluation at the LLOQ level (6.3 pg/2ml) yielded a %COV of 14.8% and %DFN of 11.5%, with n = 6 replicates different from the calibration points. The

additional quality control samples, including the dilutional control, yielded a suitable accuracy and precision. For inter-run precision and accuracy (3 replicates for 3 days), %COV was no greater than 11.4% and no more than an absolute variation in concentration of 14.6% across the quality control concentrations (data not shown).

The linear regression parameters for each calibration curve were compared to assess consistency of slope and for lack of a y-intercept. Using a $1/x^2$ weighting factor for each calibration curve, the parameters presented (tables 3-23 and 3-24) showed consistency between calibration curves. The slope parameters were statistically different from zero, while the intercept did not show a statistical difference from zero (*p*-values > 0.3 for both analytes). The zero intercept highlights the ability for the dilution factor to be appropriate for the H and NH analysis. For both analytes, the coefficient of determination (R^2) was acceptable for each calibration curve, supporting the use of a linear regression for the calibration.

Of note, "blank" and "zero" samples did not result in a significant peak for either H or NH at the respective retention time (see figure 3-15). The diluted plasma matrix did not yield a significant YOH peak in the "blank" sample. Throughout all calibration runs, the YOH peak was consistent and showed minimal variability of the absolute peak area (%COV < 5.2%). The extraction recovery was assessed at three quality control levels in triplicate of 12.5 pg, 400 pg and 750 pg (LQC, MQC, and HQC). The normalized extraction efficiency is shown in table 3-25. The recoveries obtained from this analysis were constant but showed a slightly less recovery from that of unmodified pooled plasma.

Table 3-21: Calculated concentration (pg/2ml) for norharman in modified plasma calibration curves (n=6), with precision (%COV) and accuracy (%DFN, absolute value) assessment.

pg NH	calib #1	calib #2	calib #3	calib #4	calib #5	calib #6	average	sd	%COV	%DFN
6.3	6.9	5.8	6.8	5.7	6.9	5.8	6.3	0.6	9.5	< 10.8
25	23.5	25.7	28.2	27.3	27.9	24.4	26.2	1.9	7.4	< 12.9
50	43.2	45.9	47.2	47.4	43.5	53.1	46.7	3.6	7.7	< 13.5
100	107.6	101.3	104.8	105.9	97.4	104.9	103.7	3.7	3.6	< 7.6
250	284.2	242.0	284.4	212.7	276.3	246.7	257.7	28.8	11.2	< 14.9
500	557.0	463.2	461.4	470.7	574.3	475.8	500.4	51.1	10.2	< 14.8
1000	912.8	1147.2	1094.5	880.2	910.4	1122.0	1011.2	122.3	12.1	< 14.7
12.5 (LQC)	11.4	14.4	13.9	11.6	13.4	11.5	12.7	1.4	10.7	< 15.1
400 (MQC)	362.0	378.3	340.4	459.3	364.9	366.5	378.6	41.4	10.9	< 14.9
800 (HQC)	848.1	751.4	833.6	720.4	824.7	746.7	787.5	54.1	6.9	< 9.9
2500 (AUL QC)	2686.3	2373.8	2250.9	2224.6	2554.8	2221.2	2385.3	195.1	8.2	< 11.2

Table 3-22: Calculated concentration (pg/2ml) for harman in modified plasma calibration curves (n=6), with precision (%COV) and accuracy (%DFN, absolute value) assessment.

pg H	calib #1	calib #2	calib #3	calib #4	calib #5	calib #6	average	sd	%COV	%DFN
6.3	6.6	7.1	6.0	5.5	5.8	6.7	6.3	0.6	9.7	< 13.8
25	26.1	27.3	24.2	27.0	27.2	22.7	25.8	1.9	7.4	< 9.4
50	54.4	48.1	50.7	46.0	47.0	51.3	49.6	3.1	6.3	< 8.7
100	89.4	97.2	112.2	106.0	108.0	97.2	101.7	8.5	8.4	< 12.2
250	232.4	270.0	229.2	238.6	254.9	264.4	248.2	17.2	6.9	< 8.3
500	441.3	532.1	559.1	484.6	553.2	505.2	512.6	45.0	8.8	< 11.8
1000	980.5	1095.0	1106.7	1142.2	1036.2	1002.8	1060.6	63.8	6.0	< 14.2
25 (LQC)	12.3	14.0	13.3	13.4	14.2	13.1	13.4	0.7	5.2	< 13.9
400 (MQC)	353.6	363.2	384.6	374.1	441.3	440.7	392.9	38.7	9.8	< 11.6
800 (HQC)	781.4	819.5	828.5	878.4	761.7	766.8	806.0	44.8	5.6	< 9.8
2500 (AUL QC)	2631.8	2847.5	2881.4	2388.1	2607.9	2655.5	2668.7	179.5	6.7	< 15.3

Table 3-23: Standard curve parameters for n = 6 norharman calibration curves in modified plasma. Each parameter presented as an estimate with standard error (SE).

Calibration	slope	SE	y-intercept	SE	R2
1	0.0064	0.0008	0.0091	0.0035	0.9858
2	0.0067	0.0004	(-)0.0081	0.0037	0.9817
3	0.0059	0.0007	(-)0.0067	0.0018	0.9908
4	0.0062	0.0003	0.0044	0.0023	0.9912
5	0.0055	0.0007	(-)0.0049	0.0025	0.9855
6	0.0058	0.0004	0.0029	0.0049	0.9890
average	0.0061		0.0055		0.9873
stdev	0.0004		0.0032		0.0037

Table 3-24: Standard curve parameters for n = 6 harman calibration curves in modified plasma. Each parameter presented as an estimate with standard error (SE).

Calibration	slope	SE	y-intercept	SE	R2
1	0.0097	0.0004	0.0051	0.0044	0.9802
2	0.0103	0.0005	0.0037	0.0031	0.9977
3	0.0098	0.0011	(-)0.0039	0.0022	0.9834
4	0.0105	0.0009	0.0040	0.0028	0.9822
5	0.0092	0.0005	(-)0.0031	0.0021	0.9873
6	0.0097	0.0012	0.0013	0.0027	0.9888
average	0.0099		0.0035		0.9866
stdev	0.0005		0.0016		0.0063

Table 3-25: Relative recovery from 2 ml modified diluted plasma of norharman and harman (n=3 for each concentration). (% mean ± SD).

	12.5 pg	400 pg	800 pg
Harman	82.8 ± 3.5	86.5 ± 2.8	89.2 ± 5.2
Norharman	79.4 ± 2.1	82.7 ± 4.2	86.5 ± 3.7

In comparison to the unmodified, pooled plasma matrix surrogacy for the diluted matrix was established. From the linear regression parameters (i.e., slope) obtained with calibration curves constructed of both sets of matrices, a statistical similarity was seen. Table 3-26 presents the comparison of the pooled plasma (PP) linear regression parameters to those found in the modified plasma (MP) matrix for both analytes. An unpaired, two-tailed Student's t-test was performed on the slope and y-intercept parameters, assuming unequal variances.

The y-intercept showed a statistically significant difference between matrices. While the modified, diluted plasma was not significantly different from zero, the pooled plasma matrix possessed a significant y-intercept, reflecting the constitutive H and NH concentrations in the plasma. Moreover, the lack of a significant y-intercept in the modified plasma denotes successful dilution of the matrix. The statistical comparison of the y-intercept illustrates a successful dilution of the matrix.

Table 3-26: Linear regression comparison between pooled plasma (PP) and modified plasma (MP), n = 6 each, for NH (top) and H (bottom). Statistical significance was defined at the $\alpha = 0.05$ level.

	Regression Parameter			
	Slope		Y-intercept	
calibration	PP	MP	PP	MP
1	0.0068	0.0064	0.0670	0.0091
2	0.0072	0.0067	0.0713	(-)0.0081
3	0.0065	0.0059	0.0660	(-)0.0067
4	0.0065	0.0062	0.0780	0.0044
5	0.0068	0.0055	0.0810	(-)0.0049
6	0.0064	0.0058	0.0691	0.0029
avg	0.0067	0.0061	0.0721	0.0055
stdev	0.0003	0.0004	0.0061	0.0032
p-value	*0.9399*		*<0.01*	

	Regression Parameter			
	Slope		Y-intercept	
calibration	PP	MP	PP	MP
1	0.0094	0.0097	0.0580	0.0051
2	0.0113	0.0103	0.0524	0.0037
3	0.0109	0.0098	0.0642	(-)0.0039
4	0.0118	0.0105	0.0688	0.0040
5	0.0096	0.0092	0.0721	(-)0.0031
6	0.0091	0.0097	0.0830	0.0013
avg	0.0104	0.0099	0.0664	0.0035
stdev	0.0011	0.0005	0.0108	0.0016
p-value	*0.3337*		*<0.01*	

Of primary importance is the statistical similarity in the slopes for both H and NH in both matrices. The comparison proves similarity and supports the interchangeability of the matrix for calibration purposes. In essence, for a given change in concentration of H or NH analyte, a similar change in response will be seen, regardless of using a modified or pooled plasma matrix. Moreover, "parallelism"

between the matrices was proven between the modified and the pooled plasma matrix. Further assessment of selectivity was warranted for this method, evaluating additional plasma sources for potential interferences. A similar approach was used to assess "parallelism" in patient plasma samples.

3.3.4d-5 Patient plasma experiments

In order further evaluate selectivity and parallelism, individual plasma from separate donors was used as a matrix. Selectivity should be assessed to show that the anticipated analytes are measured and that their quantification is not affected by the presence of biological matrix, known metabolites, degradation products, or co-administered drugs (Viswanathan et al., 2007). In assay wherein the intrinsic selectivity is low (e.g., HPLC with detection other than MS), it is essential to confirm using blank matrices from at least n=6 independent sources, that the matrix will not impede the assay significantly. The White Paper from the 3rd Bioanalytical Workshop has proposed determination of matrix factors from 6 independent sources of matrix as a way of assessing the matrix effect (AAPS, 2006).

In the case for drug molecules, the "blank" matrix should not produce any significant background at the retention time of the analytes. For chromatographic assays, the peak response in the blank matrix at the retention time for the analytes should be no more than 20% of the response for the LLOQ of the assay. Both statements would be violated in the assessment of selectivity of H and NH quantification, because of the endogenous nature of the analytes in plasma. Nonetheless,

selectivity of the bioanalytical assay may still be assessed by spiking known concentrations of analyte into the matrix of interest, as performed as a standard addition experiment. Standard addition methods are particularly useful for analyzing complex samples in which the likelihood of matrix effects is substantial (Skoog et al., 1998). In this approach, different weights of the analyte(s) are added to the sample matrix, which initially contains an unknown concentration of analyte. Extrapolation of a plot of response found for the standard-addition calibration concentrations to zero concentration defines the original concentration of the unspiked sample. One disadvantage of this calibration method is the fact that at least three to five aliquots need to be prepared because and increasing amount of calibrant must be added to these different aliquots. In general, this method may require a significant volume of plasma that may not be available for the intended method. Moreover, an important aspect of standard addition is that the response prior to spiking additional analyte should be high enough to provide a reasonable S/N ratio (>10), otherwise, the result will have poor precision (Snyder et al., 1997).

The major criteria in establishing "surrogacy" of the proxy matrix, is that the response factor, or slope of the calibration curves obtained in the substitute matrix, should be statistically similar. Upon approval, matrix differences between the surrogate and unmodified, sample matrix, may presumably be accounted for. The parallelism between the calibration curves of both matrices suggests that quantification via a common calibration curve would be similar between the surrogate and actual sample matrix. In essence, parallelism studies need to be performed where the response of the

assay to a range of calibration standard concentrations made up in the surrogate matrix is compared to that of a series of dilutions of patient samples. This method of deeming "surrogacy" of a proxy matrix for quantification has been successfully employed (DeSilva et al., 2003; Smolec et al., 2005). In order to evaluate the use of the HPLC-FD method along with the modified matrix, a total of six sources of unmodified plasma were used in the subsequent experiments.

Using individual plasma (n=5, presumably healthy, drugs of abuse-free, smokers and non-smokers, collected in EDTA) and pooled plasma were used to satisfy the conditions for testing selectivity. Using the finalized extraction method, spiked concentrations of H and NH was evaluated in 2-ml of human patient plasma. As the availability of significant volumes of individual human plasma was limited, full calibration curves could not be constructed in the patient plasma. For the individual plasma experiments a total of five concentrations was used for the standard addition calibration curve construction including 12.5, 100, 400, 625, and 800 pg of H and NH and 300 pg of YOH in 2-ml of plasma. Moreover, a "zero" concentration (unspiked H or NH with 300 pg of YOH) was evaluated to assess the endogenous H and NH concentrations. Individual plasma calibration curves were constructed in singlicate. The concentrations assessed in these experiments are representative of those used in previous calibration curves.

In order to assess potential interferences with YOH retention time, absolute peak area was compared to that of the pooled plasma chromatogram, where there was no observable interference. A significant observable interference was defined as a %COV

>10% of absolute peak area at the YOH retention time for all individual plasma chromatograms. In addition to the patient plasma calibration curves, a pooled plasma matrix calibration curve was constructed (n=1), using the full range of calibration concentrations.

In order to appraise the identity of the constitutive H and NH, spectral confirmation studies were performed. The emission spectra of authentic H and NH were scanned at a fixed excitation wavelength of 300 nm for both authentic (spiked) and constitutive (unspiked) patient samples at the respective retention times Comparison was made between the emission spectra scans of the samples to assess the spectra shape and emission wavelength maximum.

A five-point, linear calibration curve was constructed for each individual plasma donor using a $1/x^2$ weighing, identical to the weighted regressions performed as the other experiments. The linear regression parameters for the calibration curves, including slope and y-intercept, for the individual plasma were assessed. The y-intercept for each individual calibration curve presumably reflected the constitutive contribution of H and NH and compared to that of the "zero" concentration level, as calculated from the surrogate matrix calibration curve. The 95% confidence interval for the y-intercept (peak area ratio) should include the peak area ratio of that seen of the "zero" sample for both H and NH. This method was to ensure the accuracy of using the surrogate matrix calibration curve to calculate a concentration in unmodified, real sample.

Of primary importance was the evaluation of the slope parameter for each individual plasma calibration curve. The estimate and its corresponding 95% CI was calculated and compared across donors. The estimates for the slope parameter should be statistically similar upon evaluation of the 95% CI. Across patient calibration curves, concentrations were back-calculated using (1) the regression obtained from the patient calibration curve, with baseline correction and (2) the full calibration curve using the modified "surrogate" matrix. At each level, the concentrations were compared between matrices to appraise difference between the two quantification methods. It was defined that, if the percent difference was greater than 20%, surrogacy of the matrix was not obtained and a significant issue of selectivity was present. Individual NH and H concentrations were calculated and compared for each patient sample using the standard addition method and the surrogate matrix calibration.

Moreover, the slopes ± standard deviation between the surrogate matrix plasma (n=6), individual patient plasma (n=5), and pooled plasma (n=6) calibrations were compared via one-way analysis of variance (ANOVA) to ensure a matrix effect between the matrices was not present. Prior to statistical contrast, the residuals were evaluated for equal variance and tested accordingly if that assumption did not hold true. If significant deviations and variability in the slopes was present in the analysis, the chromatographic parameters and/or extraction method was further optimized eliminate the interfering substances.

3.3.4d-6 Patient plasma results

Using the finalized extraction method, spiked concentrations of H and NH was evaluated in 2-ml of human patient plasma from n=5, presumably healthy, drugs of abuse-free, smokers and non-smokers. Representative chromatograms for LLOQ in modified matrix, patient plasma, and "zero" modified matrix are presented in figure 3-15 below. The respective retention times for NH, H and the internals standard, YOH are 3.82, 4.43, and 8.09 minutes, respectively. The LLOQ of 6.3 pg/2ml of modified plasma (Figure 3-15a) shows distinct bands at the respective retention times of H and NH while figure 3-15c exemplifies the "zero" sample level (dilution of H and NH) in the presence of the internal standard, YOH. Unmodified patient plasma (2 ml) sample was quantified with the surrogate matrix calibration curve and resulted in 42.5 pg/2 ml of NH and 25 pg/ 2ml of H, both of which fall within reported physiological ranges of plasma β-carboline (Figure 3-15b).

The goal of patient plasma experiments was to assess selectivity of the assay methodology. Of note, the accessibility of significant volumes of individual human plasma was limited, as consequence, full calibration curves could not be constructed in the patient plasma. The corresponding calibration curve parameters are presented below.

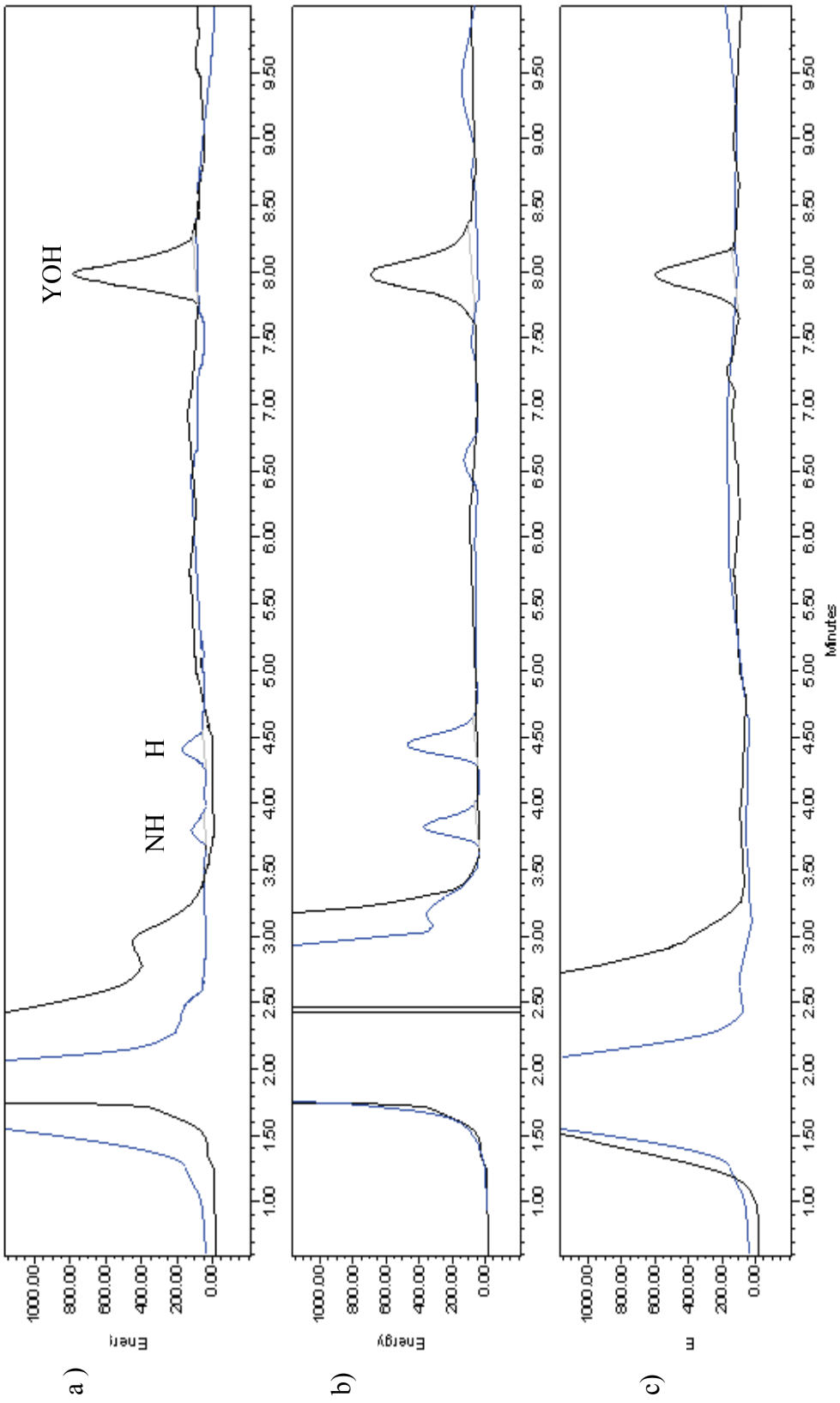


Figure 3-15: Representative chromatograms for a) lower limit of quantification (LLOQ) of 6.3 pg/2ml in surrogate matrix, b) patient plasma containing authentic NH and H, and c) "zero" surrogate matrix with internal standard, YOH.

Table 3-27: Linear regression parameters obtained from patient plasma (PP #1-5) and one modified plasma (MP) calibration curve for NH (top) and H (bottom). 95% CI denotes the upper and lower bounds in brackets for both slope and y-intercept.

Norharman

plasma	slope	95% CI	y-int	95% CI	R2
MP	0.0064	[0.0058, 0.0074]	0.01	[-0.06, 0.04]	0.9985
PP#1	0.0065	[0.0054, 0.0078]	1.40	[1.12, 1.73]	0.9882
PP#2	0.0071	[0.0051, 0.0080]	1.05	[0.91, 1.16]	0.9748
PP#3	0.0071	[0.0066, 0.0074]	0.32	[0.22, 0.41]	0.9955
PP#4	0.0073	[0.0063, 0.0081]	0.10	[0.03, 0.16]	0.9833
PP#5	0.0059	[0.0040, 0.0075]	2.00	[1.45, 2.33]	0.9722
avg	0.0067		0.81		0.9854
stdev	0.0005		0.80		0.0107

Harman

plasma	slope	95% CI	y-int	95% CI	R2
MP	0.0099	[0.0094, 0.0105]	0.00	[-0.03, 0.05]	0.9974
PP#1	0.0107	[0.0101, 0.0114]	0.37	[0.28, 0.45]	0.9872
PP#2	0.0098	[0.0092, 0.0103]	1.47	[1.43, 1.52]	0.9987
PP#3	0.0091	[0.0079, 0.0111]	0.25	[0.11, 0.41]	0.9637
PP#4	0.0089	[0.0082, 0.0096]	0.08	[0.05, 0.14]	0.9911
PP#5	0.0109	[0.0093, 0.0115]	2.68	[2.55, 2.74]	0.9884
avg	0.0099		1.01		0.9878
stdev	0.0008		1.06		0.0127

The coefficients of determinations for NH and H, in the patient plasma, were greater than 0.96 for both, suggesting the linear calibration function is acceptable for this matrix (using $1/x^2$ weighting). The y-intercepts obtained for all calibration curves, with the exception of the modified plasma, were all statistically different from zero, as deemed by the 95% CI. Moreover, the intercepts between the plasma sources resulted in significant deviations, suggesting the variable nature of the constitutive NH and H between the five patients (COV%, NH: 98.4% and H: 130%).

The slope parameters from the calibration curves were all statistically similar between the patient plasma sources and in comparison to the modified matrix. Because of the limited number of concentration points on each calibration curve (5-point) for the patient plasma, the 95% CI range was relatively large as compared to the modified plasma matrix (i.e., a full calibration curve). Of note, according to the aforementioned criteria, no significant interference was seen at the YOH, internal standard retention time. For all individual plasma chromatograms and pooled plasma chromatograms the %COV in absolute peak area for the internal standard was 7.2%.

Results of spectral confirmation studies compare the authentic to that of constitutive H and NH. According to the shape and maximum wavelength of emission spectra for both analytes, the patient plasma peaks seen at the respective retention times of H and NH (i.e., 3.82 and 4.43 minutes) are similar to those of authentic, spiked H and NH (see figure 3-16).

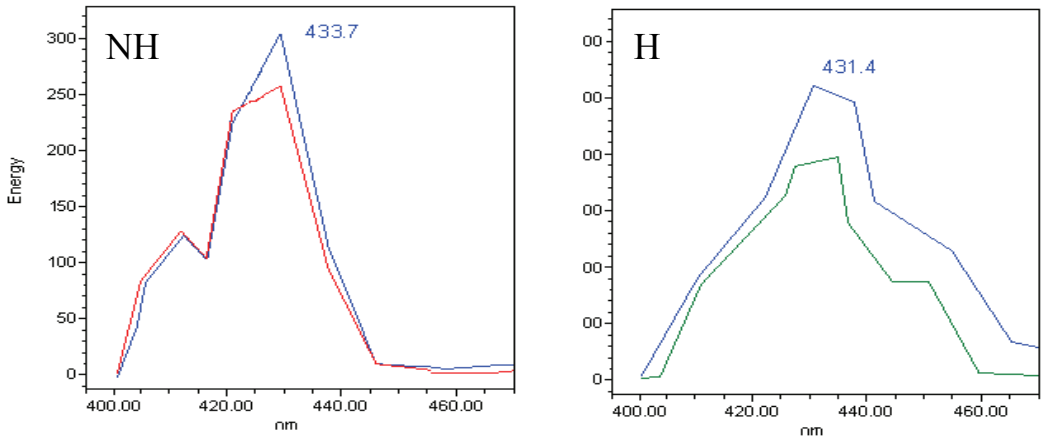


Figure 3-16: Spectral confirmation of NH and H comparing the emission spectra of 50 pg of authentic (spiked) NH and H (top spectra) to endogenous NH and H (bottom spectra).

To further evaluate the "surrogacy" of using the modified matrix, a comparison of the utility between the patient plasma and the modified plasma calibrations was appraised. This analysis compares the back calculated concentrations obtained from the modified matrix calibration curve to that of the back-calculated concentrations of the standard addition calibration of the patient plasma (see tables 3-28 and 3-29). Of importance is the calculation of the constitutive concentrations of H and NH within the patient plasma sources. Upon comparison of both calibration methods, a significant percent difference (denoted as %diff on tables) is present between both methods of quantification for both analytes. The standard addition method of calibration possesses greater inaccuracy as compared to the surrogate matrix calibration. This discrepancy maybe caused by the difference in the number of calibration points used in each method (n=5 for standard addition vs. n = 8 for the surrogate matrix calibration). This limitation of using the standard addition method for calibration purposes is due to the lack of significant volumes of patient plasma. In order to run an accurate standard addition calibration for this developed assay, at least 16-ml of patient plasma would be required.

The concentrations obtained from the surrogate matrix calibration curves yielded endogenous levels that were within reported physiological ranges. For NH, the concentration range within the individual patient samples ranged 12.6 - 309 pg per 2 ml plasma. For H the concentration range within the five patient samples ranged from 6.2 - 292 pg per 2 ml plasma.

Surrogacy of the modified matrix was further evaluated by statistical comparison of calibration curves from pooled plasma and patient plasma. One-way ANOVA was performed on the slopes of the calibration curves between three different plasma matrices, modified (surrogate), pooled, and patient plasma. The summary table of all calibration slopes is presented in the table 3-30 below. The three groups of plasma matrices, compared using the unequal variance F-test, was not significantly different for both NH and H. For NH the means were found to be statistically similar, $F(1, 15) = 1.3$, p-value = 0.067, while the same conclusion was drawn for the H calibration parameter $F(1, 15) = 0.96$, p-value = 0.341. The results are presented as F-test calculation (degrees of freedom for groups, residuals) and p-value. All statistical comparisons were performed on S-PLUS 8.0 for Windows.

Table 3-28: Concentration calculations (pg/2ml) between standard addition calibration and surrogate matrix (SM) calibration curve for Norharman (measurements, n=1).

		standard addition		*surrogate mat calib*		
patient #1	pg NH	calc conc	%DFN	SM conc	%DFN	% Diff
	12.5	222.2	10.0	208.5	-1.2	-6.1
	100	332.5	14.9	336.8	12.8	1.3
	400	598.9	1.6	597.1	-0.2	-0.3
	625	777.5	-4.5	838.8	1.9	7.9
	800	1036.0	4.7	1011.4	1.3	-2.4
	zero	189.4	**	198.5	**	4.8
patient #2	pg NH	calc conc	%DFN	SM conc	%DFN	% Diff
	12.5	178.9	11.0	176.4	5.4	-1.4
	100	274.3	10.3	271.9	6.6	-0.9
	400	545.8	-0.5	508.6	-8.3	-6.8
	625	683.8	-11.6	741.1	-5.0	8.4
	800	1067.5	12.5	1003.6	5.1	-6.0
	zero	148.7	**	155.0	**	4.2
patient #3	pg NH	calc conc	%DFN	SM conc	%DFN	% Diff
	12.5	61.0	15.6	55.5	0.9	-9.1
	100	146.9	4.7	136.7	-4.0	-6.9
	400	417.7	-5.1	461.2	4.2	10.4
	625	641.0	-3.6	676.9	1.4	5.6
	800	889.3	5.8	872.2	3.5	-1.9
	zero	40.3	**	42.5	**	5.4
patient #4	pg NH	calc conc	%DFN	SM conc	%DFN	% Diff
	12.5	31.3	24.2	23.3	-7.3	-25.6
	100	91.9	-18.5	103.6	-8.0	12.7
	400	397.0	-3.8	433.9	5.2	9.3
	625	665.0	4.3	711.6	11.6	7.0
	800	895.4	10.2	885.6	9.0	-1.1
	zero	12.7	**	12.6	**	-0.8
patient #5	pg NH	calc conc	%DFN	SM conc	%DFN	% Diff
	12.5	392.0	4.6	327.1	1.8	-16.6
	100	492.7	6.6	426.2	4.2	-13.5
	400	709.5	-6.9	665.7	-6.1	-6.2
	625	1033.8	4.7	957.6	2.5	-7.4
	800	1043.9	-10.2	1053.2	-5.0	0.9
	zero	362.3	**	309.0	**	-14.7

Table 3-29: Concentration calculations (pg/2ml) between standard addition calibration and surrogate matrix calibration curve for Harman (measurements, n=1).

		standard addition		surrogate mat calib		
patient #1	pg H	calc conc	%DFN	SM conc	%DFN	% Diff
	12.5	38.0	-3.0	43.1	1.7	13.3
	100	119.8	-5.5	131.4	1.2	9.8
	400	478.2	12.1	439.3	2.2	-8.1
	625	712.6	9.3	670.1	2.3	-6.0
	800	787.7	-4.7	863.4	4.0	9.6
	zero	26.7	**	29.8	**	11.8
patient #2	pg H	calc conc	%DFN	SM conc	%DFN	% Diff
	12.5	168.6	8.1	166.2	7.3	-1.4
	100	275.1	13.0	233.7	-3.6	-15.0
	400	573.9	5.6	545.7	0.6	-4.9
	625	791.5	3.0	780.2	1.7	-1.4
	800	941.0	-0.3	919.5	-2.4	-2.3
	zero	143.5	**	142.4	**	-0.7
patient #3	pg H	calc conc	%DFN	SM conc	%DFN	% Diff
	12.5	29.8	-20.9	38.3	2.2	28.8
	100	102.4	-18.2	111.0	-11.2	8.4
	400	525.1	23.5	477.2	12.3	-9.1
	625	791.9	21.8	757.3	16.5	-4.4
	800	843.8	2.3	787.0	-4.6	-6.7
	zero	25.1	**	25.0	**	-0.6
patient #4	pg H	calc conc	%DFN	SM conc	%DFN	% Diff
	12.5	21.2	16.0	18.8	0.6	-11.3
	100	102.7	-17.9	130.1	4.1	26.6
	400	449.5	5.7	388.9	-8.5	-13.5
	625	667.4	2.7	644.4	-0.9	-3.4
	800	855.6	3.7	757.5	-8.2	-11.5
	zero	5.8	**	6.2	**	7.0
patient #5	pg H	calc conc	%DFN	SM conc	%DFN	% Diff
	12.5	294.6	6.1	310.4	1.9	5.4
	100	338.4	-7.3	376.6	-4.0	11.3
	400	573.4	-13.8	624.5	-9.8	8.9
	625	953.8	7.1	997.2	8.7	4.6
	800	1018.9	-4.3	1078.7	-1.2	5.9
	zero	265.3	**	292.2	**	10.2

Table 3-30: Statistical comparison of calibration regression slope parameter between pooled, patient and surrogate matrix for NH and H. One-way ANOVA performed between matrices with significance at the $\alpha = 0.05$ level.

Norharman

	Slope of Regression		
calibration	Pooled	Patient	Surrogate
1	0.0068	0.0065	0.0064
2	0.0072	0.0071	0.0067
3	0.0065	0.0071	0.0059
4	0.0065	0.0073	0.0062
5	0.0068	0.0059	0.0055
6	0.0064		0.0064
avg	0.0067	0.0068	0.0062
stdev	0.0003	0.0006	0.0004

p-value *0.067*

Harman

	Slope of Regression		
calibration	Pooled	Patient	Surrogate
1	0.0094	0.0107	0.0097
2	0.0113	0.0098	0.0103
3	0.0109	0.0091	0.0098
4	0.0118	0.0089	0.0105
5	0.0096	0.0109	0.0092
6	0.0091		0.0097
avg	0.0104	0.0099	0.0099
stdev	0.0011	0.0009	0.0005

p-value *0.341*

From these results, the appropriateness of using the modified plasma matrix as a surrogate matrix was demonstrated. Parallelism between the calibration curves in different matrices suggests the interchangeable nature of using either matrix for validation purposes. Limitations of using patient plasma pertain to insufficient volumes available for constructing an accurate calibration curve. Moreover, an accurate

and precise assessment of an LLOQ for the standard addition method is dependent on the constitutive concentration of the analyte in the plasma. The utilization of pooled plasma, solely, does not address the selectivity assessment as required for any bioanalytical method development. Therefore, the surrogate matrix will be incorporated for the clinical sample analysis.

3.3.4e Validation Conclusions

In summary, the acceptance criteria for validation were met according to predetermined specifications. In the surrogate and unmodified matrices, chromatographic response was linear throughout the concentration range of 6.3 pg/2 ml (LLOQ) and 1 ng/2 ml (ULOQ). For both analytes, accuracy throughout the calibration range was acceptable with %DFN ranging from -7.6 to 14.9%. Surrogacy of the modified matrix was confirmed via statistical comparison of calibration curve slopes with those of unmodified matrix (pooled and patient plasma). For both types of calibration matrices, the slope precision (%COV) for H and NH were < 6.5% for both analytes with both analytes possessing R^2 precision of <3.7%.

Accuracy and precision estimates for the assay were in acceptable ranges for the calibration points, the LLOQ and quality control samples with %COV and %DFN meeting analytical validation criteria. Selectivity of the method was evaluated with 6 donor lots (5 individual plasma and 1 pooled plasma) and no interference was apparent with the analyte or YOH detection. Across all quality control samples, recovery was relatively high (>80.2%) and reproducible for both H and NH.

Long-term plasma stability experiments for 6 months at -80ºC resulted in negligible deviations over time for both analytes. Additional stability experiments, including stock solution, bench-top, and post-preparative resulted in negligible deviations throughout the respective tested time-spans. Under the optimized SPE experimental conditions and chromatography, endogenous H and NH were detected in plasma at concentrations comparable to reported literature values.

3.4 β-carboline assay conclusions

A robust, sensitive, selective and reproducible assay has been developed for the quantification of the endogenous β-carbolines, H and NH, in 2 ml of human plasma. This optimized assay technique involves a simple protein precipitation with SPE extraction along with 300 pg of the internal standard, YOH.

In comparison to reported methodologies for the quantification of H and NH in human plasma, this procedure has overcome the limitations aforementioned. The optimized chromatography has preserved the baseline resolution of the H and NH analytes ($Rs > 1.5$) throughout the concentration range, improving the reliability of quantification. In addition, an appropriate internal standard, YOH, was chosen in part to the similar physicochemical characteristics of H and NH. The internal standard possesses comparable HPLC retention attributes, stability in plasma, and extraction recovery efficiency. For these reasons, YOH proved to be a valuable candidate for H and NH extraction from human plasma.

Successful quantitation without a true blank matrix requires the use of surrogate matrices, especially in the case where the analytes of interests are in low concentrations. This method justified the use of a surrogate matrix for validation and practical purposes. The standard addition method, although useful, resulted in less accurate results compared to the surrogate matrix calibration method. This is presumably due to the lack of significant patient plasma volume to run a full and accurate calibration curve.

The current developed method has maintained resolution between analytes, utilizes a novel internal standard to assess sample loss from extraction and has been fully validated using and appropriate surrogate matrix, unlike currently reported assays for H and NH quantification in plasma. Moreover, this new method has maintained adequate sensitivity for physiological studies. The chromatographic separation conditions along with the optimized extraction technique and surrogate matrix calibration will be used to support clinical studies for the quantification if the β-carbolines, H and NH, in human plasma.

CHAPTER 4

BIOANALYTICAL ASSAY OPTIMIZATION AND VALIDATION FOR R/S-SALSOLINOL AND DOPAMINE IN HUMAN PLASMA

4.1 Introduction – Selection of Analytical method

Salsolinol (1-methyl-1,2,3,4-tetrahydro-6,7-dihydroxy-isoquinoline, SAL) is a dopamine-derived tetrahydroisoquinoline (TIQ) alkaloid that has been purported to play a role in the neurochemical mechanisms underlying addiction. However, results found in previous human studies on SAL plasma and urine concentrations and its enantiomeric ratio between healthy and alcoholic populations show conflicting results and show large variability. A sensitive and reliable method to determine the enantiomeric composition of endogenous SAL is required to test a possible correlation between alcoholism and R/S-SAL exposure. Moreover, as SAL is a dopamine derived TIQ alkaloid, it would be useful to determine the physiological concentrations of dopamine (DA) to assess SAL synthesis characteristics in human populations. Assessment of the DA precursor along

with the SAL product may provide valuable information on the characterization of SAL biosynthesis.

Both enantiomers of SAL are found in urine, cerebrospinal fluid (CSF), blood, and brain of humans. As physiological concentrations of SAL are reported to be in the low nanogram to low picogram / ml plasma range, and the available samples from humans are complex matrices, multi-step enhancement and preparation techniques are necessary for ultimate detection and quantification.

Few reports of bioanalytical assays for *total* SAL in biofluids and foods have been published including GC-MS (Musshoff et al., 1997), HPLC-ECD (Riggin and Kissinger, 1977; Dufay et al., 1991) and HPLC-FD (Pagel et al., 2000) methodologies. Recently, LC/MS methods using electrospray ionization (ESI) tandem mass spectrometry (MS) (Song et al., 2006) or atmospheric pressure photoionization MS (Starkey et al., 2006) have been used for SAL analysis without enantiomeric separation.

The enantiomeric discrimination of SAL became of importance when *in-vitro* and *in-vivo* pharmacological differences between R-SAL and S-SAL were identified. SAL enantiomers were first analyzed by GC and nitrogen-phosphorus detection after derivatization with N-trifluoroacetyl-L-proyl chloride (Strolin-Benedetti et al., 1989). Regular analyses of SAL enantiomers from biological matrices using this methodology have been complex and irreproducible. A GC/MS method was developed by a two-step derivatization process to SAL diastereomers with *N*-methyl-*N*-trimethyl-silyl-trifluoroacetamide (MSTFA) and (R)-(-)-2-phenylbutyryl chloride as a chiral derivatizing agent (Haber et al., 1995b; Musshoff et al., 2000). Although baseline

separation of SAL enantiomers was established, this method utilized water sensitive derivatization procedures that required evaporation of aqueous solvent to absolute dryness, frequently promoting oxidative degradation of SAL. Moreover, the resulting chiral derivatives were unstable, hindering a consistent quantifiable analysis for both R- and S-SAL.

Use of chiral HPLC employing β-cyclodextrin stationary phases or as mobile phase additives has been used to provide enantiomeric separation of SAL (Rommelspacher et al., 1995; Stammel et al., 1995; Deng et al., 1997). In addition, determination of SAL enantiomers by HPLC-ECD has been conducted as diastereoisomeric derivatives, via reaction with (S)-1-(1-naphthyl) ethyl isothiocyanate as a chiral derivatizing reagent (Pianezzola et al., 1989). Yet, poor resolution, specificity, and sensitivity as well as lack of positive identification were severe limitations in the reported HPLC/ECD methods. The reported methodologies for quantification of R- and S-SAL in a biological matrix have been sensitive but several shortcomings exist for the published techniques. A list of published records of R-SAL and S-SAL analysis in a biological matrix is presented in the table below.

Further critique of the reported bioanalytical assays is warranted as inadequacies in the chromatography, extraction, and validation of the SAL assay methods are present. Adequate chromatographic baseline resolution between the SAL enantiomers has not been accomplished with majority of the assays presented, even after sophisticated techniques used for the separation. In regard to quantification, several deficiencies are

Table 4-1: Reported methodologies for quantification of the tetrahydroisoquinolines, R- and S-SAL in biofluids.

Author, year	Analytes	Matrix	Method	Extraction	Enantio-separation	LLOQ	Internal Standard	Calibration matrix
(Baum and Rommelspacher, 1994)	DA, R/S-SAL with sulfo-conjugates	10 ml blood	HPLC – ECD	liquid-solid extraction- PSA (primary/secondary amine) + PBA cartridge in series	Nucleodex β-OH column	20 pg/ml (LOD)	none	buffer
(Musshoff et al., 1999)	DA, R/S-SAL and nor-SAL	0.2 g brain	GS-MS	SPE – PBA MSTFA (*R*)-(−)-2-phenylbutyryl chloride deriv	Nucleodex β-OH column	0.5 ng/g (LOD)	deuterated DA, SAL and nor-SAL	buffer
(Pianezzola et al., 1989)	R/S-SAL	5 ml urine	HPLC – ECD	SPE – PBA	S-1-(1-napthyl)-ethyl iso thiocyanate (S-NEIT) deriv	2.5 ng/ml	none	buffer
(Deng et al., 1997)	R/S-SAL, R/S- N-methyl SAL	1.0 g brain tissue	HPLC-FD	SPE – PSA (primary secondary amine) and PBA cartridge	β-cyclodextrin mobile phase additive and ion pairing agent	0.047 pmol/inj (LOD)	N-methyl-norsalsolinol	buffer
(Haber et al., 1999)	R/S-SAL	PBMC peripheral blood mono-nuclear cells	GC-MS	Hypaque gradient centrifugation. SPE – PBA	MSTFA and R-(−)-2-phenylbutyryl chloride	50 pg/ml (LOD)	racemic deuterated salsolinol	buffer

SAL: salsolinol, HPLC: high-performance liquid chromatography, ECD: electrochemical detection, DA: dopamine, PSA: primary-secondary amine, PBA: phenyl boronic acid, LOD: limit of detection, LLOQ: lower limit of quantification, GS-MS: gas chromatography-mass spectrometry, MSTFA: *N*-methyl-*N*-trimethyl-silyl-trifluoroacetamide

present in reported methods. Firstly, internal standards have not been used in the reported assays. Significant sample pretreatment and preparation steps utilized in these methods necessitate the use of an internal standard.

Aside from the methodological issues pertaining to R/S-SAL assays, appropriate bioanalytical validation metrics (i.e., accuracy and precision) have either not been presented or assessed. Most importantly, the calibration matrix that has been used for quantitation in published assays has been via external calibration in buffer or mobile phase (see discussion in Chapter 3). This practice of calibration completely disregards sample extraction efficiency or matrix effects that may occur during the analysis or detection.

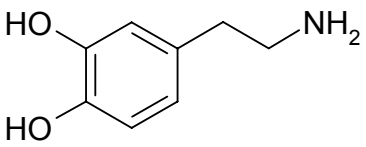
In summary, the reported bioanalytical methodologies for quantification of the SAL enantiomers in human plasma are not adequate for clinical study. In this investigation, a new robust analytical technique to determine SAL enantiomers and their precursor DA simultaneously based on chemical derivatization and chiral HPLC/ESI-tandem mass spectrometry was developed, optimized and validated (Lee et al., 2007). Presented throughout this chapter is the investigation on the optimization and validation of the already developed assay. The current method developed for R-SAL, S-SAL and DA quantification addresses the limitations associated with reported assay literature, maintaining the sensitivity required for use in human pharmacology studies.

4.2 Physicochemical Characteristics of R/S-SAL and DA

Tetrahydroisoquinolines, such as SAL (1-methyl-6,7-hydroxy-1,2,3,4-tetrahydroisoquinoline), are a class of partially aromatic compounds that are formed via the condensation of catecholamines (e.g., dopamine) with aldehydes. Recall the structure and pertinent physiochemical characteristics of salsolinol (SAL, figure 1-1).

Like many other TIQ's, SAL has an asymmetric center at the C-1 position of the heterocycle, thus leading to two stereo-isomeric forms (+)-(R)-SAL and (-)-(S)-SAL. SAL is an isoquinoline analog consisting of a catechol ring and a secondary amine that is able to be protonated at physiological pH (primarily ionized). Under acidic pH the secondary nitrogen possesses the propensity to form a quarternary ammonium species. The catechol moiety of SAL is speculated to have a pKa of ~ 9.4 in which, at physiological pH, is primarily unionized. The solubility and lipophilicity of S-SAL has not been well characterized. The logD has not been experimentally determined. Therefore, as SAL is primarily ionized at physiological pH and is moderately hydrophilic.

The precursor dopamine (4-(2-aminoethyl)benzene-1,2-diol, DA), is one of the primary catecholamine neurotransmitters in the brain. It is derived from tyrosine and is the precursor to norepinephrine and epinephrine. Dopamine is a major transmitter in the extrapyramidal system of the brain, and important in regulating movement, in which a family of receptors mediate its action. The structure and pertinent physicochemical characteristics are shown in the figure below.



Mol Weight (g/mol)	153.2 g/mol
pKa	9.8 (primary amine)
log D	-2.34
solubility	Very soluble (1000 g/L water)

Figure 4-1: Structure and physicochemical characteristics of dopamine (calculated from Advanced Chemistry Development, ACD/Labs, Software V 8.19 for Solaris © 1994-2008).

DA is an biogenic amine consisting of a catechol ring and a primary amine that is able to be protonated at physiological pH (primarily ionized). Like SAL, the catechol moiety of DA is speculated to have a pKa of ~ 9.0 in which, at physiological pH, is primarily unionized. DA is very soluble in water and the logD suggests that it is very hydrophilic in nature.

4.3 Enantiomeric Determination of R/S-SAL and DA via HPLC-ESI MS/MS

A method for the simultaneous determination of the enantiomeric concentrations of R- and S-SAL, along with the precursor, DA, was developed in the Laboratory of Molecular Signaling, National Institute of Alcohol Abuse and Alcoholism, National Institutes of Health (Lee et al., 2007). Along with a synopsis of the reported method, below summarizes the optimization of derivatization steps along with final validation of the assay in human plasma. The method was subsequently used to the support the

quantification of R/S-SAL and DA in two clinical studies involving alcohol dependent and tobacco smoking population.

4.3.1 Materials and Reagents

All chemicals were or analytical grade quality and obtained from commercial sources. Solvents used for experiments and measurements were of spectroscopic or HPLC grade and used without further purification.

1. (±)-Salsolinol hydrochloride (racemic, SAL-HCl, Sigma-Aldrich Corp., St. Louis, MO)
2. Dopamine hydrochloride (DA-HCl, Sigma-Aldrich Corp., St. Louis, MO)
3. N,N-diisopropylethylamine (DIPEA, Sigma-Aldrich Corp., St. Louis, MO)
4. Pentafluorobenzyl bromide (PFBBr, Pierce Chemical Company, Rockford, IL)
5. Deuterium-labeled (S)-SAL-d_4-HBr (1'-methyl-d_4) and (R)-SAL-d_4-HBr (1'-methyl-d_4) prepared by Cambridge Isotope Laboratories (Andover, MA)
6. Deuterium-labeled 1,1,2,2-d_4-DA-HCl (Cambridge Isotope Laboratories, Andover, MA)
7. Perchloric acid, 70% $HClO_4$, double distilled (GFS Chemicals, Columbus, OH)
8. Ethylene glycol bis-2-aminoethyl ether-tetraacetic acid, (EGTA, Sigma-Aldrich Corp., St. Louis, MO)
9. Semicarbazide hydrochloride (Fluka Chemie., Buchs, Switzerland)
10. Sodium metabisulfite (Sigma-Aldrich Corp., St. Louis, MO)

11. Monobasic sodium phosphate, $NaH_2PO_4 \bullet H_20$ (Sigma-Aldrich Corp, St. Louis, MO)
12. Dibasic sodium phosphate, $Na_2HPO_4 \bullet 7\ H_20$ (Sigma-Aldrich Corp., St. Louis, MO)
13. 10 M NaOH (VWR, Westchester, PA)
14. Hydrochloric Acid, ACS Reagent grade, 37% (Sigma-Aldrich Corp., St. Louis, MO)
15. Methanol, HPLC Grade (Burdick and Jackson, Morristown, NJ)
16. Hexane (Burdick and Jackson, Morristown, NJ)
17. Acetonitrile (Burdick and Jackson, Morristown, NJ)
18. Isopronanol, HPLC grade (Burdick and Jackson, Morristown, NJ)
19. Milli-Q water (Millipore, Bedford, MA)
20. Human plasma samples for validation - collected from healthy volunteers at the National Institutes of Health Apheresis Clinic and were either analyzed immediately or stored at -80 °C until the time of analysis.
21. Pooled plasma for validation, n = 20 males and females, drug free, nonsmokers (BioChemed Services, Winchester, VA).

4.3.2 Equipment

1. Solid Phase Extraction Manifold, 20-port with stopcocks – (Alltech, Deerfield, IL)
2. Solid phase extraction cartridges, Bond Elute phenyl boronic acid (PBA) – 100 mg, 1.0 ml (Varian Inc., Palo Alto, CA)
3. 10-µl, 100-µl, and 1000-µl Eppendorf variable volume pipette and corresponding pipette tips.

4. Instrumentation:
    a. Mass spectrometer: TSQ Quantum mass spectrometer with Electrospray Ionization source (Thermo-Finnigan, San Jose, CA)
    b. HPLC: Agilent 1100 HPLC System (Agilent, San Jose, CA)
    c. Column: Chiralpak AD-H column, 2.1 mm x 150 mm, 5 µm, (Chiral Technologies, Inc., West Chester, PA)
    d. Guard Column: Chiralpak AD-H column, 2.1 mm x 10 mm, 5 µm, (Chiral Technologies, Inc., West Chester, PA)
    e. Data acquisition: Excalibur 2.0 Software (Thermo-Finnigan, San Jose, CA)

4.3.3 Preparation of Solutions and Standards

As neither of the (S)-SAL and (R)-SAL enantiomers are available separately, standard solutions of SAL were prepared by dissolving the racemic mixture of (R/S)-SAL-HCl in methanol. Standard stock solutions of (R/S)-SAL-HCl as well as DA-HCl were prepared in methanol at a concentration of 1 mg/ml in amber, silanized vials. The deuterated internal standard stock solutions were additionally made in methanol at a concentration of 100 ng/ml for (S)-SAL-d_4-HBr and (R)-SAL-d_4-HBr and 1 mg/ml for d_4-DA-HCl. Of note, the *individual* enantiomers of R-SAL-d_4 and S-SAL-d_4 were synthesized and purchased. Stock standard solutions were stored in darkness at -20 °C until further use. The working internal standard solutions were prepared by further dilution of the stock solutions to 500 pg/ml of each SAL-d_4 enantiomer and 500 pg/ml of DA-d_4. Further dilutions used for calibration and validation are presented below in

the SAL validation section. The solutions used for the assay methodology and validation are presented below.

1. Sodium phosphate buffer (pH 8.2, 0.2 M) for derivatization procedure

   In 500 ml of Milli-Q® water 26.81 g of $Na_2HPO_4 \cdot 7\ H_20$ and 1.38 g of $NaH_2PO_4 \cdot H_20$ to a 500 ml volumetric flask. The maximum buffering capacity ~ 33% at this pH level and buffer concentration.

2. Sodium phosphate buffer (pH 8.5, 0.5 M) for solid-phase extraction

   In 500 ml of Milli-Q® water 67.04 g of $Na_2HPO_4 \cdot 7\ H_20$ and 1.725 g of $NaH_2PO_4 \cdot H_20$ to a 500 ml volumetric flask. The maximum buffering capacity ~ 18.2% at this pH level and buffer concentration.

3. Protein precipitation/Antioxidant solution (1M perchloric acid with 0.01% EGTA, 0.02% semicarbazide HCL, 0.02% sodium metabisulfite)

   Approximately 400 ml of 1M perchloric acid was made by adding 34.18 ml of 70% $HClO_4$ to 365 ml Milli-Q® water. To this solution 80 mg EGTA, 80 mg of sodium metabisulfite and 400 mg of semicarbazide HCl was added and subsequently stirred.

4. 6N NaOH and 2N NaOH for pH adjustment of sample for solid phase extraction and derivatization procedure.

   A 10 N NaOH solution was diluted accordingly to obtain the required normality.

5. 10% Pentafluorobenzyl bromide (PFBBr) in acetonitrile for derivatization

Approximately 500 µl of PFBBr was added to 4.5 ml of acetonitrile in an amber vial and vortexed.

6. 10% N,N-diisopropylethylamine (DIPEA) in acetonitrile for derivatization

   Approximately 500 µl of DIPEA was added to 4.5 ml of acetonitrile in an amber vial and vortexed.

7. 0.1 M Hydrochloric acid: Methanol (HCl:MeOH, 1:1 ratio) for elution solvent

   2.06 ml of 37% HCl was added to 248 ml of Milli-Q® water and mixed. 250 ml of methanol was added and mixed accordingly.

8. 1% acetic acid for post-column addition

   5 ml of glacial acetic acid was added to 495 ml of Milli-Q® water.

4.3.4 Method Summary for the Analysis of R/S-SAL and DA in human plasma

4.3.4a Sample Preparation

Pooled plasma and human plasma samples that were collected from healthy volunteers were stored at -80 °C until the time of analysis. Aliquots of 1.0 ml plasma were spiked with 1 ng each of (S)-SAL-d_4 and (R)-SAL-d_4 and 5 ng of DA-d_4 as internal standards. Extraction of SAL from human plasma was carried out as described by Haber et al. with a slight modification (Haber et al., 1995b). Samples were acidified with 1.0 ml of a 1 M $HClO_4$ antioxidant solution containing 0.01% EGTA, 0.02% semicarbazide hydrochloride, and 0.02% sodium metabisulfite. This solution was used primarily for protein precipitation. The additives utilized in the $HClO_4$ solution were used as a precautionary measure to inhibit artifactual formation of

SAL during the sample work up. EGTA is a chelating agent that acts as a preservative and antioxidant while sodium metabisulfite serves as a strong antioxidant. The semicarbazide component acts as an "aldehyde trapping" agent to prevent artifactual formation of SAL.

The plasma solution was subsequently centrifuged at 2000g for 15 min at 4 °C to remove precipitated proteins. In order to hydrolyze any conjugated SAL and DA, the supernatant was collected and heated for 60 min at 80 °C. More than 90-98% of SAL and DA were present as the conjugated form in plasma based on their levels determined with or without acid hydrolysis (data not shown). This assessment is consistent with the reported literature. The protein free, hydrolyzed sample was cooled and the pH of the sample was adjusted to 8.5 using ~ 100 µl of a 6 N NaOH solution and buffered with 0.5 ml of 0.5 N potassium phosphate buffer (pH 8.5). A further, more fine, adjustment of pH was performed with a less concentrated 2N NaOH, if needed.

4.3.4b Solid Phase Extraction by Phenyl Boronic Acid (PBA)

After pH adjustment, the SAL and DA in plasma were isolated by PBA solid-phase extraction. PBA has been successfully used to isolate SAL and DA from biological matrices (see table 4-1). PBA is a distinctive sorbent comprising of a phenylboronic acid covalently linked to a silica gel surface. The boronate group has a high specificity for cis-diol containing compounds like catechols, nucleic acids, low molecular weight proteins and carbohydrates. PBA utilizes a covalent retention mechanism that involves an interaction of 10-100 times greater energy than other

extraction mechanisms (Varian, 2005). PBA has proven to be especially effective in the isolation of catecholamines from biological fluids. Retention is usually strongest when the analytes' functional groups are co-planar, as in the case of SAL and DA.

The PBA solid-phase cartridge was rinsed twice with 2 ml each of methanol and water to remove any contaminants and subsequently conditioned with 2 ml of potassium phosphate buffer (0.5 M, pH 8.5). The pH 8.5 buffered sample was loaded on the PBA cartridge and washed twice with 1 ml each of water and methanol. Two 750 ml aliquots of the 0.1 M HCl/MeOH (1:1) elution solvent mixture was used to elute SAL and DA from the cartridges. The acidic component of the elution solvent was employed to break the covalent bonds between the PBA solid phase and the cis-diol groups from the SAL and DA. The elution solvent containing the analytes of interest was directly subjected to derivatization without drying and reconstituting in organic solvents.

4.3.4c SAL and DA derivatization via direct PFBBr alkylation

Derivatization of SAL and DA from the SPE column directly in the eluting solvent minimized the sample loss and oxidation. After elution, the sample was adjusted to a pH of 8.2 using 5-10 µl of 2 N NaOH and 100 µL of 0.2 N potassium phosphate buffer (pH 8.2). PFBBr was used as the primary reagent for the derivatization of both SAL and DA. DIPEA was used to prevent the formation of quaternary ammonium species from “over-alkylation” of SAL, of which would not be the product of interest for analysis. Optimization of this derivatization step was pH, temperature, and reagent concentration dependent. Further discussion on the

procedures used to optimize this step is presented in the succeeding section. As result, approximately 100 µL of 10% PFBBr and 20 µL of 10% DIPEA in acetonitrile were added. The derivatization was performed at 68 °C for 2.5 h with intermittent vortexing, to ensure completeness of the reaction. A schematic summarizing the reaction of R- and S-SAL to their respective tri-PFB derivatives is shown below.

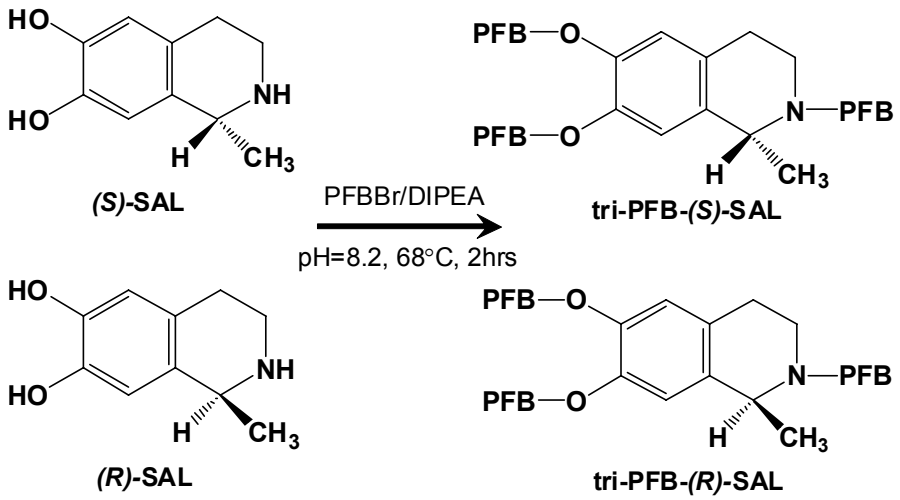


Figure 4-2: Chemical structures and derivatization reaction summary of (R)- and (S)-SAL to their respective PFB derivatives.

Resultant SAL-PFB and DA-PFB derivatives were extracted into a 500 µL hexane phase followed by simple water-hexane partitioning to remove water soluble salt components from the reaction mixture. A 200 µl aliquot of the hexane layer containing the derivatives was removed and evaporated to dryness, reconstituted in methanol, and subjected to chiral phase HPLC/ESI-MS/MS analysis. A schematic of the entire sample preparation is presented in the figure below.

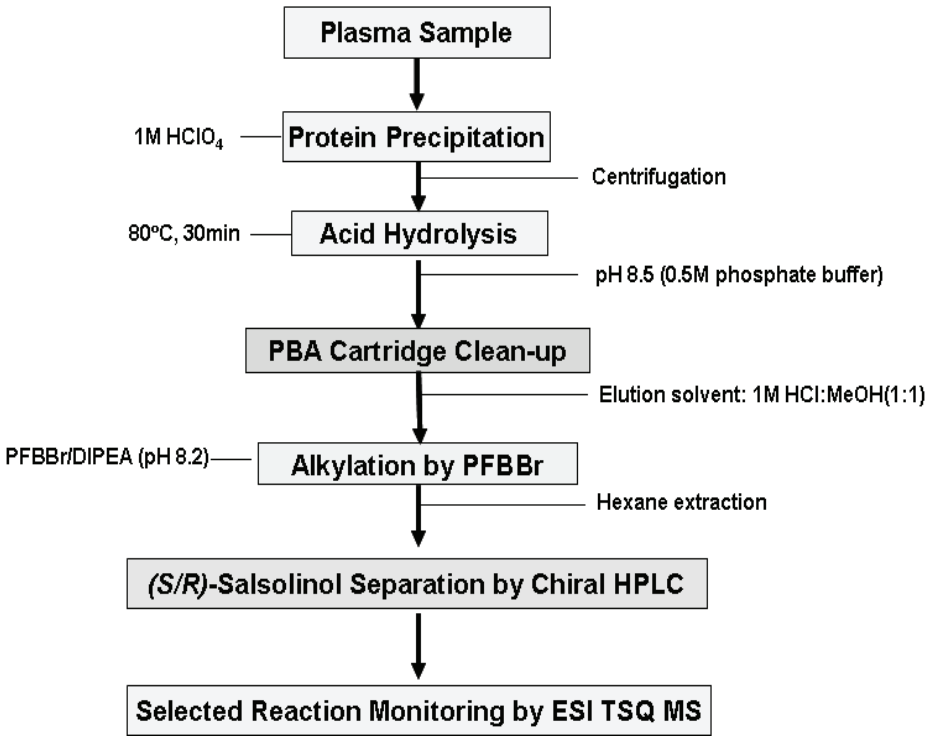


Figure 4-3: Flow chart of sample preparation for the analysis of R/S-SAL and DA from human plasma.

4.3.4d Chromatographic and Mass Spectrometric Conditions

After extraction and derivatization, of the analytes of interest were analyzed via HPLC-MS/MS analysis, with an electrospray ionization source (ESI). Simultaneous chromatographic separation of both derivatized SAL enantiomers and DA, was attained using a Chiralpak AD-H column (2.1 mm x 150 mm, 5 µm). This type of column utilizes a cellulose chiral stationary phase, specifically tris(3,5-dimethylphenylcarbamate)-amylose, to attain separation of enantiomers. The

phenylcarbamate derivative of the optically active amylose polysaccharide exhibits a high chiral recognition capability (Yashima et al., 1995). These types of columns involve a combination of attractive interactions and inclusion complexes to produce separation. Each unit of the amylose phases displays a propeller-type shape and are believed to form helical polymeric structures which combine polar, π-π interactions with inclusion complexation (Ghanem and Naim, 2006). These phases are generally used in the normal phase mode due to the water solubility of cellulose-type stationary phases. For chromatographic separation, an isocratic mobile phase consisting of isopropyl alcohol and methanol (IPA/MeOH, 3:2) at a flow rate of 0.12 ml/min was delivered by the HPLC system.

Detection after chromatographic separation of the derivatized analytes was conducted via MS/MS analysis with an ESI source in the positive ion mode. The heated capillary temperature was set at 350 °C, sheath gas (nitrogen) flow rate at 35 units, auxiliary gas at 5 units, and spray voltage at 3.5 kV. For quantitation, the mass spectrometer was operated in the multiple reaction monitoring (MRM) mode. Collision-induced dissociation (CID) was performed using argon as the collision gas at 1.5 mtorr with relative collision energy set at 35 V for SAL and 28 V for DA. Signal intensity was significantly improved via post-column addition of 1% acetic acid in water (~50 μL/ min) and was employed prior to the detection by MS.

ESI mass spectra of tri-PFB derivatives of SAL and d_4-SAL contained $[M+H]^+$ ion as the base peaks at m/z 720 and m/z 724, respectively. Chromatograms of MRM transitions m/z 720→181, 210, 358 for *(R/S)*-SAL and m/z 724→181, 210, 362 for d_4-

(S)-SAL were used upon initial analysis. For SAL enantiomers the MRM corresponding to the ring cleavage, m/z 720→210 (SAL) and m/z 724→210 (d_4-SAL), was selected to ensure the selectivity of detection. The quantitation of DA was performed by MRM using the transitions of m/z 874 → 497 (DA) and m/z 878 → 501 (d_4-DA). The ESI-MS/MS product ion mass spectra of $[M+H]^+$ produced from tri-PFB derivatives of SAL and d_4-SAL with the tetra-PFB derivatives for DA and d_4-DA are shown in the figures below.

The chromatograms of derivatized *(R/S)*-SAL and d_4-*(S)*-SAL show resolution, *Rs* > 2.2 between SAL enantiomers and that the *(S)*-form (retention time: 8.2 min) eluted prior to the *(R)*-form (retention time: 12.6 min). DA possessed a retention time of 7.9 minutes. Of importance, the inter-conversion, from R to S or vice versa, between two enantiomers did not occur throughout the analysis. The SRM chromatograms of *(R/S)*-SAL further indicated that each isomer was detected with approximately 1:1 relative peak area ratio. The deuterium labeled SAL and DA internal standards were stable during the analysis and there was no evidence of deuterium/hydrogen exchange. Separate MS and MS/MS analyses was performed to test lack of this phenomenon in an aqueous environment over a wide pH range (data not shown).

Even though monitoring all three transitions in the MRM mode for SAL yielded a higher relative intensity, the sensitivity of the assay was not improved due to a compromise in specificity when plasma samples were analyzed. Consequently, the SRM to the ring cleavage, m/z 720→210 (SAL) and m/z 724→210 (d_4-SAL), was selected to ensure the selectivity of detection. Post-column infusion of 1% acetic acid

in water (~50 μL/min) to the main column flow considerably improved the sensitivity (by > 10-fold) by enhancing ionization efficiency while circumventing the adverse effects of and acidic and aqueous environment that may affect the chiral column stability.

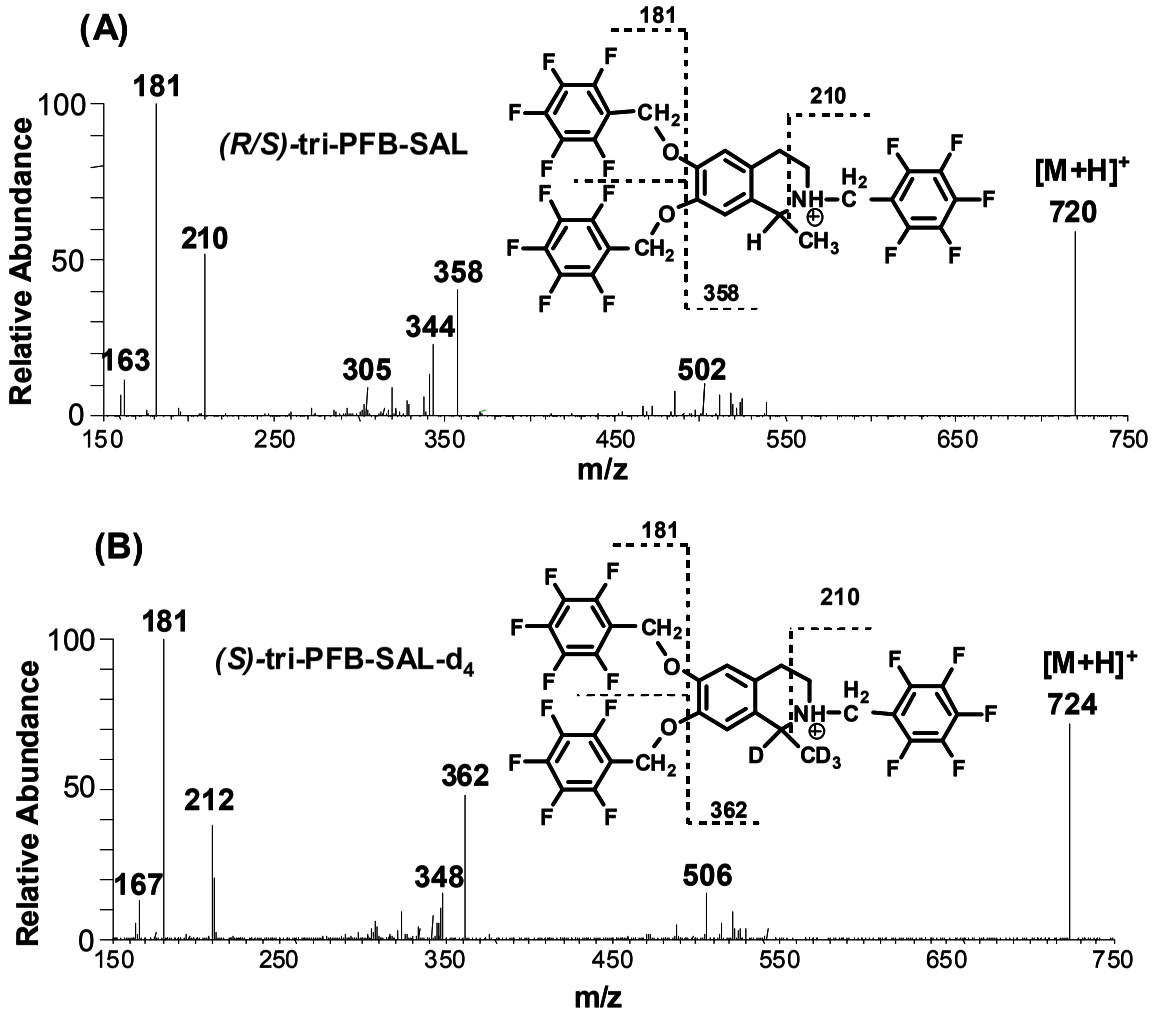


Figure 4-4: ESI-MS/MS product ion mass spectra of $[M+H]^+$ produced from tri-PFB derivatives of a) SAL and b) d_4-SAL.

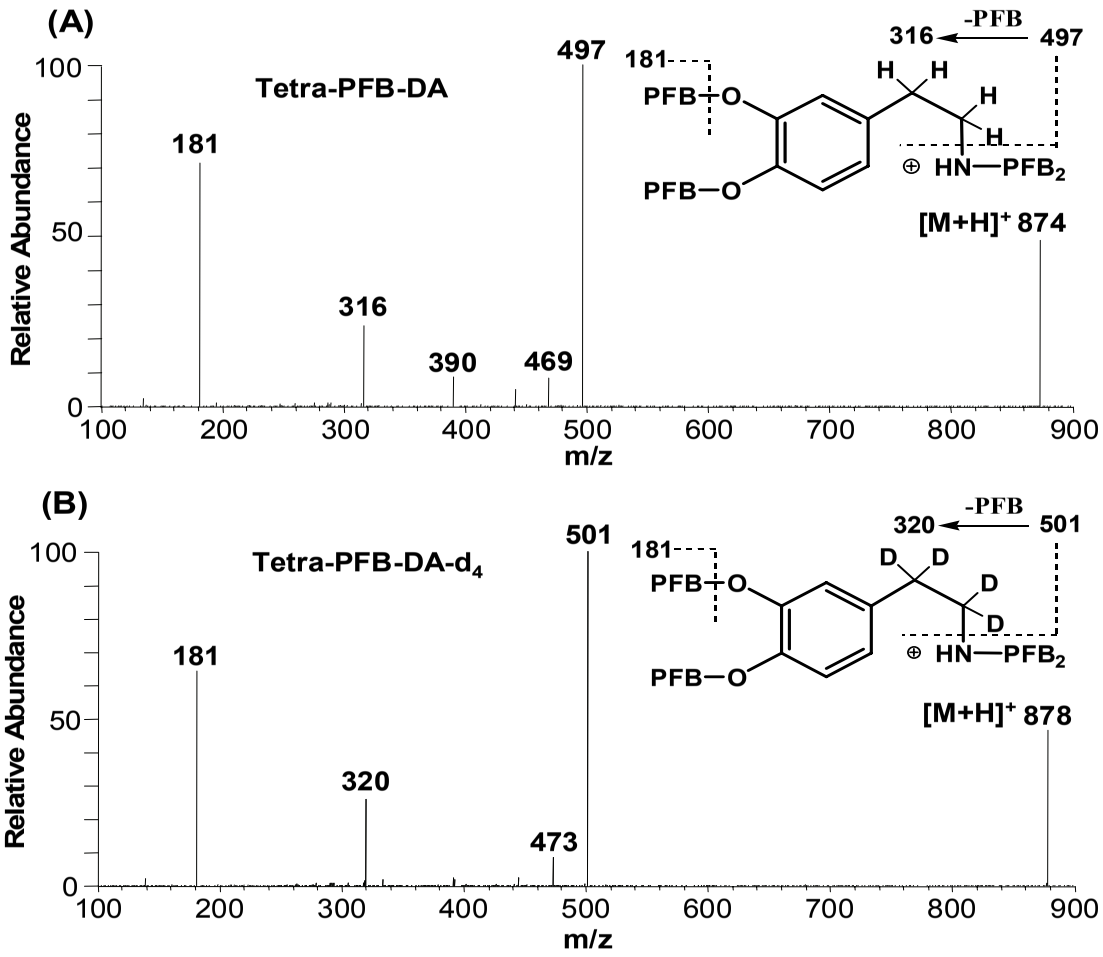


Figure 4-5: ESI-MS/MS product ion mass spectra of $[M+H]^+$ produced from tetra-PFB derivatives of a) DA and b) d_4-D

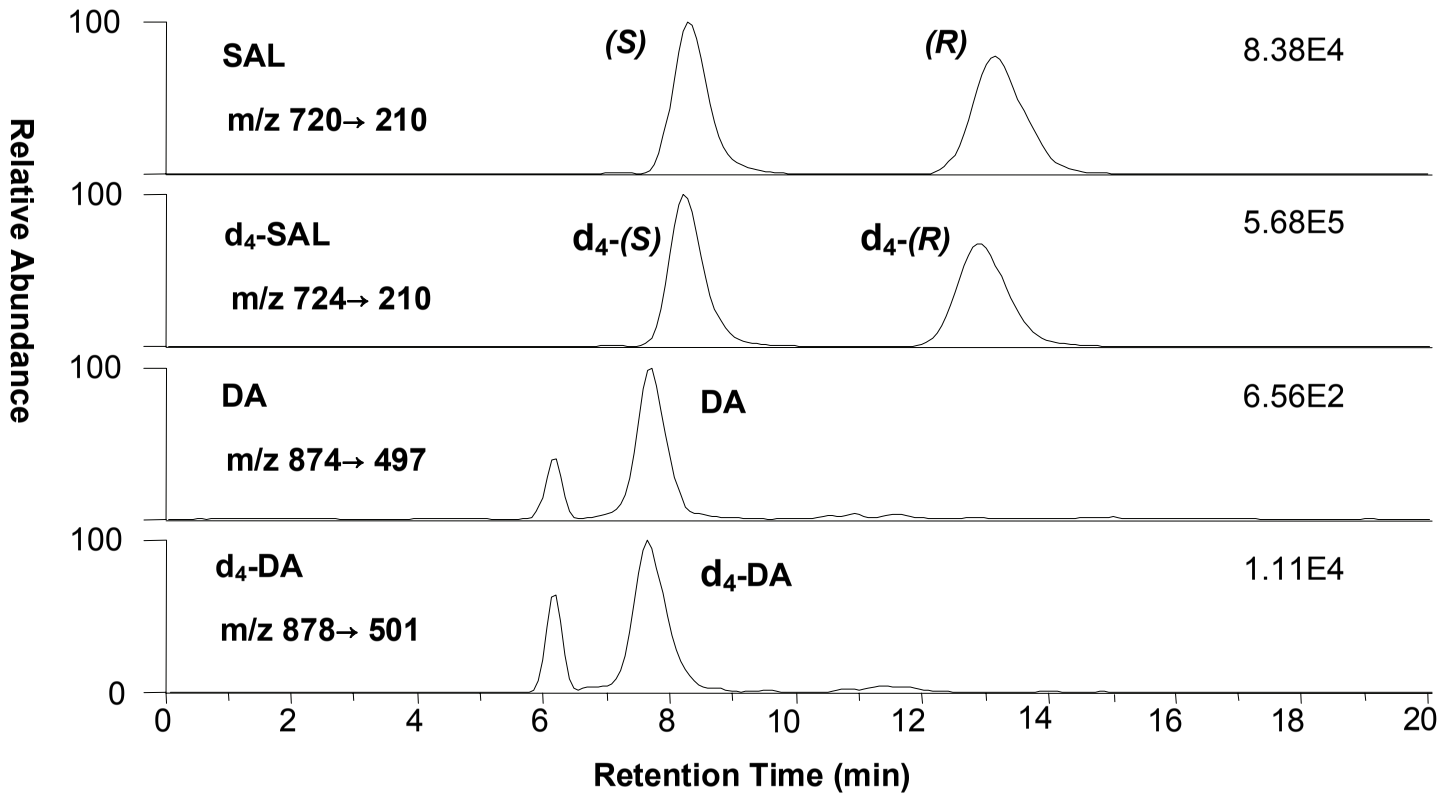


Figure 4-6: Representative SRM chromatograms and retention times of S-SAL (8.2 min) and R-SAL (12.6 min), d_4-R/S-SAL, DA (7.9 min) and d_4-DA.

4.3.4e Method Summary Discussion

Only a few analytical assays for the measurement of total (unconjugated and conjugated) R- and S-SAL in biological samples have been developed, including HPLC with electrochemical detection and GC/MS. Quantitation of the enantiomeric composition in biological samples can be difficult due to inadequate baseline separation of enantiomers, irreproducibility, and lack of internal standard use for extraction techniques. Therefore, the development of analytical techniques for the enantiomeric determination of SAL is indispensable due to its enantioselective occurrence and physiological activities underlying alcoholism.

The selectivity of this method in determining SAL and DA from plasma is superior to those already reported. Utilization of PBA SPE was able to discriminately remove catechol moieties from plasma, namely the SAL enantiomers and DA. Use of this method of extraction of SAL from plasma has been exemplified in the literature. Novel deuterium-labeled R-SAL and S-SAL along with DA allowed for the adequate compensation of analyte loss throughout the assay method. A stable isotopically labeled analogue is believed to be the most appropriate internal standard in a quantitative bioanalytical LC/MS/MS assay. It is assumed that a this type of internal standard compensates for variability in chemical derivatization, sample extraction and LC/MS/MS analysis due to its nearly identical chemical and physical properties to the unlabeled analyte. The derivatization of SAL and DA to its corresponding PFBBr analogues, along with the innovative chiral stationary phase, allowed for sufficient resolution of both SAL enantiomers, in addition to simultaneous separation of DA.

Moreover, racemization of the analytes was not observed, permitting reliable interpretation of pharmacological studies discerning specific roles for each SAL enantiomer.

Selectivity of the assay was reinforced by the detection via ESI-MS/MS. Choosing the appropriate MRM transitions improved the selectivity, whilst maintaining the sensitivity required for ultimate plasma analysis. The product ion spectra of $[M+H]^+$ acquired from the SAL derivatives possessed the major peaks at m/z 181, 210 and 358 for SAL and at m/z 181, 210 and 362 for d_4-SAL, respectively. The most prevalent product ion appeared at m/z 181 corresponding to the PFB fragment ion. The product ions at m/z 358 and 362 were derived from the loss of two PFB groups from the corresponding $[M+H]^+$ ions of SAL and d_4-SAL derivatives, respectively. The product ion at m/z 210 and 210 derived from the ring cleavage with one PFB group on the amine group, was common for SAL and d_4-SAL, respectively. Therefore, the reaction monitoring transition of m/z 720$\rightarrow$210 for SAL and m/z 724$\rightarrow$210 for d_4-SAL was used to ensure the selectivity of detection.

Detection of DA in plasma was also achievable with use of the assay method and LC-MS/MS system. The MS/MS analysis revealed the presence of four derivatives of DA corresponding to three tri-PFB-DA isomers (m/z 694) and a fully derivatized tetra-PFB-DA derivative (m/z 874). Experiments exemplified the tetra-PFB-DA is the major derivative under the conditions employed (data not shown), which was ultimately chosen as the derivative of interest for quantitative analysis of DA. The mass spectrum obtained by CID of tetra-PFB-DA produced major product ions at m/z 181, 316 and

497. The ultimate quantitation of DA was performed by MRM using the transitions of m/z 874 → 497 (DA) and m/z 878 → 501 (d_4-DA).

This novel method was developed at NIH-NIAAA Laboratory of Molecular Signaling. Prior to validation of the method, optimization of the PFBBr derivatization was carried further to ensure high reaction yield and, more importantly, consistent and reproducible derivatization products for the primary analyte(s) of interest, R- and S-SAL. Subsequently, validation of the assay methodology was performed to ensure accurate and precise results for the support of two clinical studies.

4.3.5 PFBBr Derivatization Optimization for R/S-SAL and DA

The efficient separation of the SAL enantiomers required the use of a PFBBr derivatization procedure after SPE from plasma. Derivatization involves a chemical reaction between an analyte and a reagent to change the chemical and physical properties of the analyte of interest. In turn, the method improves detectability and chromatographic separation, thereby enhancing the assay method sensitivity (Snyder et al., 1997). In the case of SAL, the key to the chiral analysis is the ability to react an optically active target with PFBBr, in order to achieve adequate resolution between the enantiomers. The derivatization of SAL enables the chiral recognition by the novel chiral column used for this analysis. The ability of the derivatized analyte and the chiral stationary phase to form transient-diastereomeric complexes utilizing hydrogen bonding, dipole stacking, inclusion complexing and π-π interactions governs the enantioseparation. Therefore, the derivatization step involved in the R- and S-SAL

analysis is of great importance. Of note, successfully resolved enantioseparation of SAL without derivatization has not been reported.

The first step for derivatization for chiral method development is to examine the chemical structure of the analyte and identify information such as solubility in different solvents, hydrogen and π-bonding capability, pKa, functional groups and inclusion-complexing capability. All characteristics, of which, determine the ability for the chiral stationary phase to resolve the sample enantiomers. The ultimate arrangement of the substituent groups, relative to the achiral center plays an important role in the enantiomer separation (Snyder et al., 1997). Recall the structure of SAL. This analyte is primarily water soluble with three important functional groups, two being the hydroxyls of the cis-diol catechols moiety of the molecule. The chiral center at the C-1 position is directly adjacent to the secondary amine present in the molecule. In most cases, the closer a functional group is to the chiral center, the more likely is chiral recognition. Therefore, derivatization of the secondary nitrogen is of greater importance than that of the hydroxyl groups of SAL.

The use of PFBBr was used for its ease and successful use for derivatization of amines and hydroxyl functional groups that are present in SAL. PFBBr converts carboxylic acids, mercaptans, phenols, and sulfonamides to halogenated derivatives that are easily detected by electron capture. Electron capturing esters are popular for gas chromatographic analyses of short chain fatty acids. Specifically, PFBBr has been effectively used as a derivatizing agent for GC analysis of polyfunctional thiols (Montanari et al., 2006). In addition, this reagent was used for the preparation of

pentafluorobenzyl esters of organic acids for determination by capillary (Cataldi et al., 1999) and GC (Husek et al., 2008). PFBBr is used in extractive alkylation (simultaneous extraction and derivatization), in conjunction with tetrabutylammonium hydrogen sulfate as the counterion an ion-pairing reagent (Sigma-Aldrich, 1999). Pentafluorobenzylation by alkylation gives derivatives of phenols, carboxylic acids, and sulfonamides to create esters, ethers, alkyl amines and alkyl amides. The specific alkylation reaction reduces molecular polarity by replacing active hydrogens with an alkyl group. The principal reaction employed for preparation of these derivatives is nucleophilic displacement (Knapp, 1979).

PFBBr is generally used to convert organic acids into esters. Of note, the primary functional moiety of interest in the secondary nitrogen of SAL because its proximity to the chiral center. As the acidity of the active hydrogen decreases, the strength of the alkylating reagent must be increased. The harsher the reaction conditions or reagents, the more limited the selectivity and applicability of this method. For these reasons, optimization of the derivatization reaction was pertinent. Some advantages of PFBBr derivatization include 1) reaction conditions can vary from strongly acidic to strongly basic, 2) some reactions can be done in aqueous solutions, and more importantly , 3) the alkylation derivatives are generally stable (Sigma-Aldrich, 1999). The disadvantages include sometimes severe reaction conditions and toxicity of PFBBr (this reagent is a strong lachrymator). Nevertheless, PFBBr is an effective reagent for alkylation of amines and acidic hydroxyls, as those part of the SAL structure.

The following experiments were designed to optimize the derivatization of SAL with PFBBr. As the primary goal of the optimization, the derivatization of the secondary nitrogen of SAL was imperative for chiral recognition.

4.3.5a Methods

Prior to optimization of the PFBBr-SAL reaction, preliminary information about the reaction was obtained from the literature. The environment of the reaction was to take place in the elution solvent from the PBA extraction (0.1 M HCl:MeOH = 1:1), which is highly acidic and primarily aqueous in nature. SAL is known to possess two types of functional groups that may be derivatized. The hydroxyl groups of the catechol moiety are primarily unionized while the secondary nitrogen is in its ionized form in this acidic milieu. This characteristic suggests that pH dependency of the reaction is important for a high and reproducible reaction yield. The physicochemical characteristics of both PFBBr and SAL differ greatly where PFBBr is highly lipophilic as opposed to the analyte. A consideration on use of a dispersion agent in order to ensure the interaction and subsequent reaction of PFBBr and SAL was necessary because of the differing characteristics. The structure of PFBBr is shown below.

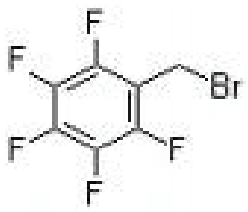


Figure 4-7: Chemical structure of pentafluorobenzyl bromide (PFBBr).

As PFBBr has been used extensively for the derivatization for GC detection, molar ratios of reagent to the functional groups have been thoroughly explored. An approximate molar ratio of 1 to 0.3 (reagent to phenols) is necessary for derivatization of the catechol portion of SAL (Sigma-Aldrich, 1999), while the secondary amine requires a molar ratio of 1.1 to 1 (reagent to secondary amine) for alkylation to occur (Moore et al., 2005).

Of importance is the alkylation of the secondary amine with the PFBBr alkyl halide to form a tertiary amine. From a methodological view, direct alkylation to the tertiary amine is straight-forward but has been somewhat limited. Direct N-alkylation of secondary amines often results in the formation of the quaternary ammonium salts and a mixture of the desired tertiary amine and the starting secondary amine (Moore et al., 2005). In turn, derivatization yields of the desired products have been low and irreproducible. The use of a Huenig base, *N,N*-Diisopropylethylamine (DIPEA), has been proven to hamper the formation of quaternary ammonium salts to yield desired tertiary amines from alkylation of secondary amines (Moore et al., 2005). This

compound is a good base but a poor nucleophile, which makes it a useful organic reagent. The recommended molar ratio of secondary amine to DIPEA to alkyl halide is (1 to 1.5 to 1:1) in acetonitrile at room temperature.

With prior information about the PFBBr reaction with the functional groups of SAL, experiments were designed to evaluate several factors to construct a reproducible and high reaction yield. In a univariate manner the following factors were explored to improve the reaction equilibrium of the alkylation in elution solvent (1M HCl: MeOH), in priority order:

1. molar ratio of total SAL to PFBBr (1:3)
2. molar ratio of SAL:PFBBr with respect to the Huenig base, DIPEA, (1 : 3 : 1.5)
3. use of a dispersion agent: (i.e., acetonitrile, chloroform, hexane, benzene)
4. pH dependency, buffer type and concentration
5. time dependency (15 min, 1, 2, 3, 6, 8, 12, and 24 hours)
6. temperature dependency (RT, 40, 50, 60, 70, 80, 100°C)

For formal evaluation, relatively high concentrations of total SAL and d_4-R/S-SAL were prepared in 1.5 ml of elution solvent (total of 2 ng for total internal standard and 4 ng of total analyte). Throughout the experiments it was assumed that smaller concentration would yield similar results. All experiments were performed in triplicate to assess precision of the reaction conditions. The formation of the desired tri-PFB-SAL product was identified by GC/NCI-MS. Reversed phase HPLC/ESI-MS was further used to confirm results. Of note, total SAL and total d_4-SAL was used for the

interpretation of results. The individual SAL enantiomers were not evaluated for reaction yield.

Prior to optimization, initial reaction conditions included those that have been reported (Knapp, 1979; Moore et al., 2005). Initial reaction conditions in the elution solvent included excess of reagent to SAL ratio (1:100), at pH 7.0, for 2 hours at 68ºC. For PFBBr: SAL reaction ratio in elution solvent, molar ratios of 1:1, 1:3, 1:10 and 1:1000 were varied from initial conditions. Conditions for reaction include without and with varying concentrations of the DIPEA Huenig base. As the physicochemical characteristics of the SAL and PFBBr differ greatly, dispersion agents such as acetonitrile, chloroform, hexane, and benzene were evaluated for improvement of reaction yields. In the elution solvent, pH ranging studies were performed by adjusting pH at the following values: 7.0, 7.5, 8.0, 8.2, 8.5, 9.1, 9.5, and 10.0. 1N NaOH was used to adjust to the desired pH and varying concentrations of phosphate and borate buffer were used to maintain pH. Reaction time and temperature dependency experiments were performed for 15 min, 1, 2, 2.5, 3, 6, 12 hours and room temperature, 40, 68, 80, 100°C, respectively. In priority order the factors were varied and optimal results were carried through for evaluation of the next factor.

Throughout each experiment the SAL was subsequently extracted with 500 µl of hexane, washed with 2 ml water to remove salts and unwanted polar products. A 200 µl aliquot was dried down and reconstituted in 20 µl of methanol and subjected to HPLC-MS/MS analysis. Further confirmation was performed by GC/NCI-MS and PCI-MS.

4.3.5b Results

Excess reagent was used for the reaction of PFBBr and SAL to result in a >90% reaction yield. Approximately 100 µl of 10% PFBBr in acetonitrile reagent was used for reproducible reaction equilibrium. The molar ratio of PFBBr to SAL for this reaction was approximately 1000:1. DIPEA was necessary to produce a precise derivatization yield in which a 20 µl of 10% DIPEA in acetonitrile reagent was used to prevent formation of the quaternary ammonium species. As both reagents were dissolved in acteonitrile, additional use of a dispersion agent was not needed for the reaction. Consistent yields were observed with or without the use of additional acetonitrile, chloroform, hexane, or benzene.

Upon evaluation, the pH dependency of the reaction yield ad reproducibility proved important. The result of the pH dependency study is shown in the figure below. The final pH utilized for the reaction was 8.2 using 100 µl of 0.2M K_2HPO_4/KH_2PO_4 buffer. Apparently, at this pH both phenol and amine groups of SAL were deprotonated for efficient alkylation, and yet degradation of SAL and PFBBr was minimal. For the reaction to occur, the pH of the environment needed to be between pH 7 and 9.5. Relatively large and precise reaction yields were observed at between pH 8.0-8.2. The reaction time and temperature dependency experiments yielded consistent results at a time of 2.5 hours at 68°C. The results of the pH ranging experiment are shown in the figure below.

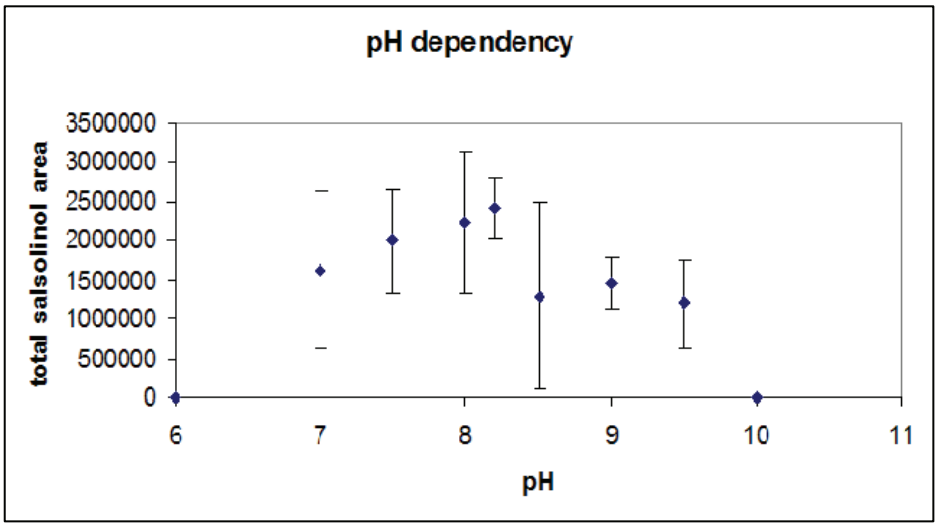


Figure 4-8: pH dependency of the PFBBr – SAL derivatization reaction in 1.5 ml 1M HCl:MeOH (1:1). Presented as pH vs. total SAL area (mean ± SD). Reaction conditions: 100 µl of 10% PFBBr and 20 µl of 10% DIPEA in acetonitrile, for 2 hours at 68°C.

Under the optimized conditions, the extracted ion chromatograms from GC/NCI-MS analysis indicated that the desired tri-PFB-SAL was the predominant product while the mono- and di-derivatized forms were the minor components. The positive ion spectrum obtained by GC/PCI-MS contained $[M+H]^+$ at m/z 720. Reversed phase HPLC/ESI-MS and HPLC/ESI-MS/MS analyses confirmed the results obtained by GC/MS analyses (data not shown). The reaction products were stable for > 8 hours at room temperature and > 48 hours in -20°C. The final desired SAL derivative is shown in the figure below.

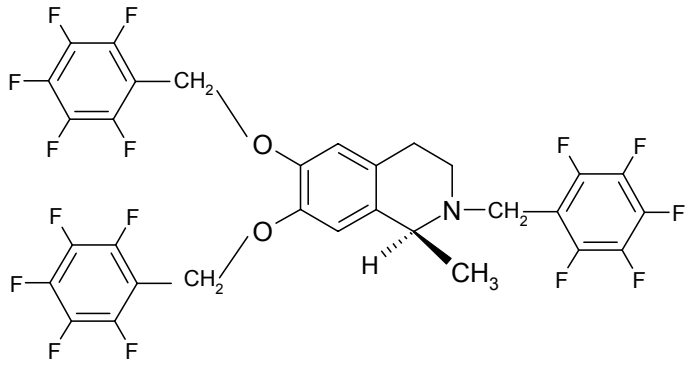


Figure 4-9: Structure of the desired S-SAL-PFB_3 (MW = 719) reaction product under optimized derivatization conditions.

4.3.5c Discussion

Derivatization conditions vary widely, depending upon the specific compounds being derivatized. If derivatization is not complete under the recommended procedures, the addition of a catalyst, use of another solvent, pH control, higher reaction temperature, longer reaction time, and/or higher reagent concentration should be evaluated. Experiments confirmed that, in presence of excess PFBBr reagent, both DIPEA and pH dependency were important for formation of the desired SAL product. The use of DIPEA for the formation of tertiary amines via alkylation has been exemplified in other reports. The distinctive role of DIPEA in preventing the quaternization is not well understood. It can be implicitly understood that this non-nucleophilic strong base forms a salt with the released hydrogen halide permitting the reaction to proceed under kinetically restricted conditions. In this case, the reaction of

the secondary amine with the starting alkyl halide may be faster than the reaction of the tertiary amine product with the starting alkyl halide. Of note, higher concentrations employed of DIPEA resulted in a decrease of reaction yield. This is presumed to be a result of an increase in unfavorable side reaction products of PFBBr with DIPEA. Moreover, DIPEA is a strong base that may cause the lack of an appropriate pH buffering in the elution solvent.

The influence of pH has a profound effect on the derivatization reaction. As the desired product involved the simultaneous deprotonation of the both the catechol and secondary amine, pH effects were expected. At lower pH, SAL functional groups are primarily protonated hindering the preferred alkylation. At higher pH (> pH 9) the reaction yield was minimal to none for the desired product. In a basic milieu, both SAL (Haber et al., 1996) and PFBBr (Gyllenhaal, 1978) are reported to be unstable which would explain the lack of the preferred reaction product. The pH ranging study suggests that a tight regulation of the pH (between 8.0 – 8.2) is necessary for the reproducible and high (>90%) reaction yields. The buffer concentration used in the reaction was strong enough to maintain and adequate buffering capacity (~30% of maximum). As a phosphate buffer was utilized for the reaction, it was imperative that a wash step be employed to the hexane extract to prevent instrumental exposure to phosphate salts. Additional inclusion of a dispersing agent or phase transfer catalysts was not necessary for the reaction equilibrium.

These experimental conditions were also chosen for the simultaneous derivatization of DA. The HPLC/ESI-MS/MS analysis revealed presence of four

derivatives of DA corresponding to three tri-PFB-DA isomers (m/z 694) and a fully derivatized tetra-PFB-DA derivative (m/z 874). Since the tetra-PFB-DA is the major derivative under the optimized SAL derivatization conditions, we chose this form for quantitative analysis of DA. The mass spectrum obtained by CID of tetra-PFB-DA produced major product ions at m/z 181, 316 and 497. The characteristic fragment ion at m/z 497 resulted from the loss of NH-$(PFB)_2$ from $[M+H]^+$ ions as depicted in figure 4-5.

4.3.5d Conclusion

In a primarily aqueous environment both SAL and DA were able to be derivatized by PFBBr to its tri-PFB and tetra-PFB products, respectively. Structural confirmation of the products was evaluated via separate analytical methods to ensure the reaction yield. The optimal conditions used for subsequent analytes in plasma included a favorable molar ratio of analyte to PFBBr to DIPEA. With an unyielding control of pH, the desired products were derivatized with high and reproducible yield. The summarized method, along with the optimized derivatization reaction was subsequently used for the validation of R/S-SAL and DA quantification in human plasma.

4.3.6 Validation of the Assay Method in Human Plasma

Discussion of analytical method validation has been discussed in the prior chapter for the β-carboline HPLC-FD assay in human plasma. The approaches for

validation of the R/S/-SAL assay presented henceforth includes the assessment of selectivity, sensitivity, accuracy, precision, reproducibility and precision. Other parameters of interests that will be investigated include those of extraction efficiency, calibration range, matrix effects, a dilution integrity, and response function.

Similar special considerations are required for the validation of the R/S-SAL assay as the β-carbolines for quantification of constitutive SAL components in a biological matrix. R/S-SAL are endogenous compounds with quantifiable baseline levels in the biological matrix of interest, therefore the nature of biomarkers posts a challenge to find analyte-free biological matrix to prepare calibrator standards. For this reason, the "surrogate matrix" approach for calibration was also used for the R/S-SAL quantification.

Along with formal validation of the R/S-SAL method, this section will investigate the similarity of the analytical concentration-responses relationships between the unadulterated sample matrix and the surrogate matrix. All reported methodologies used for the quantification of R/S-SAL and DA in biological matrices utilized a neat buffered matrix for validation purposes, without the formal assessment of a matrix effect. As the hindrance of constitutive SAL and DA poses an issue with calibration curve construction, a strategy limiting or completely eliminating the background was investigated.

Moreover, if alteration of the plasma matrix is necessary, the response behavior (i.e., slope) of the calibration curves between the unadulterated and the altered matrix needs to be statistically indistinguishable to judge matrix surrogacy. "Matrix effect"

should not be present upon testing the calibration between the two matrices. Only until surrogacy is proven, quantification using the modified matrix may be performed.

Considering the issues of endogenous R/S-SAL and DA background and the lack of a true blank matrix, the same strategy to that of the β-carbolines was employed for this assay for bioanalytical validation in human plasma. The strategy for the bioanalytical validation of the R/S-SAL and DA assay is as follows:

1. Preliminary exploration of the assay was evaluated in a pooled plasma matrix, with the parameters such as precision, calibration range, and selectivity being assessed. Moreover, the type of calibration function (i.e., linear or nonlinear) used was assessed at this point. The primary objective was to determine, in an unadulterated matrix, the precision of the bioanalytical assay throughout a given concentration range. Accuracy was expected to be confounded by the constitutive concentrations of SAL and was not scrutinized during the pooled plasma analysis. Short-term and long-term stability studies were conducted in the unadulterated pooled plasma.
2. Exploration of a suitable surrogate matrix included destruction of constitutive SAL and DA in pooled plasma, to create a "blank" matrix. The pooled plasma matrix was modified until devoid of a significant SAL and DA chromatographic signal, while minimizing the difference of composition in the true matrix. Subsequently, SAL and DA were spiked into the modified matrix to assess the validation parameters aforementioned. Full calibration curves (n = 6) and quality control samples were used to evaluate the linear range, accuracy and precision. Of

importance, the Lower Limit of Quantification (LLOQ) was determined during this step using n = 6 non-calibration point replicates. Recovery assessment was evaluated using this matrix to assess the relative recovery and, more importantly, the precision.

3. Upon calibration curve evaluation of both pooled plasma and modified plasma matrix, n = 5 individual, unmodified plasma sources were subsequently spiked with a range of SAL and DA standards. Owing to the different constitutive concentrations of SAL and DA in the individual plasma sources, it was expected that the intercepts for each calibration regression would differ. Therefore, precision was the primary assessment in this portion of the investigation.
4. To appraise surrogacy of the modified plasma matrix, parallelism studies were performed. The slopes of all calibration curves from pooled plasma matrix, modified matrix and the n = 5 different sources were to be statistically similar (parallel) in order to deem the modified matrix and appropriate "surrogate" matrix. If statistically different, the method was considered to have a significant matrix effect and issues of selectivity. In the case that selectivity was of concern, MS detection or chromatographic re-optimization experiments were conducted to rid the interference.
5. For all successive experiments, including ultimate sample analysis, the calibration curves and quality control samples were to be constructed using the "surrogate" plasma matrix.

Comprehensive discussion for the use and validation of a "surrogate matrix" is summarized in the previous chapter.

4.3.6a Materials and Reagents

All chemicals were or analytical grade quality and obtained from commercial sources. All solvents used for the R/S-SAL and DA validation analysis were of spectroscopic or HPLC grade. The validation required the materials and reagents listed in section 4.3.1 of this chapter.

4.3.6b Equipment

The analytical equipment used for validation of the assay is presented in section 4.3.2 of this chapter.

4.3.6c Preparation of Solutions and Standards

(S)-SAL and (R)-SAL enantiomers are not separately available, standard solutions of SAL were prepared by dissolving the racemic mixture of (R/S)-SAL-HCl in methanol. Standard stock solutions of (R/S)-SAL-HCl as well as DA-HCl were prepared in methanol at a concentration of 1 mg/ml (1μg/μl) in amber, silanized vials. Further dilutions were created to obtain lower concentrations, if needed. The deuterated internal standard stock solutions were additionally made in methanol at a concentration of 100 ng/ml for (S)-SAL-d_4-HBr and (R)-SAL-d_4-HBr and 1 mg/ml for d_4-DA-HCl. Of Stock standard solutions were stored in darkness at -20 °C until further use. The

working internal standard solutions were prepared by further dilution of the stock solutions to 500 pg/ml of each SAL-d_4 enantiomer and 500 pg/ml of DA-d_4. From the primary stock solutions, 1-ml volumes of calibrators and quality control standards were created to ensure that the mass required for each level was transferred into the matrix via 10 µl.

Table 4-2: Preparation of SAL calibrator and quality control standards. Standards were prepared at concentrations to ensure that a 10 µl volume was transferred into the matrix of interest. SAL concentrations reported are individual enantiomers.

STD	Final STD conc	amount of STD	methanol
4000 pg	400.0 pg/µl	800 µl (500 pg/µl)	200 µl
2000 pg	200.0 pg/µl	400 µl (500 pg/µl)	600 µl
1200 pg	120.0 pg/µl	240 µl (500 pg/µl)	760 µl
1000 pg	100.0 pg/µl	200 µl (500 pg/µl)	800 µl
750 pg	75.0 pg/µl	150 µl (500 pg/µl)	850 µl
500 pg	50.0 pg/µl	100 µl (500 pg/µl)	900 µl
200 pg	20.0 pg/µl	40 µl (500 pg/µl)	980 µl
160 pg	16.0 pg/µl	32 µl (500 pg/µl)	968 µl
100 pg	10.0 pg/µl	20 µl (500 pg/µl)	980 µl
50 pg	5.0 pg/µl	10 µl (500 pg/µl)	990 µl
20 pg	2.0 pg/µl	4 µl (500 pg/µl)	996 µl
10 pg	1.0 pg/µl	2 µl (500 pg/µl)	998 µl

Table 4-3: Preparation of DA calibrator and quality control standards. Standards were prepared at concentrations to ensure that a 10 µl volume was transferred into the matrix of interest.

STD	Final STD conc	amount of STD	methanol
20000 pg	2000.0 pg/µl	20 µl (10 ng/µl)	980 µl
10000 pg	1000.0 pg/µl	1000 µl (1 ng/µl)	0 µl
8000 pg	800.0 pg/µl	800 µl (1 ng/µl)	200 µl
5000 pg	500.0 pg/µl	500 µl (1 ng/µl)	500 µl
4000 pg	400.0 pg/µl	400 µl (1 ng/µl)	600 µl
2000 pg	200.0 pg/µl	200 µl (1 ng/µl)	800 µl
1000 pg	100.0 pg/µl	100 µl (1 ng/µl)	900 µl
600 pg	60.0 pg/µl	60 µl (1 ng/µl)	940 µl
500 pg	50.0 pg/µl	50 µl (1 ng/µl)	950 µl
200 pg	20.0 pg/µl	20 µl (1 ng/µl)	980 µl
100 pg	10.0 pg/µl	10 µl (1 ng/µl)	990 µl

All other solution prepared for the analysis is presented in section 4.3.3 of this chapter.

4.3.6d Methods

Validation of the bioanalytical assay was performed using optimized ideal extraction and derivatization conditions. The HPLC-ESI-MS/MS parameters are denoted in method summary previously reported in this chapter and by Lee and colleagues (Lee et al., 2007). A table summarizing the parameters is presented in the table below.

Table 4-4: Experimental conditions used for validation of the HPLC-ESI MS/MS bioanalytical assay for R/S-SAL and DA in a human plasma matrix.

Variable	Condition
HPLC System	Agilent 1100 system (autosampler/pump)
Column w/guard column	Chiralpak AD-H (2.1x 150 mm, 5 μm) protected with a guard column
Solvents A and B (normal phase)	
%A	50% isopropyl alcohol
%B	50% methanol
Flow rate	Isocratic, 150 μl/min
Column, sample temperature	room temperature, 5ºC
Injection volume	10 μl
Run time	20 minutes
Post column addition	1% CH_3COOH in H_2O, ~50 μl/min
MS/MS System	Thermo-Finnegan TSQ Ultra
Ionization	ESI – positive ion mode
Capillary temperature	150°C
Sheath, Auxiliary gas	N_2 flow rate, 35 units, 5 units
Spray voltage	3.5 kV
Collision-induced dissociation (CID)	Argon @ 1.5 mTorr in the second (rf only) quadrupole
Relative collision energy	35 eV
Detection (Selective Reaction Monitoring)	R/S-SAL m/z 720→210 d_4-R/S-SAL m/z 724→210 DA m/z 874→497 d_4-DA m/z 878→501

Another major distinction from the previous reports incorporated the use of an antioxidant/aldehyde trapping agent. Several investigators noted a major concern in the quantitative analysis of TIQ alkaloids is the suppression of artifactual formation during sample preparation. The successful use of semicarbazide as an aldehyde trapping reagent and ascorbic acid as an antioxidant in decreasing artifactual formation has been exemplified (Rommelspacher et al., 1984; Adell and Myers, 1994; Fekkes et al., 2004). Therefore, the protein precipitation/antioxidant solution describes in section 4.3.3 was implemented in the extraction of R/S-SAL from human plasma to circumvent any issues pertaining to artifactual formation.

Stock solution stability for R/S-SAL and DA, deuterated and non-deuterated were performed and did not vary at -20°C for 6 months. Stock solution and benchtop stability for both analytes and internal standard at two different concentrations was assessed for 6 hours at room temperature and no variation was seen. The plasma samples used for validation were subjected to three freeze and thaw cycles during which no appreciable degradation of R/S-SAL or DA was observed (<2%, data not shown).

4.3.6d-1 Pooled plasma experiments

Using pooled plasma (presumably healthy, fasting drug free, non smokers, collected in EDTA) initial partial validation experiments were explored to evaluate the linear concentration range for R/S-SAL along with precision and stability of the method. Of note, evaluation of DA in pooled plasma was not performed. Further

calibration assessment of DA was involved in subsequent testing with surrogate and individual plasma matrices.

The linearity of the developed method was evaluated by preparing n=3 standard curves for the R/S-SAL analytes in duplicate (2 x 1 day) with 1 ng of d_4-R/S-SAL. The concentrations of R/S-SAL used for a seven-point standard curve were 20, 50, 100, 500, 1000, and 2000 and 4000 pg/ml for each enantiomer. Moreover, along with at least 8 non-zero standards, a blank sample (matrix processed without internal standard) and a zero sample (matrix processed with internal standard) were processed. A 10 µl volume of standard calibrator solutions were used at each concentration level to ensure that the volume of the stock solution added was <10% of the total matrix volume. Experiments were performed using 1-ml of human pooled plasma. Plots of peak area ratio (R-SAL/d_4-R-SAL and S-SAL/d_4-S-SAL) against analyte concentration were constructed. Plots of the residual against concentration assessed the behavior of the response variance across the calibration range. If the residuals for the linear regression analysis were heteroschedastic in nature, an appropriate weighting factor was investigated (1/x and $1/x^2$).

The calibration function (e.g., linear or weighted-linear) was established through observation of reverse calculated standard concentrations of which were reverse predicted from the curve. The appropriateness of the concentration of internal standard for R/S-SAL was based on the peak height of the internal standard being approximately at the mean of analyte response of the concentration range tested. Moreover, the analyte to internal standard ratio at the upper and lower limits were not to exceed 10 or

be less than 0.1, respectively, in order to minimize error associated at the extremes of the linear calibration curve.

The linear range was subsequently tested in the pooled plasma with n = 3 replicates (1 calibration curves x 3 days) to assess precision of the method. Moreover, quality control samples for SAL were created at the following levels: Low QC: 125 pg/ml, Medium QC 1: 600 pg/ml, Medium QC 2: 1200 pg/ml and High QC: 2500 pg/ml for each SAL enantiomer. The QC concentrations were used to assess relative recovery from the pooled plasma. The comparison of an extracted to unextracted analyte to internal standard ratio, at each concentration, was used to calculate the relative recovery. Experiments were performed in triplicate.

It was suspected that a systematic bias (positive y-intercept) would be present in the calibration curve because of the endogenous R/S-SAL. Therefore, accuracy and precision calculations were performed on the analyte to internal standard ratio, across the concentration range. Accuracy and precision assessment and acceptance criteria for the calibration range along with the QC samples were calculated as seen in the previous chapter.

For long-term stability, standard solutions using a high, low, and zero (unspiked) control concentrations in biological matrix were portioned and stored under the conditions of study sample storage (-80˚C). Measurements were taken in triplicate over a six-month period on three separate occasions. For each sample, the ratio of the analyte to internal standard was recorded and concentration back-calculated and compared between measurements and across occasions for statistical deviations

(accuracy and precision within ±15 % nominal value). Freeze-thaw stability was assessed over three cycles, in which the initial freeze was 24 hours and subsequent cycles was held in -80˚C for 12 hours.

Of primary importance, the linear regression parameters for the calibration curves, including slope and y-intercept, for the pooled plasma validation were assessed upon evaluating the estimates and corresponding 95% confidence interval (CI) along with the precision of the slope and y-intercepts (% COV) for R/S-SAL.

4.3.6d-2 Pooled plasma results

Using 1-ml of plasma, a linear concentration range between 10.0 pg/ml and 4.0 ng/ml was seen for both R-SAL and S-SAL and between 100 pg/ml and 20 ng/ml for DA. According to the "blank" sample (no IS) measurements, there were no significant peaks associated with the retention time and SRM of the SAL-d_4 and DA-d_4, exemplifying a lack of interference with the internal standard. Significant peaks at the representative R-SAL, S-SAL and DA retention times were present (data not shown). Using the calibration concentration range of R/S-SAL with 1 ng d_4-R/S-SAL, (each enantiomer) n = 3 calibration curves were constructed in pooled plasma with quality control samples. Area ratios of the calibration points and quality control samples with precision estimates (%COV) are presented in the following tables for S-SAL (table 4-5) and R-SAL (table 4-6).

The individual linear regression parameters for each calibration curve for both R/S-SAL and DA are presented with a goodness of fit (R^2) metric for linearity. An

average calibration curve is presented for both S-SAL (figure 4-10) and R-SAL (figure 4-11). Of note, the calibration curve for both SAL was constructed upon calculation of an average response (across n=3 calibration levels).

Table 4-5: Peak area ratio for S-SAL in pooled plasma (n=3), with precision assessment (%COV) and calibration curve parameters ($1/x^2$ weighting).

pg S-SAL	calib #1	calib #2	calib #3	average	sd	%COV
20	0.583	0.572	0.616	0.590	0.023	3.88
50	0.771	0.788	0.792	0.784	0.011	1.42
100	1.172	1.155	1.303	1.210	0.081	6.69
500	1.732	1.653	1.782	1.722	0.065	3.78
1000	2.447	3.069	2.826	2.781	0.313	11.27
2000	4.810	4.530	4.784	4.708	0.155	3.29
4000	8.920	8.489	8.122	8.511	0.399	4.69
125 (LQC)	1.023	1.044	1.230	1.099	0.114	10.37
600 (MQC1)	1.827	1.745	1.801	1.791	0.042	2.33
1200 (MQC2)	2.625	2.598	2.929	2.837	0.184	6.47
2500 (HQC)	5.233	5.001	5.627	5.287	0.316	5.99
zero	0.433	0.455	0.412	0.433	0.022	4.96
slope	2.5300	2.6060	2.5982	2.5781	0.0418	
y-int	0.5127	0.4256	0.4872	0.4251	0.0448	
R2	0.9928	0.9990	0.9891	0.9936	0.0050	

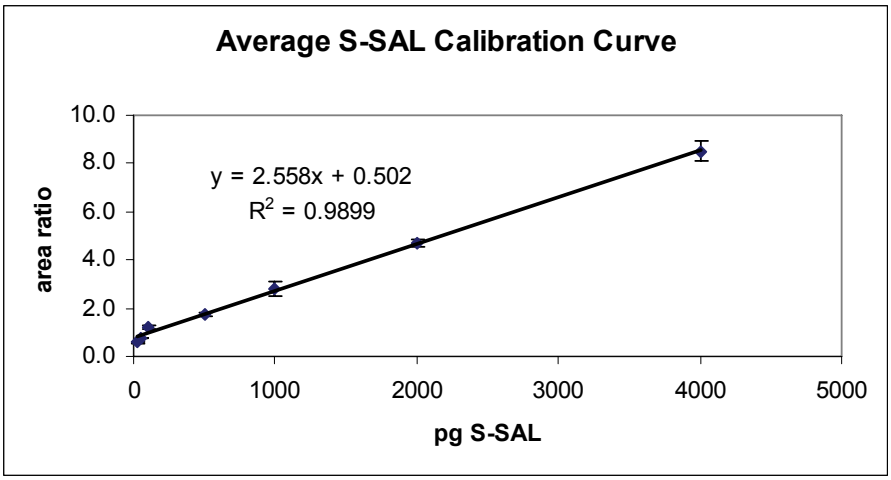


Figure 4-10: Average calibration curve (n=3) for S-SAL in pooled plasma. Linear regression performed on the average of the response across concentrations. Each point represents the average response ± SD.

Table 4-6: Peak area ratio for R-SAL in pooled plasma (n=3), with precision assessment (%COV) and calibration curve parameters ($1/x^2$ weighting).

pg R-SAL	calib #1	calib #2	calib #3	average	sd	%COV
20	0.896	0.853	0.802	0.850	0.047	5.54
50	1.094	1.172	1.266	1.177	0.086	7.32
100	1.523	1.255	1.272	1.350	0.150	11.11
500	2.171	2.526	2.727	2.475	0.281	11.37
1000	3.083	3.192	3.626	3.300	0.287	8.71
2000	4.945	4.992	4.819	4.919	0.089	1.82
4000	9.798	9.517	10.244	9.853	0.367	3.72
125 (LQC)	1.583	1.526	1.552	1.554	0.029	1.84
600 (MQC1)	2.292	2.335	2.371	2.333	0.040	1.70
1200 (MQC2)	3.227	3.524	3.223	3.325	0.173	5.19
2500 (HQC)	5.934	6.102	6.533	6.190	0.309	4.99
zero	0.671	0.626	0.653	0.650	0.023	3.51
slope	2.1120	2.2810	2.0182	2.1371	0.1332	
y-int	0.6270	0.6521	0.6132	0.6308	0.0197	
R2	0.9988	0.9892	0.9817	0.9899	0.0086	

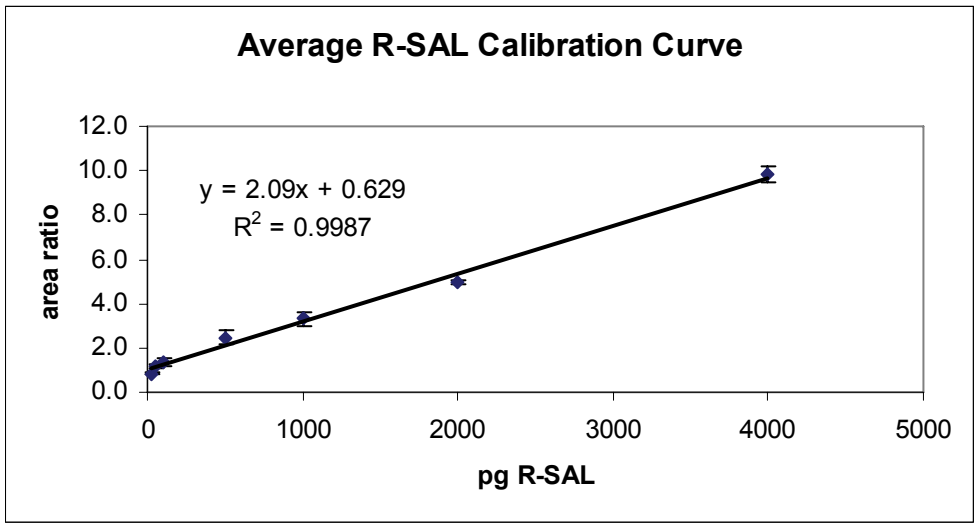


Figure 4-11: Average calibration curve (n=3) for R-SAL in pooled plasma. Linear regression performed on the average of the response across concentrations. Each point represents the average response ± SD.

A linear regression, utilizing a $1/x^2$ weighting factor, was performed on the average response for each concentration level. The calibration levels all showed acceptable precision for S-SAL and R-SAL with %COV less than 11.3% and 11.4%, respectively. The precision of the quality control samples ranged from 2.3% to 10.4% for S-SAL while the R-SAL quality control precision resulted in a %COV ranged 1.7% to 5.2%.

The "zero" level, consisting of 1-ml plasma with only internal standard, yielded a significant instrumental response for both SAL enantiomers, suggestive of the noteworthy constitutive nature of these analytes in the matrix.

Regarding the linear regression of the calibration curves, a weighting factor of $1/x^2$ was required correct for the unequal variance associated with the residuals (data

not shown). In turn, a weighted linear regression was performed on each individual calibration curve for both analytes. Linearity of the calibration curves, as assessed by the coefficient of determination (R^2), was acceptable for both analytes with S-SAL possessing R^2 ranging between 0.989 – 0.999 and R-SAL having a range between 0.982 - 0.998. For both analytes, the 95% CI for each individual calibration slopes included the estimate of the other individual calibration slopes, signifying similarity between the slope calibration parameter of each curve. Of major note, each calibration curve yielded a significant y-intercept for both analytes, indicating a significant background signal. The y-intercepts were significantly different from zero with S-SAL having an intercept (mean ± SD) of 0.425 ± 0.045 (p-value = 0.042) and R-SAL having an intercept of 0.631 ± 0.019 (p-value = 0.034). Moreover, the y-intercepts obtained from the linear regressions for both analytes were reproducible.

Of note the intercepts obtained from the weighted linear regression were not significantly different from that obtained from the "blank" sample for both S-SAL and R-SAL (p-value for both enantiomers > 0.14). According to the analysis, and using the baseline corrected calibration curves, the pooled plasma contained 259 pg of S-SAL and 430 pg of R-SAL per 1-ml plasma, which is within reported physiological range.

Long-term stability, in pooled plasma was evaluated at three different levels of High (2500 pg/1ml), Low (600 pg/1ml) and zero (unspiked R and S-SAL) over a six month period. Of note, the theoretical concentrations for the High and Low controls are those of spiked and constitutive, additive. The concentrations were measured, using baseline corrected calibration curves, at 15 days, 1 and 6 months after standard pooled

plasma preparation. At each time-point the analysis was performed in triplicate. Over a six-month period, there was a negligible variation in concentration for both SAL enantiomers (<2%). Freeze-thaw stability assessment did not yield any significant changes in R-SAL or S-SAL at all concentration levels evaluated through three-cycles, with %DFN and %COV not exceeding 4% for both analytes at all concentration levels.

The relative recovery, across the quality control concentrations, did not show much variation within concentrations (see table below). A slight increase of the recovery for both enantiomers was observed at the highest concentration level. Of note, the analyte to internal standard ratio obtained from the extraction procedure includes constitutive R- and S-SAL, contributing to the relative recovery. Therefore, the recoveries obtained from the analysis are expected to be larger than if the endogenous analyte was not present in the matrix.

Table 4-7: Relative recovery from 1 ml pooled plasma of R-SAL and S-SAL (n=3 for each concentration). (% mean ± SD).

	50 pg	200 pg	1000 pg
S-SAL	82.2 ± 2.6	84.1 ± 5.7	94.6 ± 2.4
R-SAL	83.1 ± 2.7	82.2 ± 4.6	90.8 ± 4.2

The information obtained from the pooled plasma analysis was used to design a more formal validation using a modified matrix for assessing surrogacy. Using 1-ml of matrix, the calibration range between 10 and 4000 pg of both R-SAL and S-SAL with 1.0 ng of deuterated internal standard for each enantiomer was used for validation

purposes. Quality control samples used for subsequent tests included 125 pg (LQC), 600 pg (MQC1) and 1200 pg (MQC2), and 2500 pg (HQC). The results of minimal deviation for long-term and freeze-thaw stability tests proved the chemical robustness of analyzing R- and S-SAL in the plasma matrix.

4.3.6d-3 Surrogate matrix experiments

Similar experiments aforementioned were performed using a modified pooled plasma matrix, with an emphasis on both accuracy and precision assessment. The matrix modification employed the destruction of constitutive R/S-SAL and DA in the pooled plasma via alkalization of the plasma to pH~10 and kept under refrigeration for 24 hours. Upon destruction of the constitutive SAL and DA from pooled plasma, the pH was readjusted to a physiological pH and the assay was performed per protocol.

The linearity of the developed method was evaluated by preparing n = 6 standard curves for the R/S-SAL and DA analytes in duplicate (2 x 3 days) with 1 ng of d_4-R/S-SAL and 5 ng d_4-DA internal standard. Similar concentrations of R/S-SAL were used for the standard curve constructed in the pooled plasma. In addition DA concentrations used for the calibration curve included 100, 200, 500, 1000, 2500, 5000, and 10000 pg/ml. Moreover, along with at least 7 non-zero standards, a blank sample (matrix processed without internal standard) and a zero sample (matrix processed with internal standard) were processed to assess for effectiveness of SAL and DA destruction. Experiments were performed using 1-ml of modified human plasma. Plots of peak area ratio (R-SAL/d_4-R-SAL, S-SAL/d_4-S-SAL and DA/d_4-DA) against

analyte concentration were constructed. Calibration data were analyzed similarly to that of the pooled plasma experiments (i.e., upon evaluation of the residuals of the linear fit).

The calibration function (e.g., linear or weighted-linear) was established through observation of reverse calculated standard concentrations of which were reverse predicted from the curve. The linear range was subsequently compared to that obtained in pooled plasma. Quality control samples for SAL were created at the same levels previously mentioned. For DA the following QC levels were tested: Low QC: 600 pg/ml, Medium QC 1: 2000 pg/ml, Medium QC 2: 4000 pg/ml and High QC: 8000 pg/ml. The QC concentrations were used to assess relative recovery from the modified plasma. The comparison of an extracted to unextracted analyte to internal standard ratio, at each concentration, was used to calculate the relative recovery. Experiments were performed in triplicate.

Accuracy and precision calculations were performed on the back-calculated concentrations from the regression, across the concentration range for both R/S-SAL and DA. Unlike the pooled plasma experiments, a formal assessment of the Lower Limit of Quantification (LLOQ) was determined during this step using n = 6 replicates of 20 pg/ml in modified matrix, separate from the calibrators. The linear regression parameters for the calibration curves, including slope and y-intercept, for the modified plasma validation were assessed upon evaluating the estimates and corresponding 95% confidence interval (CI) along with the precision of the slope and y-intercepts (% COV) for R/S-SAL and DA. Recovery assessment was evaluated using this matrix to assess

the relative recovery and the precision or the recovery. The room temperature stability measurements, as mentioned before, was also performed in this in modified matrix.

A more formal evaluation for precision and accuracy was conducted by evaluation of n = 6 each of the LLOQ, LQC, MQC1, MQC2, and HQC sample concentrations in one day for within-run assessment. Between run-precision and accuracy was assessed in triplicate over three days for the same controls. The accuracy (%DFN) and the precision (%COV) between and within-runs were found acceptable at a level of within ±15% for each. All quality control samples, including those that failed with no assignable cause, were used for the final calculation.

As the case for the calibration curves obtained for the pooled plasma, the linear regression parameters for the calibration curves, including slope and y-intercept, for the modified plasma validation were assessed and compared to that obtained in the pooled plasma experiments. For approval of the matrix modification via constitutive analyte destruction, the mean of the y-intercepts should not be statistically different from zero (as deemed by a one-sided unpaired Student's t-test). The individual confidence intervals of the y-intercept estimates should include zero for each individual calibration curve.

The slope of the linear regressions within all calibration curves should not be statistically different from one another (as deemed by a 95% CI). Moreover, the mean of all calibration slopes between the pooled plasma and the modified plasma matrix were evaluated for "parallelism" (i.e., the average slopes obtained between each matrix should

be statistically similar). Lack of similarity was evaluated by a two-sided unpaired Student's t-test.

4.3.6d-4 Surrogate matrix results

The modified matrix was constructed from destruction of endogenous R/S-SAL and DA from pooled plasma. Criteria to evaluate the removal of the analytes included devoid of an analytical response at the respective retention times of R/S-SAL and DA. Using this destruction technique, both R and S-SAL along with DA were successfully removed. The blank plasma spiked with d_4-SAL and d_4-DA showed no MRM peaks corresponding to endogenous SAL and DA (See figure below).

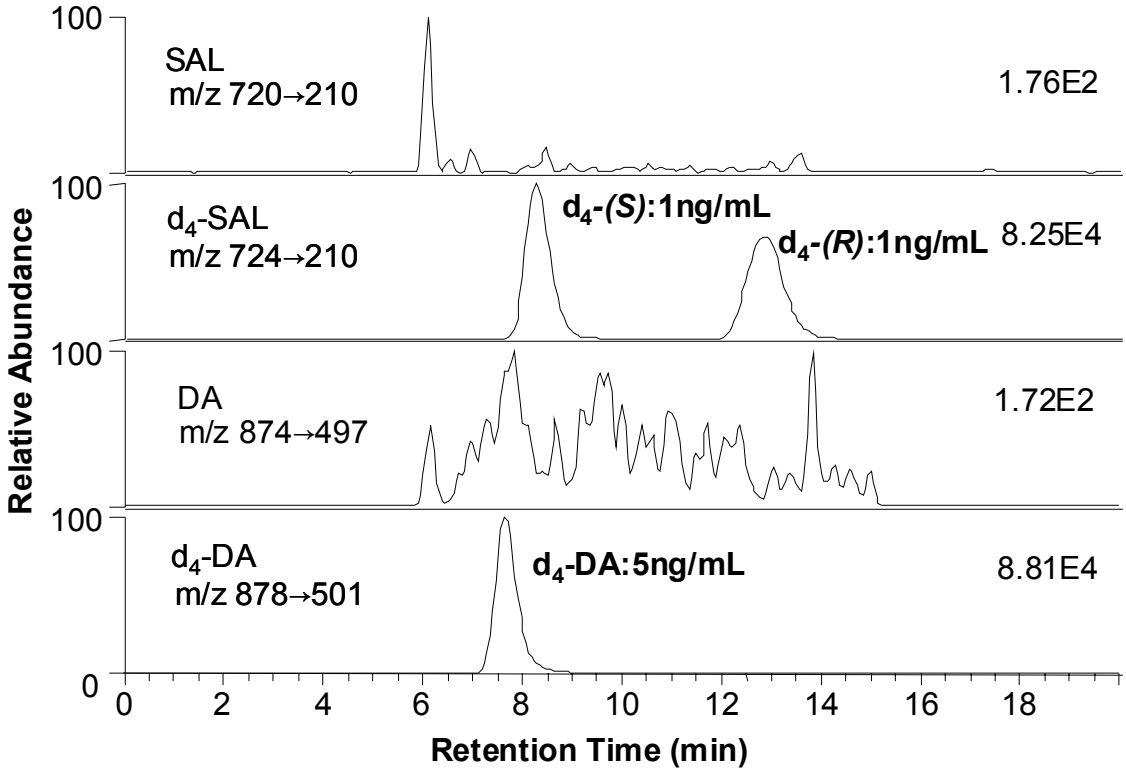


Figure 4-12: SRM chromatogram of SAL and DA prepared with 1 ml modified plasma. (spiked with 1 ng each of d_4-*(S)*- and d_4-*(R)*-SAL and 5 ng d_4-DA. No endogenous SAL or DA was detected.

Full calibration curves (n=6 for R/S-SAL, n=5 for DA) were constructed using 1-ml of modified matrix along with corresponding quality control samples. For S-SAL, throughout the concentration range the imprecision (%COV) was less than 14.4% and the inaccuracy (%DFN, absolute value) was less than 7.9% (see table 4-8). In addition, the analysis of R-SAL yielded acceptable results with a imprecision < 14.4% and an inaccuracy of < 9.6% (table 4-9). DA yielded acceptable results with an imprecision < 10.3% and an inaccuracy of < 12.4% (table 4-10). Of note, the analysis met the criteria in which >75% of all standards must be within ±15 % COV, with the exception of the LLOQ where a ±20 % COV is acceptable.

In separate experiments, further evaluation at the SAL LLOQ level (20 pg/1ml) yielded an imprecision %COV of < 11.7% and %DFN of < 6.5%, with n = 6 replicates of both R- and S-SAL. For DA %COV of < 6.8% and %DFN of < 5.2%. All quality control samples for all analytes yielded a suitable accuracy and precision. For inter-run precision and accuracy (3 replicates for 3 days), %COV was no greater than 12.7% and no more than an absolute variation in concentration of 14.1% across the quality control concentrations for all analytes (data not shown). The LLOQ of 20 pg/ml for R/S-SAL and 100 pg/ml for DA in the modified plasma is shown in the figure below.

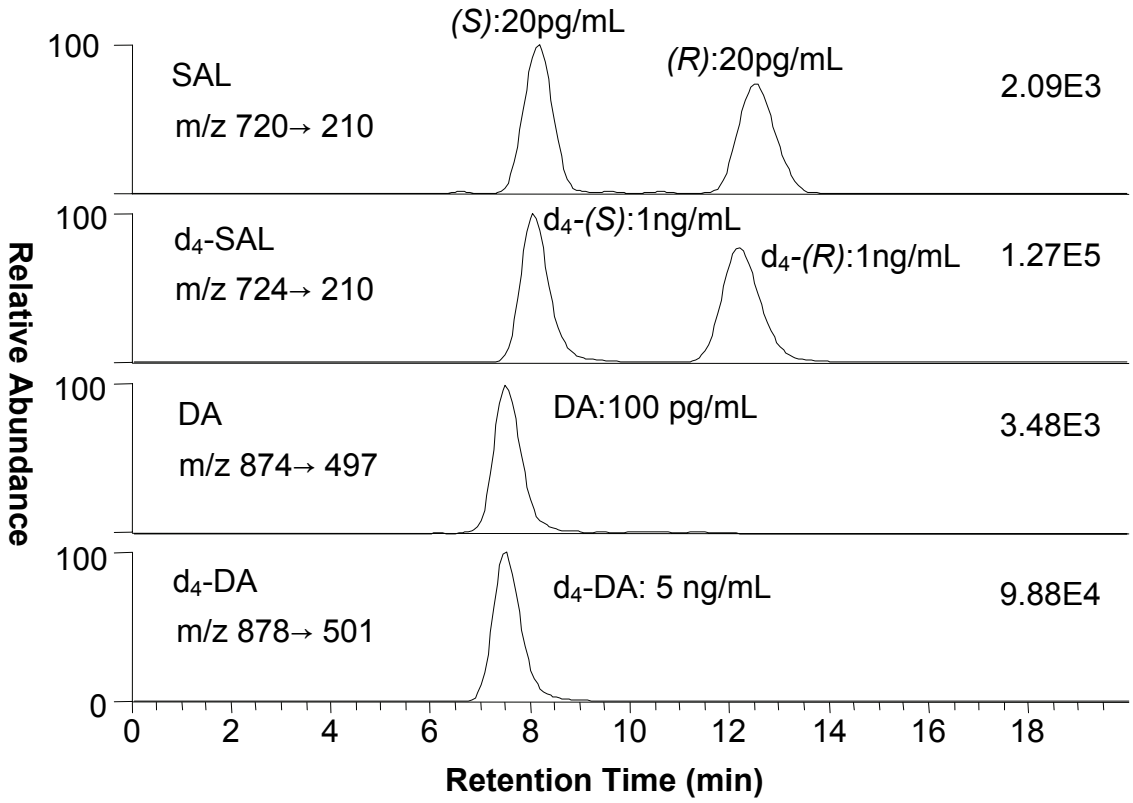


Figure 4-13: SRM chromatogram of SAL and DA prepared with 1 ml modified plasma at the LLOQ level (spiked 20 pg R and S-SAL with 1 ng each of d_4-*(S)*- and d_4-*(R)*-SAL and 100 pg of DA with 5 ng d_4-DA).

The linear regression parameters for each calibration curve were compared to assess consistency of slope and for lack of a y-intercept. Using a $1/x^2$ weighting factor for each calibration curve, the parameters presented showed consistency between calibration curves. The slope parameters for S/R-SAL and DA were statistically different from zero, while the intercept did not show a statistical departure from zero (*p*-values > 0.23 for all analytes). The zero intercept signifies the effective destruction of the constitutive SAL and DA. For all analytes, the coefficient of determination (R^2) was acceptable for each calibration curve, supporting the use of a linear regression for the calibration (see tables 4-11, 4-12 and 4-13 below).

The extraction efficiency (recovery) was assessed at three quality control levels in triplicate. The recoveries obtained from this analysis were constant across concentrations of all analytes with recoveries > 82% with less than 6% variation for all analytes.

In comparison to the unmodified, pooled plasma matrix surrogacy for the modified matrix was established. From the linear regression parameters (i.e., slope) obtained with calibration curves constructed of both sets of matrices, a statistical similarity was seen. An unpaired, two-tailed Student's t-test was performed on the slope and y-intercept parameters, assuming unequal variances, for all analytes between pooled plasma and modified plasma matrices. For S-SAL, R-SAL and DA the p-values were 0.35, 0.51, and 0.22 respectively. Statistical significance was defined at the $\alpha = 0.05$ level.

Table 4-8: Calculated concentration (pg/1ml) for S-SAL in modified plasma calibration curves (n=6), with precision (%COV) and accuracy (%DFN) assessment.

pg S-SAL	calib #1	calib #2	calib #3	calib #4	calib #5	calib #6	average	sd	%COV	%DFN
20	22.0	24.3	18.7	17.9	23.4	19.7	21.0	2.6	12.5	5.0
50	55.2	51.6	48.9	44.7	50.2	56.7	51.2	4.4	8.5	2.4
100	113.5	104.8	98.3	95.7	90.2	94.6	99.5	8.4	8.4	-0.5
500	517.2	487.0	498.3	488.5	507.4	434.6	488.8	28.9	5.9	-2.2
1000	1120.0	1082.4	1023.5	976.2	1136.5	1102.9	1073.6	61.7	5.7	7.4
2000	2201.9	1982.7	1872.8	1977.3	1827.4	1965.0	1971.2	129.5	6.6	-1.4
4000	3762.4	4362.5	4222.5	4102.6	4092.8	4402.9	4157.6	232.3	5.6	3.9
125 (LQC)	126.7	133.7	125.2	143.2	129.7	133.6	132.0	6.5	4.9	5.6
600 (MQC1)	663.0	588.2	611.5	603.9	599.8	687.1	625.6	39.8	6.4	4.3
1200 (MQC2)	1222.8	1132.5	1098.9	1143.0	1107.0	1232.2	1156.1	57.7	5.0	-3.7
2500 (HQC)	2686.3	2373.8	2250.9	2873.3	2983.5	2773.1	2656.8	287.3	10.8	6.3

Table 4-9: Calculated concentration (pg/ml) for R-SAL in modified plasma calibration curves (n=6), with precision (%COV) and accuracy (%DFN) assessment.

pg R-SAL	calib #1	calib #2	calib #3	calib #4	calib #5	calib #6	average	sd	%COV	%DFN
20	23.0	21.4	19.3	19.8	21.3	20.5	20.9	1.3	6.3	4.4
50	54.9	55.2	48.4	49.7	57.3	55.5	53.5	3.6	6.7	7.0
100	118.2	112.9	106.7	89.5	92.7	102.1	103.7	11.2	10.8	3.7
500	539.4	512.7	454.7	444.8	475.6	488.2	485.9	35.6	7.3	-2.8
1000	982.7	993.5	976.4	923.4	929.6	977.4	963.8	29.6	3.1	-3.6
2000	1892.0	1983.4	2008.5	2201.9	2398.2	2372.1	2142.7	213.4	10.0	7.1
4000	4213.0	4491.1	4840.5	4682.8	3202.4	4883.2	4385.5	629.9	14.4	9.6
125 (LQC)	134.9	117,3	122.6	111.4	127.9	134.0	126.2	9.6	7.6	0.9
600 (MQC1)	608.0	712.4	654.1	676,3	661.6	603.4	647.9	44.6	6.9	8.0
1200 (MQC2)	1229.4	1309.7	1333.3	1258.6	1345.5	1434.2	1318.5	72.0	5.5	9.9
2500 (HQC)	2631.8	2847.5	2881.4	2536.1	1982.5	1972.4	2475.3	406.8	16.4	-1.0

Table 4-10: Calculated concentration (pg/ml) for DA in modified plasma calibration curves (n=6), with precision (%COV) and accuracy (%DFN) assessment.

pg DA	calib #1	calib #2	calib #3	calib #4	calib #5	average	sd	%COV	%DFN
100	94.7	94.8	98.5	102.5	88.7	95.8	5.1	5.3	-4.2
200	202.4	213.9	212.0	198.7	204.4	206.3	6.5	3.1	3.1
500	546.9	529.4	488.3	459.5	487.9	477.3	35.2	7.4	-4.5
1000	1160.5	1092.6	1291.4	1067.0	1009.2	1124.1	108.1	9.6	12.4
2500	2873.7	2542.3	2637.2	2201.4	2827.5	2616.4	268.8	10.3	4.7
5000	4482.0	4929.0	4450.4	4492.7	4872.1	4645.2	234.4	5.0	-7.1
10000	10826.0	9827.4	10325.0	11029.4	10920.3	10585.6	502.4	4.7	5.9
600 (LQC)	698.6	625.1	672.4	681.9	629.4	593.4	32.7	5.5	-1.1
2000 (MQC1)	2019.5	2219.4	2091.4	2111.3	2240.9	2211.5	92.4	4.2	10.6
4000 (MQC2)	4428.4	4092.8	4493.5	3874.7	3888.0	4155.5	292.9	7.0	3.9
8000 (HQC)	7829.0	7726.6	7490.6	7029.5	7726.5	7560.4	321.7	4.3	-5.5

Table 4-11: Standard curve parameters for n = 6 S-SAL calibration curves in modified plasma. Each parameter presented as an estimate with standard error (SE).

Calibration	slope	SE	y-intercept	SE	R2
1	2.5820	0.0922	(-)0.0124	0.0275	0.9892
2	2.6129	0.0721	(-)0.0833	0.0359	0.9917
3	2.4672	0.0733	0.0127	0.0223	0.9748
4	2.4422	0.0261	0.0245	0.0425	0.9933
5	2.5342	0.0526	(-)0.0101	0.0266	0.9898
6	2.3272	0.0882	0.0229	0.0355	0.9913
average	2.4943		0.0200		0.9884
stdev	0.1046		0.0064		0.0068

Table 4-12: Standard curve parameters for n = 6 R-SAL calibration curves in modified plasma. Each parameter presented as an estimate with standard error (SE).

Calibration	slope	SE	y-intercept	SE	R2
1	2.1452	0.0883	(-)0.0251	0.0139	0.9934
2	2.2316	0.0728	0.0247	0.0227	0.9897
3	2.0928	0.0557	(-)0.0133	0.0182	0.9912
4	2.1330	0.0623	0.0154	0.0266	0.9855
5	2.2516	0.0611	(-)0.0088	0.0019	0.9914
6	2.2199	0.0572	0.0122	0.0214	0.9937
average	2.1790		0.0174		0.9908
stdev	0.0639		0.0065		0.0030

Table 4-13: Standard curve parameters for n = 5 DA calibration curves in modified plasma. Each parameter presented as an estimate with standard error (SE).

Calibration	slope	SE	y-intercept	SE	R2
1	0.9962	0.0251	0.0133	0.0237	0.9823
2	0.9881	0.0099	(-)0.0092	0.0241	0.9899
3	0.9928	0.0172	(-)0.0221	0.0035	0.9846
4	0.9821	0.0133	0.0258	0.0102	0.9915
5	0.9954	0.0352	(-)0.0288	0.0196	0.9977
average	0.9909		0.0196		0.9892
stdev	0.0059		0.0088		0.0061

The y-intercept showed a statistically significant difference between matrices. While the modified plasma was not significantly different from zero, the pooled plasma matrix possessed a significant y-intercept, reflecting the constitutive R/S-SAL and DA concentrations in the plasma. Moreover, the lack of a statistically significant y-intercept in the modified plasma denotes successful removal of the constitutive background signal.

Of primary importance is the statistical similarity in the slopes for all analytes in both matrices. The comparison supports the interchangeability of the matrix for calibration purposes. Moreover, "parallelism" between the matrices was demonstrated between the modified and pooled plasma matrix. Further assessment of selectivity was warranted for this method, evaluating additional plasma sources for potential interferences. A similar approach was used to assess "parallelism" in patient plasma samples.

4.3.6d-5 Patient plasma experiments

In order further evaluate selectivity and parallelism, individual plasma from separate donors was used as a matrix. Further selectivity should be assessed to show that the anticipated analytes are measured and that their quantification is not affected by the presence of any interferences in the biological matrix (see discussion in chapter 3). Although the intrinsic selectivity of this assay is high (compared to other types of analytical methods, e.g., HPLC-FD), further confirmation using matrices from at least n = 5 independent sources was evaluated for matrix effects.

Nonetheless, selectivity of the bioanalytical assay will be assessed by spiking known concentrations of analyte into the matrix of interest, as performed as a standard addition experiment. In this approach, different masses of R-SAL, S-SAL and DA are spiked to the sample matrix, which initially contains an unknown concentration of analyte. Extrapolation of a plot of response found for the standard-addition calibration concentrations to zero concentration defines the original concentration of the unspiked sample.

The primary criteria in establishing "surrogacy" of the modified matrix, is that the response factor, or slope of the calibration curves obtained in the substitute matrix, should be statistically similar to that of unmodified matrix and "parallelism" between the calibration curves of both matrices be present. In order to evaluate the use of the HPLC-ESI-MS/MS method along with the modified matrix, a total of five sources of unmodified plasma were used in the subsequent experiments.

Individual patient plasma samples (n=5, presumably healthy, drugs of abuse-free, smokers and non-smokers, collected in EDTA) were used to test the conditions for selectivity. Using the finalized extraction method, spiked concentrations of R- and S-SAL along with DA was evaluated in 1-ml of human patient plasma. As the availability of significant volumes of individual human plasma was limited, full calibration curves could not be constructed in the patient plasma. For the individual plasma experiments a total of four concentrations were used for the standard addition calibration curve construction including 20, 160, 600, and 1250 pg of both R- and S-SAL using 1 ng of the deuterated R/S-SAL internal standard in 1-ml of plasma. In these experiments DA

selectivity was assessed with 200, 600, 2000, and 4000 pg of DA using 5 ng of the DA internal standard. Moreover, a "zero" concentration (unspiked SAL and DA with internal standard) was evaluated to assess the endogenous R/S-SAL and DA concentrations. Individual plasma calibration curves were constructed in singlicate. The concentrations assessed in these experiments are representative of those used in previous calibration curves.

A four-point, linear calibration curve was constructed with each individual donation of plasma using a $1/x^2$ weighing, identical to the weighted regressions performed as the other experiments. The linear regression parameters for the calibration curves, including slope and y-intercept, for the individual plasma were assessed. The y-intercept for each individual calibration curve was presumably the amount of constitutive contribution of R/S-SAL and DA and compared to that of the "zero" concentration level, as calculated from the surrogate matrix calibration curve. The 95% confidence interval for the y-intercept (peak area ratio) should include the peak area ratio of that seen of the "zero" sample for all analytes. This method was to ensure the accuracy of using the surrogate matrix calibration curve to calculate a concentration in unmodified, real sample.

Of primary importance was the evaluation of the slope parameter for each individual plasma calibration curve. The estimate and its corresponding 95% CI was calculated and compared across donors. The estimates for the slope parameter should be statistically similar upon evaluation of the 95% CI. Individual R/S-SAL

concentrations were calculated and compared for each patient sample using the surrogate matrix calibration.

Moreover, the slopes ± standard deviation between the surrogate matrix plasma (n=6), individual patient plasma (n=5), and pooled plasma (n=3) calibrations were compared via one-way analysis of variance (ANOVA) to ensure a matrix effect between the matrices was not present. Prior to statistical comparison, the residuals were evaluated for equal variance and tested accordingly if that assumption did not hold true. If significant deviations and variability in the slopes was present in the analysis, the HPLC-MS/MS parameters and/or extraction method was further optimized eliminate the interfering substances.

4.3.6d-6 Patient plasma results

Using the finalized extraction method, spiked concentrations of R/S-SAL and DA were evaluated in 1-ml of human patient plasma from n=5, presumably healthy, drugs of abuse-free, smokers and non-smokers. Representative chromatograms for an individual patient plasma are present in figure 4-18 below. The calculated concentrations were obtained from the linear regression from a calibration curve in modified plasma. The respective retention times for S-SAL, R-SAL and DA are 7.92, 12.3, and 7.42 minutes, respectively.

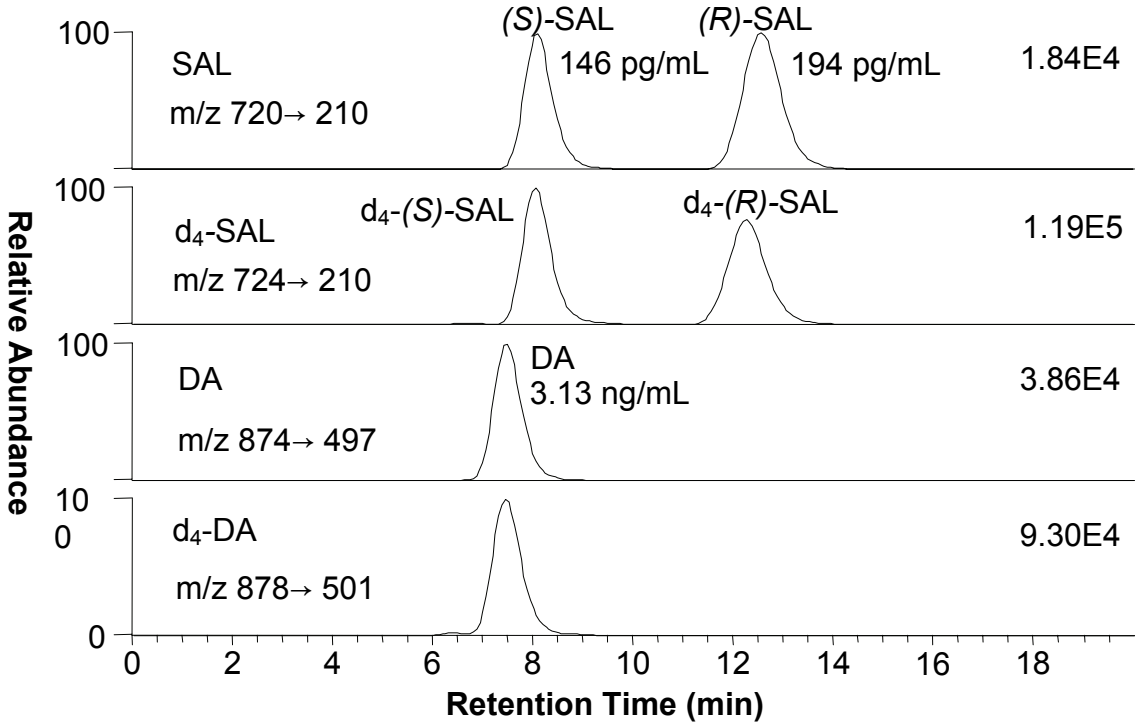


Figure 4-14: Representative SRM chromatograms of endogenous SAL and DA found in 1.0 ml human plasma spiked with 1 ng each of d_4-*(S)*- and d_4-(*R*)-SAL and 5 ng of d_4-DA as internal standards. The concentrations of *(S)*-SAL, *(R)*-SAL and DA were determined to be 146 pg/ml, 194 pg/ml and 3.13 ng/ml, respectively.

The goal of patient plasma experiments was to assess selectivity of the assay methodology. Of note, the accessibility of significant volumes of individual human plasma was limited, as consequence, full calibration curves could not be constructed in the patient plasma. The corresponding calibration curve parameters are presented below.

Table 4-14: Linear regression parameters obtained from patient plasma (PP #1-5) calibration curve for S-SAL, R-SAL and DA. 95% CI denotes the upper and lower bounds in brackets for both slope and y-intercept.

S-SAL

plasma	slope	95% CI	y-int	95% CI	R2
PP#1	2.553	[2.231, 2.774]	0.56	[0.44, 0.67]	0.9734
PP#2	2.511	[2.229, 2.825]	1.32	[1.15, 1.56]	0.9883
PP#3	2.736	[2.483, 2.955]	1.72	[1.63, 1.84]	0.9921
PP#4	2.637	[2.232, 3.036]	0.64	[0.56, 0.72]	0.9853
PP#5	2.441	[2.136, 2.750]	2.23	[2.10, 2.37]	0.9798
avg	2.576		1.29		0.9838
stdev	0.114		0.71		0.0073

R-SAL

plasma	slope	95% CI	y-int	95% CI	R2
PP#1	2.167	[1.940, 2.305]	0.49	[0.35, 0.54]	0.9811
PP#2	2.189	[1.998, 2.283]	1.65	[1.43, 1.81]	0.9859
PP#3	2.059	[1.824, 2.187]	1.93	[1.78, 2.17]	0.9955
PP#4	2.114	[2.056, 2.170]	0.91	[0.75, 1.09]	0.9993
PP#5	2.154	[2.155, 2.387]	2.77	[2.40, 2.97]	0.9784
avg	2.137		1.55		0.9880
stdev	0.051		0.89		0.0091

DA

plasma	slope	95% CI	y-int	95% CI	R2
PP#1	0.986	[0.954, 1.092]	2.31	[2.16, 2.54]	0.982
PP#2	0.991	[0.975, 1.105]	3.81	[3.33, 4.07]	0.9777
PP#3	0.994	[0.970, 1.068]	0.63	[0.44, 0.82]	0.9833
PP#4	0.979	[0.958, 1.070]	1.02	[0.83, 1.24]	0.9875
PP#5	0.984	[0.953, 1.035]	1.42	[1.29, 1.56]	0.9899
avg	0.987		1.84		0.9841
stdev	0.006		1.27		0.0048

The coefficients of determinations for S-SAL, R-SAL and DA in the patient plasma were greater than 0.97, suggesting the linear calibration function is acceptable for this matrix (using $1/x^2$ weighting). From evaluation of the 95% CI, the y-intercepts obtained for all calibration curves, were all statistically different from zero. Moreover, the intercepts between the plasma sources resulted in significant deviations, suggesting the variable nature of the constitutive S/R-SAL and DA between the five patients.

Slope parameters from the calibration curves were all statistically similar between the patient plasma sources and in comparison to the modified matrix. Because of the limited number of concentration points on each calibration curve (4-point) for the patient plasma, the 95% CI range was relatively large as compared to the modified plasma matrix (i.e., a full calibration curve).

The concentrations obtained from the surrogate matrix calibration curves yielded endogenous levels that were within reported physiological ranges. For S-SAL, the concentration range within the individual patient samples ranged from 32 - 224 pg/ml plasma. For R-SAL the concentration range within the five patient samples ranged from 38 - 304 pg/ml plasma. As for DA, the concentrations ranged from 1.06 – 2.05 ng/ml plasma.

Surrogacy of the modified matrix was further evaluated by statistical comparison of calibration curves from pooled plasma and patient plasma. One-way ANOVA was performed on the slopes of the calibration curves between three different plasma matrices, modified (surrogate), pooled, and patient plasma. The summary table of all calibration slopes is presented in the table below. The three groups of plasma

matrices, compared using the unequal variance F-test, were not significantly different for all analytes. For S-SAL the means were found to be statistically similar, $F(2, 13) = 1.14$, p-value = 0.355; while the same conclusion was drawn for the R-SAL calibration parameter $F(2, 13) = 0.506$, p-value = 0.615 and DA was $F(2,10) = 0.523$, p-value = 0.608. The results are presented as F-test calculation (degrees of freedom for groups, total) and p-value. All statistical comparisons were performed on S-PLUS 8.0 for Windows.

From these results, the appropriateness of using the modified plasma matrix as a surrogate matrix was demonstrated. Parallelism between the calibration curves in different matrices suggests the interchangeable nature of using either matrix for validation purposes. The surrogate matrix will be incorporated for the clinical sample analysis.

Table 4-15: Statistical comparison of calibration regression slope parameter between pooled, patient and surrogate matrix for S-SAL, R-SAL and DA. One-way ANOVA performed between matrices with significance at the $\alpha = 0.05$ level.

S-SAL

	Slope of Regression		
calibration	Pooled	Patient	Surrogate
1	2.5300	2.553	2.5820
2	2.6010	2.511	2.6129
3	2.5980	2.736	2.4672
4		2.637	2.4422
5		2.441	2.5342
6			2.3272
avg	2.5763	2.5756	2.4943
stdev	0.0402	0.1143	0.1046

p-value *0.355*

R-SAL

	Slope of Regression		
calibration	Pooled	Patient	Surrogate
1	2.1120	2.167	2.1452
2	2.2810	2.189	2.2316
3	2.0182	2.059	2.0928
4		2.114	2.1330
5		2.154	2.2516
6			2.2199
avg	2.1371	2.1366	2.1790
stdev	0.1332	0.0512	0.0639

p-value *0.615*

DA

	Slope of Regression		
calibration	Pooled	Patient	Surrogate
1	0.9790	0.986	0.9962
2	0.9980	0.991	0.9881
3	0.9846	0.994	0.9928
4		0.979	0.9821
5		0.984	0.9954
avg	0.9872	0.9868	0.9909
stdev	0.0098	0.0059	0.0059

p-value *0.608*

4.3.6e Validation conclusion

In summary the acceptance criteria for validation were met according to predetermined specifications. In the surrogate and unmodified matrices, chromatographic response was linear throughout the concentration range of 20 pg/1 ml (LLOQ) and 4000 pg/1 ml (ULOQ) for S- and R-SAL, while for DA the range was between 100-10000 pg/ml. Accuracy and precision estimates for the assay were in acceptable ranges for the calibration points, the LLOQ and quality control samples with %COV and %DFN meeting analytical validation criteria. Selectivity of the method was evaluated with 5 donor lots and no interference was apparent with the analyte or internal standard. Across all quality control samples, recovery was relatively high (>80.2%) and reproducible for both H and NH.

Of importance, surrogacy of the matrix was demonstrated by the statistically indistinguishable slopes between modified and unadulterated matrix. "Parallelism" of the slopes confirms the interchangeability of calibration matrices.

Long-term plasma stability experiments for 6 months at -80ºC resulted in negligible deviations over time for both analytes. Additional stability experiments, including stock solution, bench-top, and post-preparative resulted in negligible deviations throughout the respective tested time-spans. Under the optimized SPE, derivatization and HPLC-MS/MS conditions endogenous R/S-SAL and DA in plasma at concentrations comparable to reported literature values.

4.4 HPLC-MS/MS Assay Conclusion

A robust, sensitive, selective and reproducible assay has been developed for the quantification of the endogenous tetrahydroisoquinolines S and R-SAL along with their precursor DA, in 1 ml of human plasma. A direct single-step pentafluorobenzyl (PFB) derivatization scheme in an aqueous media, without extractive alkylation using phase transfer catalysts, was devised for the enantioseparation of SAL with simultaneous detection of DA. This procedure permitted high derivatization yields with minimal sample degradation and low chemical background. As stable PFB derivatives, SAL enantiomers were baseline separated on a chiral phase HPLC column. Coupling with ESI-MS/MS analysis in the SRM mode allowed the detection of SAL enantiomers and DA with increased specificity and sensitivity. In the presence of deuterium-labeled internal standards, this approach allowed accurate and reliable quantitative analysis of enantiomeric (*R*/*S*)-SAL and DA in human plasma.

In comparison to reported methodologies for the quantification of R and S-SAL in human plasma, this procedure has surmounted the limitations aforementioned. The optimized chromatography has preserved the baseline resolution of the both enantiomers (Rs > 2.1) throughout the concentration range, improving the reliability of quantification. Quantification with the isotopically labeled internal standards of R and S-SAL with DA yielded suitable assay performance results for the quantitative bioanalytical HPLC-MS/MS assay.

CHAPTER 5

CLINICAL STUDY #1 – EFFECTS OF GENDER AND SMOKING ON BASELINE TIQ'S AND β-CARBOLINES

5.1 Specific Aims

The primary objective of this investigation was to evaluate the effects of smoking and gender on the baseline levels of TIQ and β-carboline concentrations in the blood of non-, light- and heavy-smokers. In this study, the baseline measurement in smoking subjects is considered to be concentration measurements 30 minutes after cigarette smoke inhalation. In particular, aims of this study included:

1) To determine information on the baseline concentrations of plasma TIQ's and β-carbolines in the blood of non-, light-, and heavy-smokers and assess if differences exist between these groups.

2) To evaluate if gender has an effect on baseline concentrations of the plasma TIQ's and β-carbolines.

3) To classify subjects according to smoking history and nicotine dependence with the Fagerström Test for Nicotine Dependence (FTND) into nonsmokers, light smokers, and heavy smokers.

4) To assess a possible association between the concentration of plasma TIQ's and β-carbolines with the smoking history and dependence of the volunteers.

5) Statistical assessment of inter- and intra-individual variability in the plasma TIQ's and β-carbolines along with smoking history/nicotine dependence scores.

5.2 Study Design

In order to address the objectives aforementioned, the study was designed as an observational, two-period outpatient clinical study in which each study period was separated by at least one week. To determine the effects of smoking and gender on TIQ and β-carboline plasma concentrations, the study integrated a classification scheme to stratify the volunteers into nonsmoking (NS), light-smoking (LS) and heavy-smoking (HS) groups and with respect to gender, respectively.

Forty-one (41) healthy male and female volunteers, aged 21-35, were recruited, including 19 nonsmokers, 11 light-smokers, and 11 heavy-smokers. Although this was a pilot study, the sample size for the study was determined *a priori*, via a two-way ANOVA power analysis (with gender and smoking status as factors). The number of subjects in each smoking status group required to show a difference of 50% in TIQ and β-carboline baseline concentrations between smoking groups with an α value of 0.05 and a power of $1 - \beta = 0.8$, was ten (10), assuming a coefficient of variation (CV%) of

50%. To evaluate the effects of smoking, the Fagerström Test for Nicotine Dependence (FTND) was administered to determine their dependence on smoking in order to classify the potential subjects into three groups: nonsmokers, light-smokers, and heavy-smokers (Fagerström and Schneider, 1989). In this study, smokers were required to smoke one complete cigarette prior to sampling of the biological specimen. As a separate analysis, a comparison of these results will be made to an additional eighteen (18) subjects from a separate study, balanced for smoking status and gender, that abstained from smoking for at least 15 hours prior to biological sampling (Leu, 2002).

5.3 Experimental methods

5.3.1 Subjects

Forty-three (43) male and female volunteers were screened for the study (11 NS females, 9 NS males, 7 LS females, 5 LS males, 6 HS females and 5 HS males). A total of forty-one (41) healthy volunteers successfully completed the study (10 NS females, 9 NS males, 6 LS females, 5 LS males, 6 HS females and 5 HS males). Subjects were required to be between the ages of 21-35 and be healthy. The recruitment of volunteers who participated in the study involved VCU-IRB approved advertisements that were placed around the Richmond, VA metro area. Potential volunteers called on their own accord, and information regarding the logistics of the study was given over the telephone. Upon their permission, a confidential health survey was administered to determine initial study qualification. Information such as

gender, ethnicity, height and weight, present disease states, concurrent medications or dietary supplements, drugs of abuse and alcohol usage, smoking history and the Fagerström Test for Nicotine Dependence (FTND) was gathered from each subject participant. In order to participate, the potential subject could not possesses any significant health disease state, not be currently taking any prescribed medications, nor be using any other drugs of abuse other than nicotine from cigarette smoking. Alcohol intake was informally assessed during the telephone interview.

5.3.2 Procedures

5.3.2a Smoking Classification

According to the number of cigarettes smoked per day and the FTND score, each patient was classified into a smoking status classification including nonsmoker (NS), light-smoker (LS), and heavy-smoker (HS). The FTND is a standard instrument to assess the intensity of this physical addiction (Fagerström and Schneider, 1989). This test is designed to help physicians document the indications for prescribing medication for nicotine withdrawal. The survey is measured on a scale of 0-10 with the higher the FTND score, the more intense the patient's physical dependence on nicotine.

Specifically, the requirements listed in table 5-1 were necessary for subjects to be classified in each group. In the situation that the smoking history and FTND scores put the subject into two different categories (specifically for LS and HS), preference was given to the smoking history (number of cigarettes per day) over the FTND score.

Table 5-1: Smoking status classification criteria.

Subject Group	Inclusion/Exclusion Criteria
Nonsmoker **(NS)**	1. No current tobacco product use 2. Not a smoker for the past 5 years 3. If previously a smoker, did not smoke more than once a year continuously and < 10 cigarettes/year 4. FTND score = 0
Light-smoker **(LS)**	1. Current smoker of cigarettes (No other tobacco products) 2. Smokes at least 10 cigarettes/day 3. Smoked for at least 1 year continuously 4. FTND score 1-7
Heavy-smoker **(HS)**	1. Current smoker of cigarettes (No other tobacco products) 2. At least more than 20 cigarettes/day 3. Smoked for at least 1 year continuously 4. FTND score > 7

5.3.2b Admission to the Clinical Research Unit

Upon qualification of the telephone screening, each patient was categorized into a smoking status classification and visits to the clinical center were scheduled. Prior to participation, the subject was required to abstain from prescription/over-the-counter/herbal medications or caffeinated products for 72-hours prior to each visit (with exception of oral contraceptives prescription for females) and no alcoholic beverages 12-hours prior to each visit. The subject was given a chance to decline participation in the study or, if necessary, to seek his/her personal physician's advice as to whether to discontinue any medications he/she may be on.

The study involved two visits in which the volunteer presented to the Virginia Commonwealth University Health Systems General Clinical Research Center (VCUHS-

GCRC). Before participation in the study procedures, each subject signed a VCU-IRB approved Informed Consent Form (Appendix E) attesting that the study procedures were explained to them and that their participation in the study was voluntary.

Both clinical visits took place between 8 – 10 AM with sampling of biological specimens and additional observations were performed before 10 AM (see figure 5-1 for study flow chart). During the first screening visit, each volunteer completed forms including: Comprehensive Medical History, Subject Entry Probe, Smoking History, Annual Alcohol Intake (AAI, Appendix C), and a TIQ/BC Food and Beverage Inventory (Appendix D). The food and beverage inventory, including caffeinated beverages, were logged by self-report prior to sampling to explore the influences of dietary factors on TIQ and β-carboline levels at baseline. All female subjects were requested to give information pertaining to the date of their last menses. Of important note, subjects who were light- and heavy-smokers were asked to smoke one (1) complete cigarette within 30 minutes prior to biological sampling, for standardization purposes.

Additionally, during each visit, the subject was tested for abstention of alcohol via an alcohol breathalyzer and breath carbon monoxide test for assessment of exposure to cigarette smoke. A 60-ml blood sample for estimation of TIQ and β-carboline plasma concentrations was drawn during each visit. The volunteer also provided a urine sample for drugs of abuse screen.

Figure 5-1: Activity Flow for Clinical Study #1

Screening/Visit #1

Activity
Outpatient Admission to GCRC
Informed Consent Form
Subject Entry Probe
Weight and Height Recording
Medical History and Smoking History
Zuckerman Sensation Seeking Scale
Temperament and Character Inventory
Annual Alcohol Intake
Breathalyzer Test
Breath Carbon Monoxide Test
SMOKE*
Vital Signs
Blood Sample
Vital Signs
Urine Sample
EXIT

Visit #2

Activity
Outpatient Admission to GCRC
Subject Entry Probe
Breathalyzer Test
Breath Carbon Monoxide Test
SMOKE*
Vital Signs
Blood Sample
Vital Signs
Urine Sample
EXIT

Official enrollment and subject number assignment was allocated after the screening visit upon meeting particular inclusion/exclusion criteria. The criteria for enrollment were as follows:

1) Demographics: Participating subjects were required to be between the ages of 21-35 years of age and be a healthy male or female. Female subjects could not be pregnant during the clinical study and were to be using acceptable methods of contraception (abstinence, barrier methods, or oral contraceptives). However, females that were not using oral contraceptives must have had regular menstrual cycles of 28-32 days on average and must not have dysmenorrhea. Subjects were required to be within ± 15% ideal body weight according to their height.

2) Medical history: During the initial screening visit, a six-page medical evaluation form, in which the patient self-reported questions regarding personal medical history, family medical history, personal habits, current medications, social history, and current symptoms, was administered. The subject had to have no history of clinically significant renal, hepatic, cardiovascular, gastrointestinal, pulmonary, neurological, and psychiatric diseases upon evaluation of the medical history questionnaire. Moreover, subjects had to have no history of alcohol or illicit drug abuse. Classification of the health status was based solely on the medical history form. A physical exam was not performed on these subjects prior to participation.

3) Laboratory results: The primary laboratory tests performed on each subject were urine drugs of abuse test, urine human chorionic gonadotropin to test for

pregnancy of female subjects and a spot urine creatinine. All pregnancy tests and urine drug screens had to be negative for further participation in the study.

4) Vital Signs: Sitting systolic and diastolic blood pressure (after five minutes of sitting), heart rate and oral temperature had to be within normal limits.

5) Medications: Subjects who qualified for the study were not allowed to take any prescription or over-the-counter medications. Females that were currently taking oral contraceptives were permitted to participate in the study.

Upon successful screening, the volunteer was subsequently scheduled for a second visit to the clinical unit. The same abstentions applied, as what was followed for the first screening visit. During the second visit, only the breath tests, blood and urine sample were repeated. A pregnancy test was given to female subjects and repeated if the time elapsed between the two visits exceeded one week.

5.3.2c Blood/urine sampling and safety measurements

During participation in the protocol, a total of one (1) blood sample was drawn during each study period. The total amount of blood from each session was 60-ml and a total of 120-ml over the entire duration of the study. During the two outpatient periods, the subjects' vital signs was recorded before and after sampling and was monitored for appearance of any adverse events by the VCU-GCRC nursing staff. A medical monitor was available to monitor for signs of adverse events associated with blood drawing such as mental confusion, dizziness, and weakness. If necessary, adverse events were followed up until resolution.

Of note, subjects who were light- (LS) and heavy-smokers (HS) were asked to smoke one (1) complete cigarette within 30 minutes prior to biological sampling. The exact time before sampling was noted as well as the type of cigarette smoked. A 60-ml blood sample was collected from the non-dominant forearm in a reclined, seated position during the study. Blood was collected into four 10-ml purple top vacutainer tubes containing K_2-ethylenediaminetetraacetic acid (K_2-EDTA) and was immediately centrifuged for 10 minutes at 4 °C at 3000 rpm to obtain plasma (Sorvall RC 3C Plus Centrifuge, Kendro Laboratory Products, Newton, CT). Approximately 6-ml of plasma was placed into a Sarstedt Tube (Newton, NC) along with a 2-ml solution containing 60% $HClO_4$, ascorbic acid, semicarbazide HCl in aqua distillata for consequent SAL quantification analysis. The anti-oxidant/aldehyde-trapping reagent solution was added to ensure the prevention of *in-vitro* artifactual formation of SAL. An aliquot of approximately 10-ml of plasma was placed in a Sarstedt Tube for future β-carboline analysis. Both sets of plasma samples were stored at -80°C until analysis.

5.3.2d Breath sampling

As part of protocol, subjects were required to abstain from alcohol for 12-hours prior to participation in each visit. Moreover, smokers were required to smoke one complete cigarette prior to blood sampling. Therefore, breath alcohol (BrAC) and breath carbon monoxide (CO) tests were performed to ensure that the subject was abstinent from alcohol (BrAC of 0.0 mg / 210ml), and to measure tobacco smoke exposure, respectively.

5.3.2e Diet and alcohol intake

As diet and alcohol intake are speculated to contribute to circulating TIQ's and β-carbolines, a dietary survey and alcohol consumption scale were given to each subject. These "pen and paper" style scales were administered to further characterize the subjects according to dietary intake of TIQ's and β-carbolines. Subjects were asked to abstain from caffeinated beverages and alcoholic beverages for 72-hours and 12-hours, respectively, prior to each clinical visit.

The food survey incorporated a list of foods that are known to have a considerable composition of TIQ's and β-carbolines. Of major note, this survey is not a validated instrument. The subject was required to mark the type of TIQ and/or β-carbolines containing food they eat along with the frequency of intake. The TIQ/BC food inventory may be seen in Appendix D. From the information provided, a calculation of weekly dietary intake of TIQ and/or β-carbolines in nanograms was reported.

Alcohol consumption was assessed for each subject participant via the Annual Alcohol Inventory (AAI, (Khavari and Farber, 1978). This survey assesses the frequency and type (e.g., beer, wine and spirits) of alcohol consumed in a twelve question format. Based on the responses, annual absolute alcohol intake can be estimated via a mathematical calculation. This process is thought to capture both regular and binge drinking consumption but does not diagnose alcoholism. The survey takes approximately 1-2 minutes to complete and was performed once per protocol at screening.

5.3.2f Discharge from VCU-GCRC

For each visit, subjects were discharged from the VCU-GCRC after approximately 2 hours of start of protocol procedures. The volunteers were followed up the following day to assess for any discomfort from the protocol procedures, primarily the needle stick site. The Flow Sheet that summarizes all procedures performed per protocol can be seen in Figure 5-1.

5.3.3 Blood Sample Analysis

The samples containing TIQ's and β-carbolines were kept at -80°C until analysis. β-carboline analysis was conducted by the investigator using resources and equipment from the PK/PD Research Laboratory, Department of Pharmaceutics, Virginia Commonwealth University, Richmond, VA. The β-carbolines, harman and norharman were analyzed via a validated HPLC-FD method (see chapter 3). In brief, 2-ml of patient plasma sample was subjected to protein precipitation with subsequent solid phase extraction (SPE). Quantification for both analytes was conducted using yohimbine as an internal standard and detected via HPLC-FD.

TIQ samples were analyzed and assayed by the investigator using resources and equipment with generous permission granted from the Laboratory of Molecular Signaling, National Institute on Alcohol Abuse and Alcoholism-NIH, Rockville, MD. The assay procedure incorporated the use of 1-ml of patient plasma sample in addition to the equivalent volume of 1-ml antioxidant solution (total volume of 2-ml). Phenyl boronic acid SPE followed by PFBBr derivatization was used to isolate R-SAL, S-SAL

and DA. The final sample was chromatographically resolved via a chiral column and subjected to ESI-MS/MS detection. Deuterated internal standards of each analyte were incorporated into the samples for adequate quantification (see Chapter 4 for further details).

5.3.4 Statistical Analysis

5.3.4a Descriptive statistics

Descriptive statistics, including mean, standard deviation, and coefficient of variation were calculated for each endpoint. In addition, intra-individual variability (e.g., COV%) was calculated for all measured endpoints, i.e., TIQ and β-carboline concentrations, for each volunteer.

In the case that assumptions of normal distribution and equal variance was not met, raw data were log-transformed to comply with the parametric assumptions of equal variance across groups and normal distribution of the residuals. Appropriate summary statistics, using the log-transformed data, such as, median, COV%, percentiles and ranges were computed.

5.3.4b Inferential statistics

Inter-individual variability (i.e., COV% and range) was calculated for the above endpoints as well as all the rating scale scores for each of the three groups and across all groups. The effects of smoking status and gender were evaluated via two-way ANCOVA, incorporating comparison of the factors smoking history and gender. The

effect of sampling occasion (visit) was additionally explored. Specifically, TIQ and β-carboline individual concentrations were evaluated using the following model:

$$Y_{ijk} = \mu + \delta_i + \pi_j + (\delta\pi)_{ij} + m\gamma_k + \varepsilon_{ijk}$$

i = NS, LS, HS

j = M, F

k = baseline covariate

where Y_{ijk} was the response (concentration of TIQ or β-carboline) of the ith smoking status and jth gender relative to their kth covariate. μ is the overall mean, δ_i is the effect of the ith smoking status, π_j is the effect of gender, $(\delta\pi)_{ij}$ is the interaction between the effect of the ith smoking status and the jth gender, γ_k is the effect of the kth baseline covariate possessing a *m* slope and, ε_{ijk} is the random error associated with the Y_{ijk} response. The error term is assumed to be independent and randomly distributed with a mean of 0 and variance σ^2 respective to their effects and independent from each other.

Further exploratory analysis was performed. Both TIQ and β-carboline individual concentrations were correlated with the measures of cigarette exposure (# cigarettes smoked/day) and dependence (FTND) using Pearson's product-moment correlation to evaluate whether these variables are better descriptors for TIQ and β-carboline exposure than the pre-specified smoking status.

Moreover, both TIQ and β-carboline mean concentrations were correlated with the measures dietary intake (TIQ/BC Food Inventory) and alcohol consumption (AAI) using Pearson's product-moment correlation and linear regression to evaluate if these

are significant covariates that need to be implemented into the full model. Associations are to be considered significant with a p-value of <0.05 and a coefficient of determination (R^2) > 0.2**.** Multiple covariate analysis was performed similar to as defined for exposure if there was more than one significant covariate.

All endpoints were tested and compared across all groups in S-PLUS 8.0 (Insightful Corporation, Seattle, WA). The full statistical model was implemented incorporating covariates, if required. The residuals were tested for normality using Quantile-Quantile (Q-Q) plots and further tested using the Shapiro-Wilk test, where α was set to 0.05 such that any p-values > 0.05 indicated that the data was normally distributed. If the data were not normally distributed, the data were log-transformed and the full model was repeated. If the interaction or the baseline covariate was found not to contribute significantly to the full model, the interaction and/or the baseline were removed and a simpler model was used. The level of significance was set a-priori at 0.05. Any statistically significant differences found via ANCOVA were further investigated via Scheffé test to isolate factor differences. All ANCOVAS performed are included in Appendix L.

5.4 Results

5.4.1 Clinical Results

5.4.1a Subject Demographics

A total of forty-three subjects qualified for the study after successfully passing

the initial telephone screening. Forty-one subjects completed both observational periods according to protocol procedures. Two subjects were disqualified from the study due to a positive urine drug screen. Into each smoking status group, 19 NS, 11 LS and, 11 HS were recruited and completed protocol, with twenty-two females and nineteen males comprising the gender distribution within the entire population. The distribution of gender and smoking status for subjects who completed the protocol is as follows: 10 NS females, 9 NS males, 6 LS females, 5 LS males, 6 HS females and 5 HS males. Final subject demographics are included in Table 5-2. The patient summary is included in Appendix G. The subjects were of a mean age of 25.1 years old, ranging from 21-32 years. Females weighed an average of 64.1 kg (range 48.7 – 86.2), while males weighed an average of 80.1 kg (range 66.5 – 107.4). No differences in weight and age were found between smoking groups. Twenty-four Caucasians, fifteen Asians, one Hispanic and one African-American completed the study.

Table 5-2: Clinical Study #1 Subject Demographics

Nonsmokers

Subject #	Race	Gender	Age	Weight	FTND	# Cig/day	pack years	mean CO ppm
1	White	F	21	68	0	0	0	2
2	Black	F	22	52.7	0	0	0	3
3	White	F	31	86.2	0	0	0	2.2
4	Asian	F	26	52.2	0	0	0	1
5	White	F	26	73.3	0	0	0	2.1
6	Asian	F	30	65.3	0	0	0	2
7	White	F	25	65.1	0	0	0	1.8
8	Asian	F	26	52.6	0	0	0	2
9	Asian	F	28	51.8	0	0	0	2.1
10	Asian	F	25	56.8	0	0	0	2.2
11	Hispanic	M	26	70.2	0	0	0	1.6
12	White	M	22	88.1	0	0	0	2
13	White	M	25	71.3	0	0	0	1.8
14	Asian	M	24	71.3	0	0	0	3.2
15	Asian	M	26	67.5	0	0	0	2.2
16	White	M	22	103.6	0	0	0	2.1
17	Asian	M	26	90.8	0	0	0	2
18	Asian	M	30	83.7	0	0	0	1.7
19	Asian	M	23	72.5	0	0	0	2.1
Total Mean			25.5	70.7	0.0	0.0	0.0	2.1
Total SD			2.8	14.6	0.0	0.0	0.0	0.5
Mean Female			26.0	62.4	0.0	0.0	0.0	2.0
SD			3.1	11.4	0.0	0.0	0.0	0.5
Mean Male			24.9	79.9	0.0	0.0	0.0	2.1
SD			2.5	12.3	0.0	0.0	0.0	0.5

Table 5-2: Subject Demographics (continued)

Light-smokers

Subject #	Race	Gender	Age	Weight	FTND	# Cig/day	pack years	mean CO ppm
21	White	F	23	49.5	4	16	2.6	8.0
22	White	F	22	60.9	0	5	1.8	12.2
23	White	F	21	86.0	2	10	1.7	14.1
24	White	F	21	58.7	1	10	3.2	13.2
25	White	F	31	54.1	1	5	1.5	14.5
26	White	F	22	70.2	1	4	1.4	11.7
31	Asian	M	24	67.7	6	10	2.2	16.5
32	White	M	32	78.5	3	17	2.1	14.1
33	Asian	M	26	81	0	5	2.4	9.5
34	Asian	M	24	81.1	2	6	1.4	12.2
35	Asian	M	21	66.5	1	7	1.7	13.4
Total Mean			24.3	68.6	1.9	8.6	2.0	12.7
Total SD			3.9	12.0	1.8	4.5	0.6	2.4
Mean Female			23.3	63.2	1.5	8.3	2.0	12.3
SD			3.8	13.1	1.4	4.6	0.7	2.4
Mean Male			25.4	75.0	2.4	9.0	2.0	13.1
SD			4.1	7.3	2.3	4.8	0.4	2.6

Heavy-smokers

Subject #	Race	Gender	Age	Weight	FTND	# Cig/day	pack years	mean CO ppm
41	White	F	21	51.2	4	20	9.5	18.4
42	White	F	23	59.0	5	20	11.2	19.5
43	White	F	21	48.7	6	30	13.2	11.7
44	White	F	24	79.7	7	25	11.5	17.5
45	White	F	28	83.6	7	30	7.4	23.7
46	White	F	31	83.6	4	30	11.2	11.9
51	White	M	23	107.4	7	15	5.8	13.7
52	White	M	24	78.8	8	15	12.2	19.5
53	White	M	25	79.8	6	17	10.2	16.8
54	Asian	M	25	79.7	7	20	7.5	20.4
55	White	M	31	82.6	6	20	10.5	22.7
Total Mean			25.1	75.8	6.1	22.0	10.0	17.8
Total SD			3.5	16.9	1.3	5.8	2.3	4.0
Mean Female			24.7	67.6	5.5	25.8	10.7	17.1
SD			4.0	16.5	1.4	4.9	2.0	4.6
Mean Male			25.6	85.7	6.8	17.4	9.2	18.6
SD			3.1	12.2	0.8	2.5	2.6	3.5

Of note, no statistically significant difference was observed with respect to weight and age between smoking groups. With regard to smoking status and frequency of cigarette smoking, figures 5-2, 5-3 and 5-4 show FTND scores, mean cigarette consumption per day and breath carbon monoxide (CO), respectively. Both nonsmoking (NS) males and females scored a mean of 0 (± 0 SD) on the FTND and self reported mean number of cigarettes smoked per day, as expected. LS females scored a mean FTND of 1.5 (± 1.4 SD) while LS males scored a slightly higher value of 2.4 (± 2.3 SD). HS females scored a 5.5 (± 1.4 SD) compared to HS males who scored on average a 6.6 (± 0.9 SD). Of note, the FTND difference between the genders was not significantly different (two-tailed unpaired t-test, p-values were greater than 0.088 for both LS and HS status). With respect to the number of cigarettes smoked per day, LS females and LS males had similar results with averages of both groups being 8.3 (± 4.6 SD) and 9.0 (± 4.8 SD) cigarettes smoked per day for females and males, respectively. HS subjects smoked on average a two-fold more number of cigarettes per day than the LS counterparts. In this group, female smokers possessed a higher average than that of males with an average of 25.8 (± 4.9 SD) for females and 17.4 (± 2.5 SD) for males. As expected, smokers had both higher FTND and number of cigarettes smoked per day than that of nonsmokers.

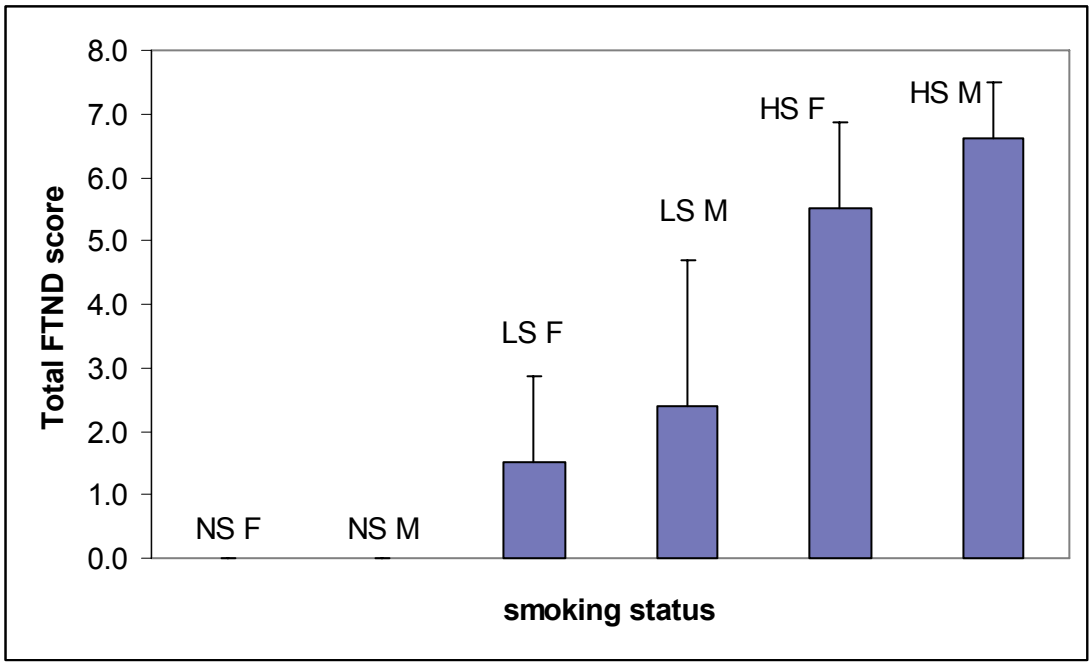


Figure 5-2: Mean (± SD) Fagerström Test for Nicotine Dependence (FTND) Score vs. Smoking Status (F: female, M: male)

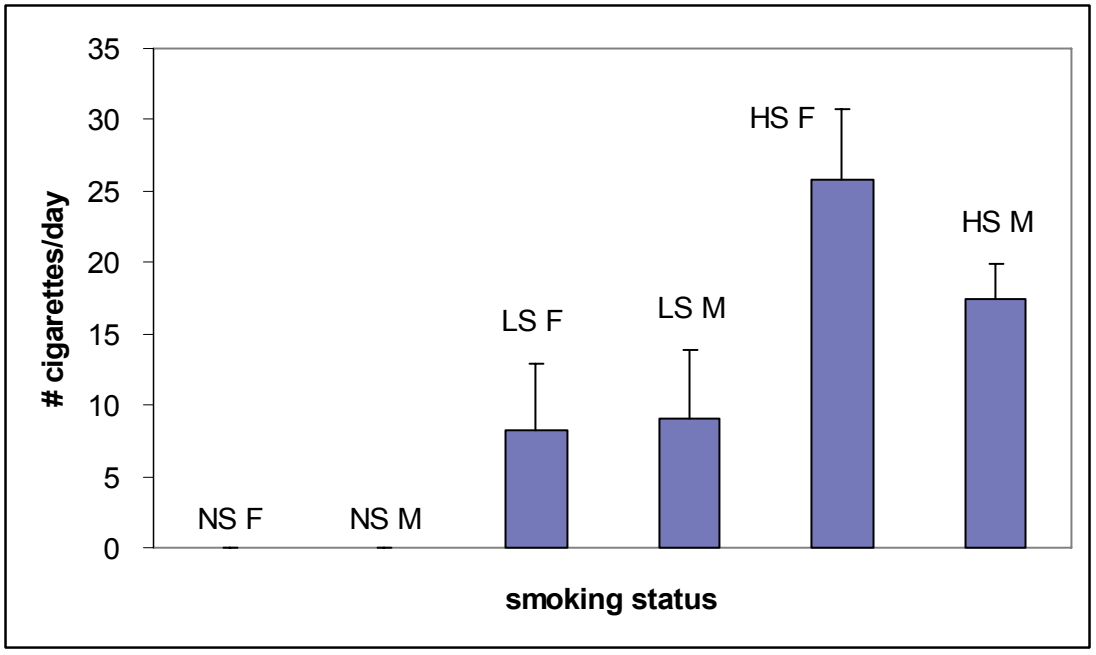


Figure 5-3: Mean (± SD) Cigarettes smoked per day vs. Smoking Status (F: female, M: male)

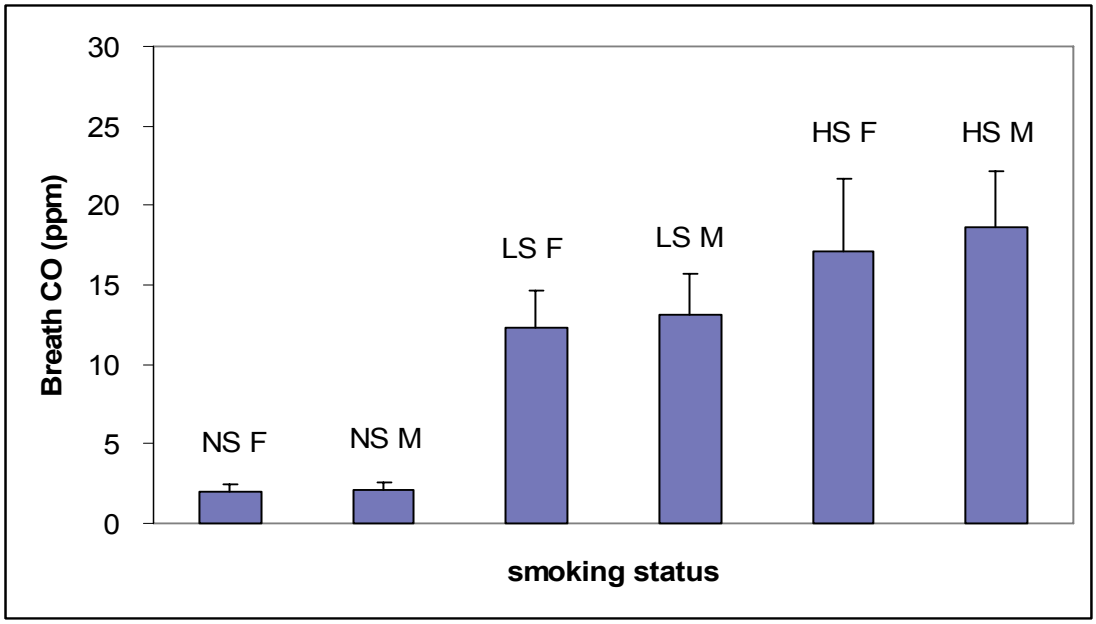


Figure 5-4: Mean (± SD) Expired Carbon Monoxide vs. Smoking Status (F: female, M: male)

With respect to breath carbon monoxide (CO), negligible differences were observed between genders within smoking groups. NS males and females possessed a mean CO level of 2.0 (± 0.5 SD) and 2.1 ppm (± 0.5 SD), respectively. This positive value of expired CO in the NS group is presumed to stem from incidental environmental exposure. Conversely, LS and HS possessed significantly higher levels of expired CO, seemingly due to recent cigarette smoking (within 30 minutes) by the smoking subjects. The mean CO measured in LS males and females was 13.1 (± 2.6 SD) and 12.3 (± 0.5 SD) while CO measurement in HS males and females resulted in 18.6 (± 3.5 SD) and 17.1 (± 4.6 SD), respectively.

5.4.1b Alcohol Intake and Food Inventory

In addition to the demographic and TIQ and β-carboline plasma measurements, information including alcohol intake and weekly exposure to dietary total SAL, harman and norharman was recorded via the Annual Alcohol Intake (Khavari and Farber, 1978) and TIQ/BC Food Inventory (Appendix C and D). As dietary intake of ethanol and/or TIQ and β-carboline containing foods may affect the overall exposure, these assessments may provide clues into the variability associated with the plasma measurement of SAL, harman and norharman. If alcohol intake and/or dietary exposure were found to significantly correlate with SAL or β-carboline exposure, AAI or Food Intake was considered a significant covariate and implemented into the statistical model.

With regard to ethanol intake, the AAI was administered to each subject on a single occasion. The total amount of alcohol consumed, in terms of milligrams of pure ethanol, was calculated upon evaluation of the usual and maximum frequencies along with usual and maximum volumes of three different types of alcoholic beverages including, wine, beer and spirits. From the assessment a total quantity of alcohol was calculated in milliliters with subsequent calculation of the absolute yearly ethanol content, accounting for the different percentage of ethanol in each type of beverage. Of primary note, this is a self-report measure of overall estimate of total ethanol consumption. It is not a diagnostic tool for alcoholism disease status nor is it sensitive to detect change in drinking patterns. The descriptive results of the AAI may be viewed in the table below in milligrams of annual absolute ethanol consumed.

In regard to dietary exposure of the analytes, the food survey incorporated measurements of type of food, average frequency and average portion size of each known food source to have significant quantities of SAL or β-carboline. A numerical value for each food type was calculated for each subject accounting for frequency and portion size. The ordinal value was subsequently multiplied by the absolute amount of SAL or β-carboline present in that food source. Absolute amounts within those food sources that have known, measureable quantities of SAL and/or β-carboline were calculated using reported literature values (Hirst et al., 1985; Collins et al., 1990; Pfau and Skog, 2004). SAL, harman and norharman amounts are reported as weekly intake of analyte in nanograms. The descriptive results of the TIQ/BC Food Inventory may be viewed table 5-3 below in average nanograms of weekly total SAL, norharman and harman consumed.

Table 5-3: Descriptive statistics of alcohol intake and dietary consumption

			Non-smoker	Light-smoker	Heavy-smoker	GRAND
AAI (mg annual ethanol)	Male	Mean	1219	3219	6196	3055
		%COV	127	71	52	99
		Median	717	4744	6516	1266
	Female	Mean	915	1160	2084	1301
		%COV	111	100	159	151
		Median	507	804	910	702
	Overall	Mean	1059	2096	3953	2114
		%COV	121	96	102	125
		Median	668	1004	1159	970
Dietary harman (ng/week)	Male	Mean	1334	1529	997	1296
		%COV	45	90	43	65
		Median	1197	1971	1218	1218
	Female	Mean	1383	1318	1398	1369
		%COV	63	42	17	48
		Median	1147	1243	1386	1203
	Overall	Mean	1360	1414	1216	1335
		%COV	55	70	32	56
		Median	1168	1393	1334	1218
Dietary norharman (ng/week)	Male	Mean	2112	2293	1699	2051
		%COV	36	33	28	58
		Median	1796	2957	1826	1826
	Female	Mean	2074	1977	1998	2027
		%COV	63	42	14	48
		Median	1720	1864	2000	1805
	Overall	Mean	2092	2121	1862	2038
		%COV	51	70	21	53
		Median	1753	2090	1998	1826
Dietary total SAL (ng/week)	Male	Mean	2136	3822	2092	2568
		%COV	65	90	46	91
		Median	1122	4928	1562	1562
	Female	Mean	2905	1712	1424	2176
		%COV	87	110	101	100
		Median	1988	1269	965	1559
	Overall	Mean	2541	2671	1728	2358
		%COV	89	107	73	95
		Median	1663	1555	1421	1562

Estimated annual ethanol exposure (AAI) resulted in substantial differences between genders within smoking groups. Results here are reported as median and range. NS males and females possessed a median annual ethanol exposure of 717 mg/year (range: 0 – 4614 mg/year) and 506 mg/year (26.5 – 2758 mg/year), respectively. Conversely, LS and HS possessed significantly higher AAI scores. The median mg of ethanol estimated in LS males and females was 4744 mg/year (184 – 4764 mg/year) and 804 mg/year (57.9 – 3214 mg/year) while in HS males and females resulted in 6516 mg/year (969 – 10264 mg/year) and 910 mg/year (129 – 9120 mg/year), respectively. Nonsmokers possessed a median mg of annual ethanol consumed of 668 mg/day (range 0 – 4614 mg/day) while the estimated LS and HS annual ethanol intake was 1004 mg/day (58 – 5399 mg/day) and 1159 (128 – 10624 mg/day). Figure 5-5 represents the distribution of the mg of ethanol intake, per smoking status and gender.

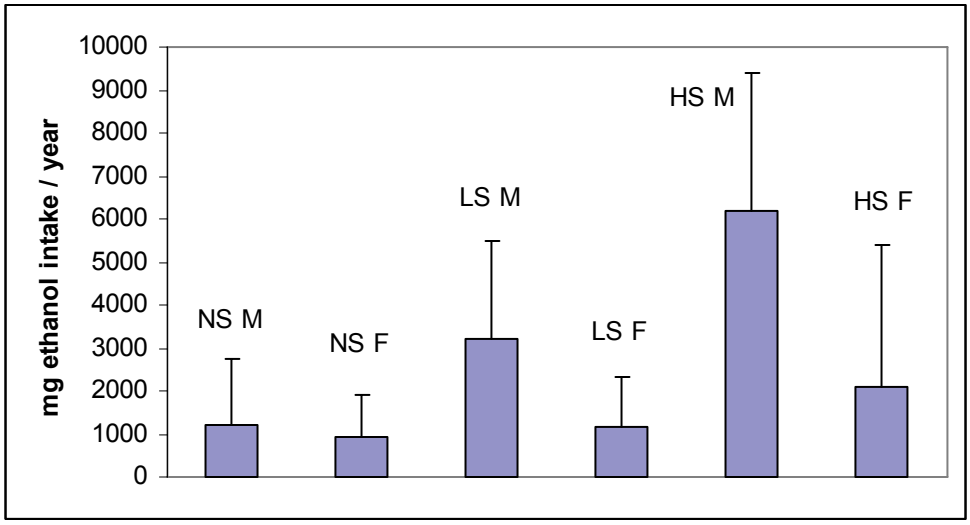


Figure 5-5: Estimated annual absolute alcohol intake in mg ethanol / year

A systematic trend was observed between smoking groups and gender for the total amount of ethanol consumed per annum. On average, females had a lower estimated ethanol intake compared to males as a whole and across smoking groups. Interestingly smoking status showed a significant trend with a resultant increase in estimated ethanol consumption with increasing level of smoking status. This result was expected as smokers are known to consume twice as much alcohol as do non-smokers (DiFranza and Guerrera, 1990). Formal evaluation of AAI as a significant covariate will be explored further as alcohol intake may affect the exposure of plasma SAL and β-carbolines.

Dietary TIQ and β-carboline exposure measured via the TIQ/BC food inventory yielded estimates that were statistically similar across smoking groups. For weekly dietary harman intake, NS males and females possessed an estimated median weekly dietary harman exposure of 1168 ng/week while in LS and HS, estimated median dietary exposure was 1393 ng/week and 1334 ng/week, respectively. For weekly dietary norharman intake, estimated median weekly dietary exposure for NS was 1752 ng/week while in LS and HS, estimated median dietary exposure was 2089 ng/week and 1998 ng/week. Within all smoking status groups, a large range of dietary intake of harman and norharman was observed.

In the case of weekly dietary SAL exposure, the individual enantiomeric composition of the food sources was not available. Therefore, dietary total SAL was estimated. It has been purported that, in food sources, the enantiomeric ratio is 1:1, as previously discussed. For weekly dietary SAL intake, estimated median weekly dietary

exposure for NS was 1663 ng/week (range: 243 – 9182 ng/week) while in LS and HS, estimated median dietary exposure was 1555 ng/week (range: 109 – 8729 ng/week) and 1421 ng/week (range: 109 – 4322 ng/week).

The average dietary intake between smoking status and gender groups for harman, norharman, and SAL are shown in the figures 5-6, 5-7, and 5-8, respectively.

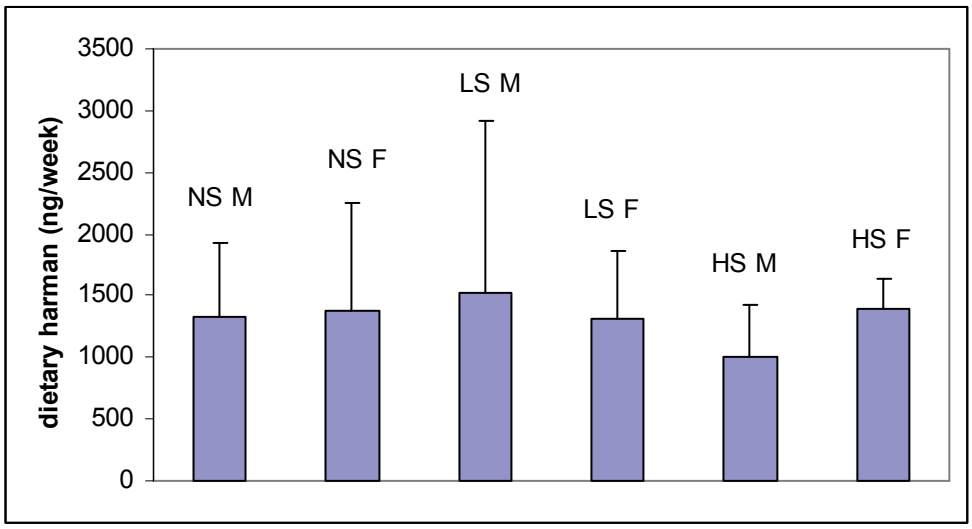


Figure 5-6: Estimated dietary harman intake in ng / week (mean ± SD)

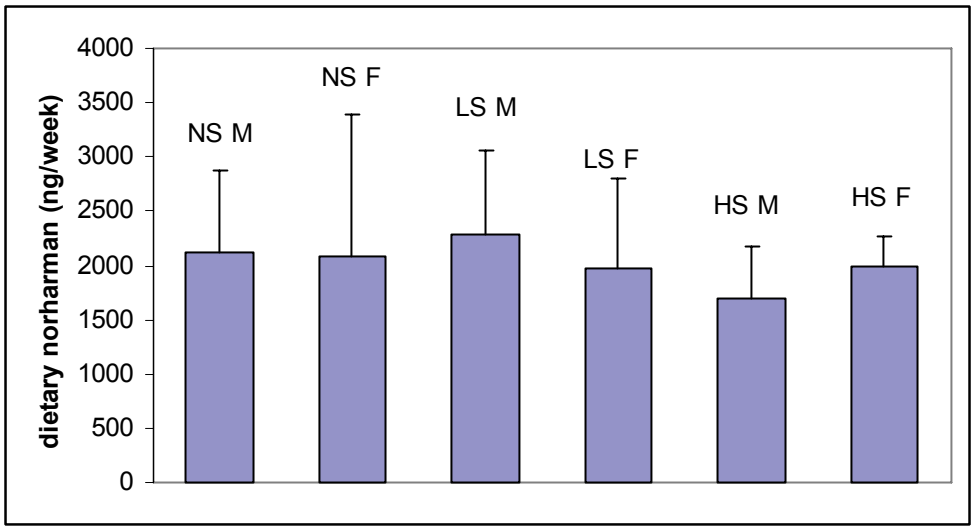


Figure 5-7: Estimated dietary norharman intake in ng / week (mean ± SD)

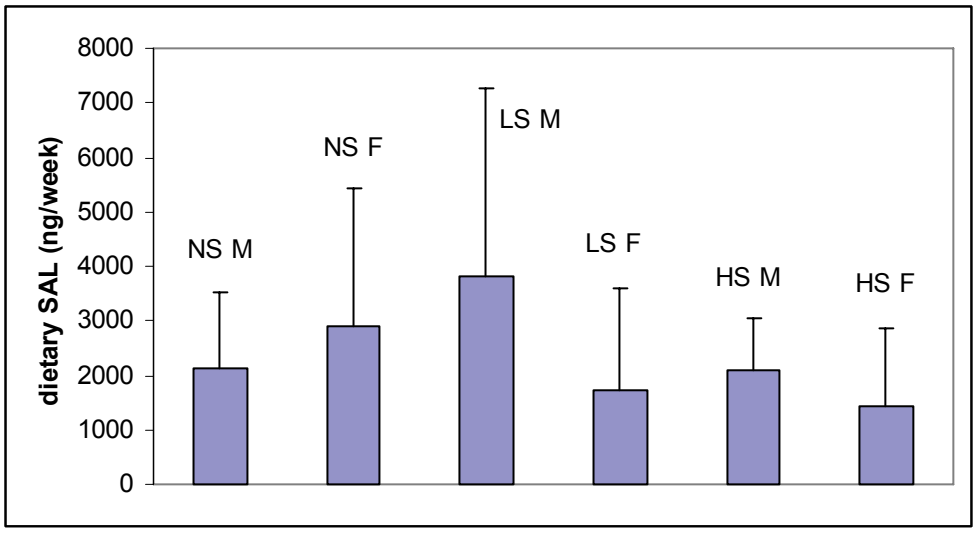


Figure 5-8: Estimated dietary total salsolinol (SAL) intake in ng / week (mean ± SD)

With the exception of dietary SAL, no statistically significant difference was observed between the smoking status groups. The LS group possessed, on average, a

higher estimated consumption of dietary SAL than NS and HS groups but the variability associated with this estimate is large (%COV of 107%).

5.4.1c Adverse Events

Due to the minimally invasive nature of this study, the investigation progressed without major complications. Out of all subject completions, only one subject complained of bruising at the injection site but was subsequently resolved within 12 hours upon follow up telephone call. During the first visit, three subjects possessed asymptomatic systolic hypotension after blood draw. For the second visit, four different individuals had similar signs. At the end of each visit, all subjects, including those with mild hypotension, were alert and able to ambulate independently and return to daily routines.

5.4.2 Primary Analysis for β-carbolines – Effects of smoking and gender

5.4.2a β-carbolines – Within Subject Variability

Prior to formal statistical comparison of H and NH between smoking and gender factors, within-subject variability of both β-carbolines for each subject was evaluated. This analysis was performed to assess the baseline stability of H and NH over two observational time points with the second observation being collected within one week of the first clinical visit. The intra-subject variability (%COV and inter-quartile ranges) for NH and H may be seen in the tables 5-4 and 5-5 below.

Table 5-4: Within Subject Variability for Plasma Norharman (pg/ml)

Sample #	GEN	NH avg	stdev	%COV	median	25% IQR	75% IQR
Nonsmokers							
1	F	9	11	123	9	5	13
2	F	5	1	31	5	4	5
3	F	19	3	15	19	18	20
4	F	7	0	4	7	7	7
5	F	28	3	10	28	27	29
6	F	5	0	5	5	5	5
7	F	45	28	62	45	35	55
8	F	21	3	14	21	20	22
9	F	2	0.1	10	2	2	2
10	F	25	11	43	25	21	28
11	M	23	7	31	23	21	26
12	M	7	2	24	7	7	8
13	M	60	36	60	60	47	72
14	M	24	20	83	24	17	31
15	M	51	33	64	51	39	62
16	M	15	1	10	15	14	15
17	M	62	10	17	62	58	65
18	M	4	1	25	4	4	4
19	M	30	28	92	30	20	40
Light Smokers							
21	F	89	4	5	89	87	90
22	F	39	23	58	39	31	47
23	F	36	5	15	36	34	38
24	F	17	7	41	17	15	20
25	F	45	12	26	45	41	50
26	F	84	30	36	84	73	94
31	M	333	85	26	333	303	364
32	M	81	5	6	81	79	83
33	M	40	8	21	40	37	43
34	M	140	17	12	140	134	146
35	M	43	1	2	43	42	43
Heavy Smokers							
41	F	236	124	52	236	193	280
42	F	289	117	40	289	247	330
43	F	109	15	14	109	104	114
44	F	161	181	112	161	97	225
45	F	284	177	62	284	221	347
46	F	238	109	46	238	199	277
51	M	67	53	78	67	49	86
52	M	33	12	35	33	29	37
53	M	119	116	98	119	78	160
54	M	181	3	2	181	180	182
55	M	145	150	104	145	92	198

Table 5-5: Within Subject Variability for Plasma Harman (pg/ml)

Sample #	GEN	H avg	stdev	%COV	median	25% IQR	75% IQR
Nonsmokers							
1	F	12	13	115	12	7	16
2	F	2	4	165	5	4	6
3	F	26	4	16	29	28	31
4	F	7	1	8	8	8	8
5	F	19	3	17	21	20	22
6	F	4	0	8	4	4	4
7	F	74	29	39	54	43	64
8	F	13	13	101	22	18	27
9	F	2	0	14	2	2	2
10	F	13	5	41	9	7	11
11	M	12	16	138	23	17	28
12	M	10	2	19	8	8	9
13	M	16	3	18	14	13	15
14	M	7	13	176	16	12	21
15	M	88	7	8	93	90	95
16	M	23	1	5	22	21	22
17	M	96	17	18	84	78	90
18	M	5	0	7	6	5	6
19	M	4	3	72	5	5	6
Light Smokers							
21	F	129	5	4	125	124	127
22	F	76	33	43	53	42	65
23	F	55	11	19	62	58	66
24	F	5	47	1026	38	21	55
25	F	4	62	1668	47	26	69
26	F	139	23	17	155	147	163
31	M	36	2	5	37	37	38
32	M	102	57	56	62	42	82
33	M	68	70	103	118	93	142
34	M	144	31	22	121	110	132
35	M	68	5	8	64	62	66
Heavy Smokers							
41	F	422	41	10	393	379	408
42	F	12	5	40	15	13	16
43	F	386	181	47	258	194	322
44	F	63	106	168	138	100	175
45	F	55	63	115	99	77	121
46	F	49	110	224	127	88	166
51	M	36	4	12	39	38	41
52	M	11	13	121	20	15	24
53	M	356	217	61	202	126	279
54	M	46	65	142	91	68	114
55	M	4	30	760	26	15	36

For NH and H concentrations, it can be noted that the variability between sampling occasions ranged from a %COV 1.8 to 123 % for NH and %COV 3.6 to 1668% for H, suggesting that baseline concentrations of these β-carboline analytes are not always stable or reproducible between occasions. Inter-quartile ranges for the between occasion variability is the more appropriate assessment for variability between two observations but, for simplicity sake, %COV were computed and compared. For non-smokers %COV ranged from 3.7 – 123% for NH and 7.4 – 165% for H. Presumably, these persons have not been recently exogenously exposed to H and NH, inferring that factors, other than smoking status or gender may contribute to the variability in baseline between occasions. For the LS population, %COV 2.1 – 58% for NH and %COV 3.6 – 1668% for H were observed, while the HS population possessed %COV 1.8 – 112% for NH and %COV 9.7 – 760% for H. The large variability between occasions observed within the smoking status populations may be due to the different times of sampling with respect to cigarette smoking. Although each subject was sampled within 30 minutes of smoking, the sampling times varied considerably within this time frame for each subject. As it has been noted by two researchers, plasma half-lives of 51 minutes (Breyer-Pfaff et al., 1996) and 25-30 minutes (Rommelspacher et al., 2002) for harman and norharman, respectively, have been estimated from human studies. Because of short plasma half-lives, and the time it takes for the subject to smoke a complete cigarette and arrive to the clinic for a blood draw is variable, it was difficult to consistently sample at a reproducible time. Of note, both genders possessed the similar amount of within-subject variability with males possessing a %COV 1.8 –

103.7% for NH and %COV 4.7 to 760% for H were observed while in the female counterparts, a %COV 2.1 – 123.0% for NH and %COV 7.2 to 1668.1% for H were observed. Only two subjects in the LS female group possessed very large within subject variability (%COV >1000%) for H concentrations. This is seemingly due to the large sampling time differences with respect to smoking a cigarette, between occasions.

As large within-subject variability was observed with majority of the subjects, individual subject concentrations were used, as opposed to average subject concentrations for providing descriptive statistics of the overall measure of central tendency and variability. This was to ensure that the intra-subject variability was not masked during descriptive and inferential statistical analysis.

Tables 5-6 and 5-7 exemplify the descriptive statistics for the concentrations observed for each smoking status and gender. Measures of central tendency, such as mean and median, along with the variability including %COV and range are presented for each factor. Of note, the distribution for the number of subjects is unbalanced with respect to smoking status and gender within each group. The distribution of gender and smoking status for subjects who completed the protocol is as follows: 10 NS females, 9 NS males, 6 LS females, 5 LS males, 6 HS females and 5 HS males. It is important to note that each smoking status: gender group contains at least 5 subjects.

Table 5-6: Descriptive statistics for Norharman concentrations (pg/ml) divided into smoking status and gender.

NORHARMAN

		Non-smoker	Light-smoker	Heavy-smoker	GRAND
Female	Mean	16	52	220	81
	Stdev	15	30	119	107
	%COV	93	57	55	132
	Median	17	47	183	32
	MIN	2	12	33	2
	MAX	65	105	410	410
Male	Mean	31	127	109	77
	Stdev	26	119	86	87
	%COV	84	93	79	113
	Median	23	81	73	42
	MIN	3	34	25	3
	MAX	85	394	251	394
GRAND	Mean	23	86	169	79
	Stdev	22	89	117	98
	%COV	95	105	69	123
	Median	17	54	160	38
	MIN	2	12	25	2
	MAX	85	394	410	410

Table 5-7: Descriptive statistics for Harman concentrations (pg/ml) divided into smoking status and gender.

HARMAN

		Non-smoker	Light-smoker	Heavy-smoker	GRAND
Female	Mean	16	80	172	76
	Stdev	17	53	148	103
	%COV	107	66	86	136
	Median	10	74	137	32
	MIN	2	4	12	2
	MAX	74	172	422	422
Male	Mean	30	80	76	55
	Stdev	33	47	105	66
	%COV	111	59	139	119
	Median	14	68	44	36
	MIN	4	22	4	4
	MAX	98	167	356	356
GRAND	Mean	23	80	128	66
	Stdev	27	49	136	88
	%COV	117	61	107	132
	Median	12	71	52	34
	MIN	2	4	4	2
	MAX	98	172	422	422

The overall average, across all patients for NH and H were 79.2 (123.4%) and 66.4 pg/ml (132.4%) suggesting that the plasma β-carboline concentrations are quite variable between all patients. The significant differences between the median and mean NH and H concentrations suggest that the data follow non-normal distribution. Upon comparison of the median concentrations and ranges, females were observed to have a median NH concentration of 32 pg/ml while median H concentrations were 31.8 pg/ml. Their male counterparts had a similar median NH concentration of 41.8 pg/ml and median H concentrations were 36.3 pg/ml. With respect to smoking status, median concentrations along with ranges are presented in the preceding table.

Median H concentrations of female NS, LS and HS were 10.4, 73.8 and 136.5 pg/ml, respectively while median NH concentrations were 16.8, 44.6 and 183.3 pg/ml. Males within NS, LS and HS groups had median H concentrations of 13.5, 70.5, and 51.9 pg/ml and median NH concentrations of 17.2, 54.4, and 159.6 pg/ml, respectively.

5.4.2b β-carbolines - Primary Factors of Smoking and Gender Analysis

The distribution of the data within each smoking group for both H and NH followed a non-normal distribution upon evaluation of quantile-quantile (Q-Q) plots. The large discrepancy between the median and mean concentrations within each group also exemplifies the non-normality of the distributions. Further evaluation of normality of the data via the Shapiro-Wilk test resulted in p-values all less than 0.03 for both analytes across all groups, suggesting that the data were indeed non-normally distributed. Moreover, unequal variance was present with the data upon visual

inspection of the residuals. For these reasons, log-transformed data were used for the primary analysis.

Separate evaluation of the effects of smoking status and gender were performed on both log H and log NH concentrations. Correlation analysis between the analytes suggested that the association between the two analytes was moderate (r = 0.65, see figure 5-9 below), therefore both analytes are treated separately for statistical analysis.

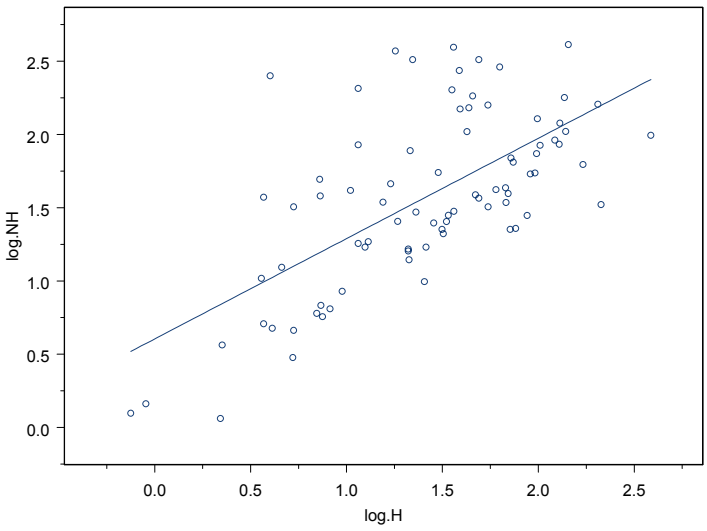


Figure 5-9: Correlation between log H and log NH. Correlation coefficient for the association is r = 0.652.

Two-way ANOVA was performed on the log transformed H and NH evaluating the factors of smoking status and gender. Evaluation of the effects of sampling visit was performed and was further explored upon significance of the effect. Box-plots showing the median and distribution, including outliers for the effects of gender and smoking status are presented below for both H (figure 5-10) and NH (figure 5-11). The

horizontal line in the interior of the box is located at the median log β-carboline, while the "x" denotes the mean concentration. The height of the box is equal to the interquartile distance or IQD, which is the difference between the third and first quartiles of the data. The whiskers include 99.3% of all data while the outliers are presented as horizontal lines outside of the whiskers.

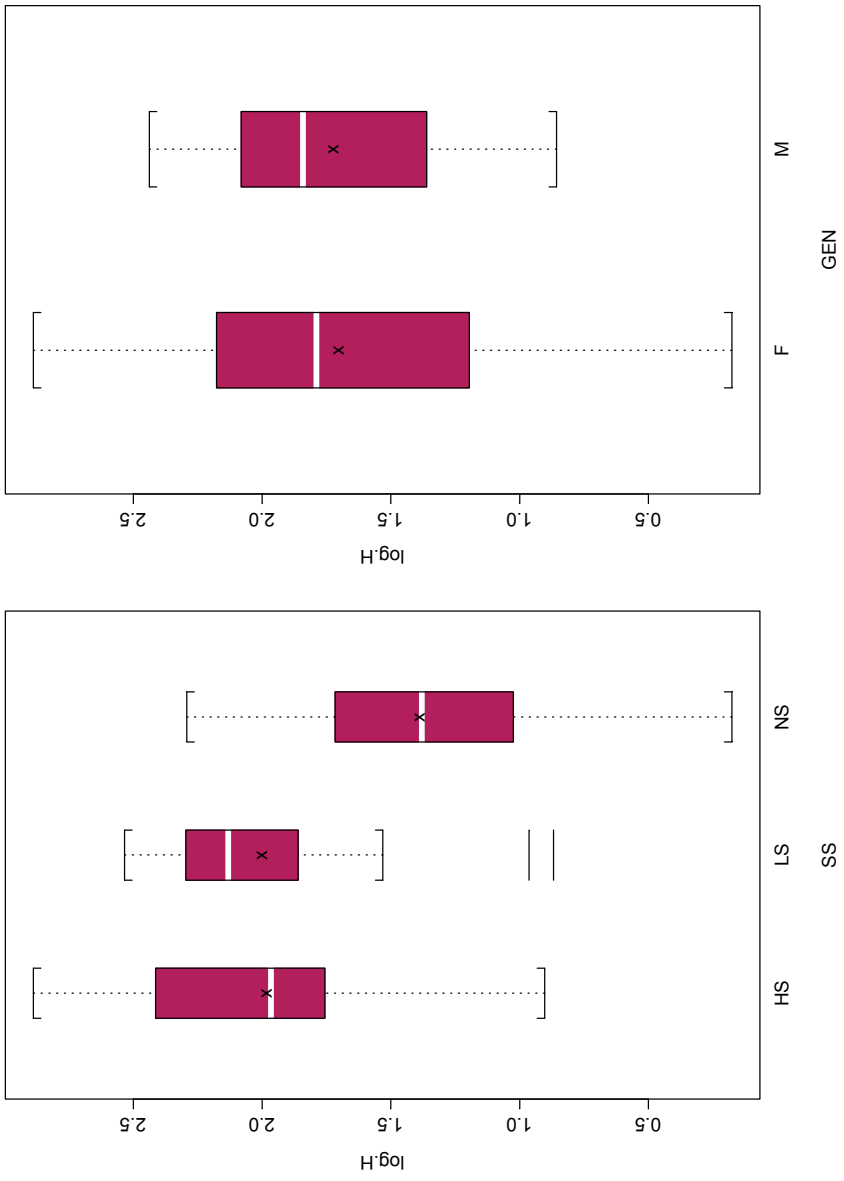


Figure 5-10: Boxplots of log H for smoking status (SS, left plot) and gender (GEN, right plot)

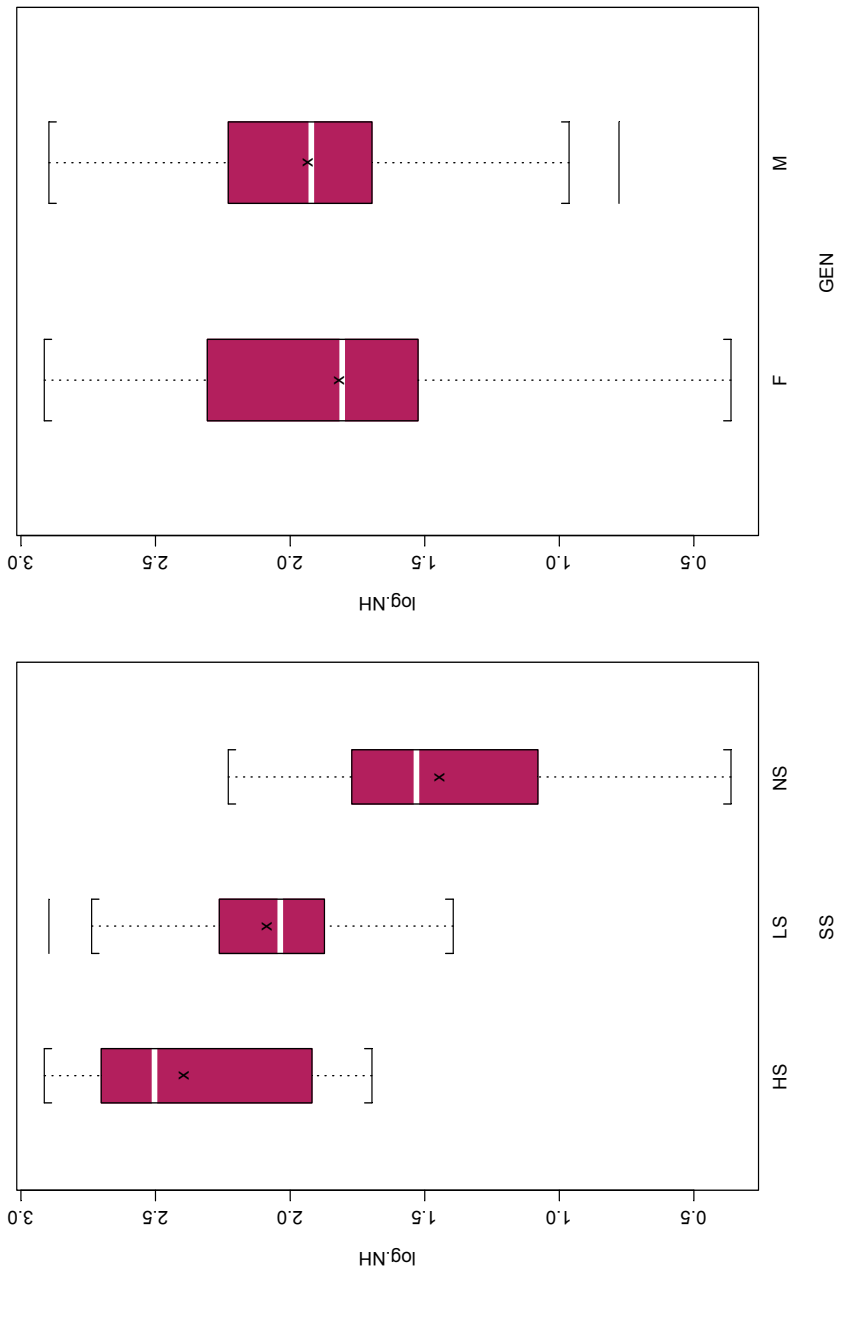


Figure 5-11: Boxplots of log NH for smoking status (SS, left plot) and gender (GEN, right plot)

Two-way ANOVA was performed to evaluate the effects of gender and smoking on log H concentrations. Statistical data are presented as F test for two-way ANOVA (*df* for factors, *df* for residuals = F-statistic, p-value).

A significant effect of smoking status (SS) was observed with respect to log H concentrations with F (2, 75) = 16.7, p-value = 1.0×10^{-6}. The effect of gender (GEN) was not significant with the test statistic resulting in F (1, 75) = 0.09, p-value = 0.756. No interaction between the SS and GEN factors was observed (F (2, 75) = 3.02, p-value = 0.074). An effect of sampling visit was not observed (p-value = 0.08). The coefficient of determination (R^2) for the entire model was 0.312 (p-value = 7.4×10^{-6}). Thus, the factors of SS and GEN account for approximately 31.2% of the variability associated with the log H concentration measurements.

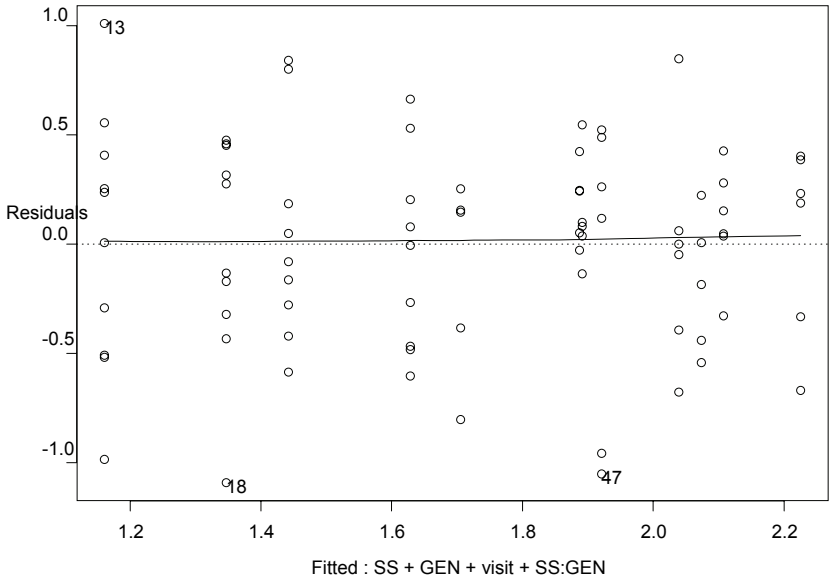
In order to determine where the difference within smoking status resides, a *post-hoc* multiple comparison test was performed. The Scheffé method for multiple comparisons found that the primary differences were observed between the HS and NS groups as well as the LS and NS groups. Via this comparison, a significant difference was not observed between the HS and LS smoking status groups.

The full two-way ANOVA output for Log H can be seen in figure 5-12 below along with the residual plot of the model fit (figure 5-13). Upon visual evaluation, the residuals show an even spread of the distribution of data suggesting the lack of unequal variance. The results of the multiple comparisons are presented below the ANOVA table where a significant difference between groups is denoted by asterisks.

```
      *** Analysis of Variance Model ***

Short Output:
Call:
   aov(formula = log.H ~ SS + GEN + visit + SS:GEN, data =
       SAL.BC..Final.Bioanalysis.Data.VCU.Smoking.031808, na.action =
       na.exclude)


          Df Sum of Sq  Mean Sq  F Value     Pr(F)
       SS  2   7.40497 3.702483 16.69658 0.0000010
      GEN  1   0.02159 0.021590  0.09736 0.7558877
    visit  1   0.71119 0.711188  3.20715 0.0773521
   SS:GEN  2   1.34362 0.671812  3.02958 0.0742898
Residuals 75  16.63132 0.221751

Multiple R-Squared: 0.3116
F-statistic: 8.715 on 4 and 77 degrees of freedom, the p-value is 7.41e-006


95 % simultaneous confidence intervals for specified
linear combinations, by the Scheffé method

critical point: 2.86
response variable: log.H
rank used for Scheffé method: 3

intervals excluding 0 are flagged by '****'

      Estimate Std.Error Lower Bound Upper Bound
HS-LS   -0.032     0.143      -0.440       0.376
HS-NS    0.571     0.127       0.209       0.933 ****
LS-NS    0.603     0.127       0.241       0.965 ****
```

Figure 5-12: Two-way ANOVA output, with multiple comparisons, for the effects of SS and GEN on Log H plasma concentrations.

Figure 5-13: Residual plot for two-way ANOVA fit for Log H.



With respect to Log NH concentrations, a significant effect of smoking status (SS) was observed with F (2, 75) = 41.67, p-value = 0.3×10^{-7}. The effect of gender (GEN) was not significant with the test statistic resulting in F (1, 75) = 2.11, p-value = 0.150. No interaction between the SS and GEN factors was observed (F (2, 75) = 2.7, p-value = 0.065). An effect of sampling visit was not observed (p-value = 0.74). The coefficient of determination (R^2) for the entire model was 0.498 (p-value = 6.2×10^{-11}). Thus, the factors of SS and GEN account for approximately 49.8% of the variability associated with the log NH concentration measurements.

The Scheffé method for multiple comparisons found that the primary differences were observed between the HS and NS groups as well as the LS and NS groups. Via

this comparison, a significant difference was not observed between the HS and LS smoking status groups, as in the same case for H.

The full two-way ANOVA output for Log NH may be seen in the figure 5-14 below along with the residual plot of the model fit (figure 5-15). Upon visual evaluation, the residuals show an even spread of the distribution of data suggesting the lack of unequal variance.

```
*** Analysis of Variance Model ***

Short Output:
Call:
aov(formula = log.NH ~ SS + GEN + visit + SS:GEN, data =
       SAL.BC..Final.Bioanalysis.Data.VCU.Smoking.031808, na.action =
       na.exclude)

Residual standard error: 0.4090246
Estimated effects may be unbalanced

           Df Sum of Sq  Mean Sq  F Value     Pr(F)
       SS   2  13.94415 6.972076 41.67382 0.0000000
      GEN   1   0.35342 0.353417  2.11246 0.1502757
    visit   1   0.01848 0.018483  0.11048 0.7405308
   SS:GEN   2   1.90789 0.953946  5.70197 0.0649521
Residuals 75  12.54758 0.167301


Multiple R-Squared: 0.4976
F-statistic: 19.06 on 4 and 77 degrees of freedom, the p-value is 6.246e-011



95 % simultaneous confidence intervals for specified
linear combinations, by the Scheffé method

critical point: 2.86
response variable: log.NH
rank used for Scheffé method: 3

intervals excluding 0 are flagged by '****'

      Estimate Std.Error Lower Bound Upper Bound
HS-LS    0.276     0.124     -0.0778       0.631
HS-NS    0.924     0.110      0.6100       1.240 ****
LS-NS    0.648     0.110      0.3330       0.962 ****
```

Figure 5-14: Two-way ANOVA output, with multiple comparisons, for the effects of SS and GEN on Log NH plasma concentrations.

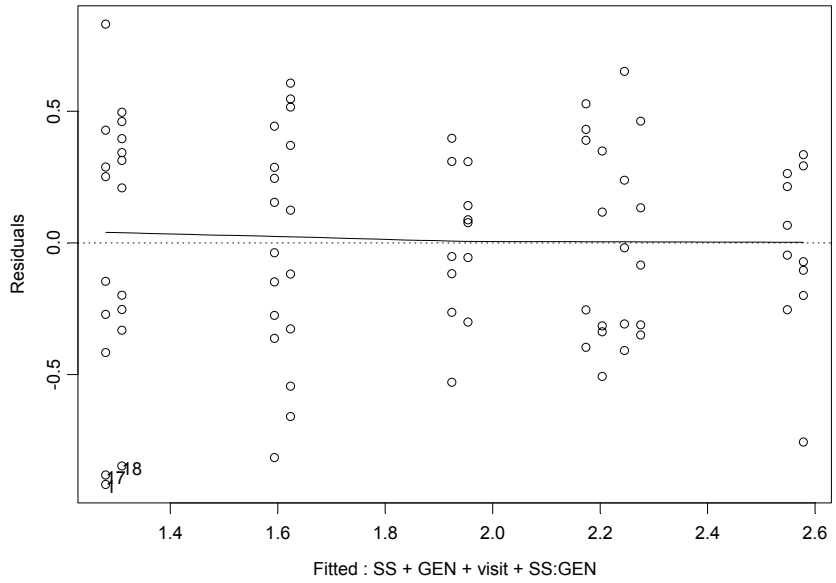


Figure 5-15: Residual plot for two-way ANOVA fit for Log NH.

A significant effect of smoking status was observed for both log H and log NH plasma concentrations. A statistically significant effect of gender was not observed in the exposure and an interaction between the factors was not observed for either analyte. According to the two-way ANOVA, the following rank was observed with plasma H concentrations (geometric means) across smoking groups and gender. NS F < NS M < HS M < LS M < LS F < HS F. The *post-hoc* multiple comparisons via Scheffé test showed that the major difference that was observed with the smoking groups were the HS – NS group and the LS – NS groups. According to this analysis, no significant difference was observed between the two smoking groups of LS and HS. On average, the LS group had a slightly larger mean than that of the HS groups for log H.

ANOVA results assigned the following rank with respect to observed plasma NH concentrations across smoking groups and gender. NS F < NS M < LS F < HS M < LS M < HS F. The *post-hoc* multiple comparisons via Scheffé test showed that the major difference that was observed with the smoking groups were the HS – NS group and the LS – NS groups. A statistically significant difference was not observed between the two smoking groups of LS and HS. On average, across smoking groups, the HS group > LS group > NS group for log NH. See figures below.

For both NS and LS smoking status groups, males possessed higher concentrations than females. The female HS group yielded higher average concentrations than that of their male counterparts for both H and NH. The fact that HS females smoked more cigarettes per day on average compared to males may provide an explanation for this divergence in plasma concentrations.

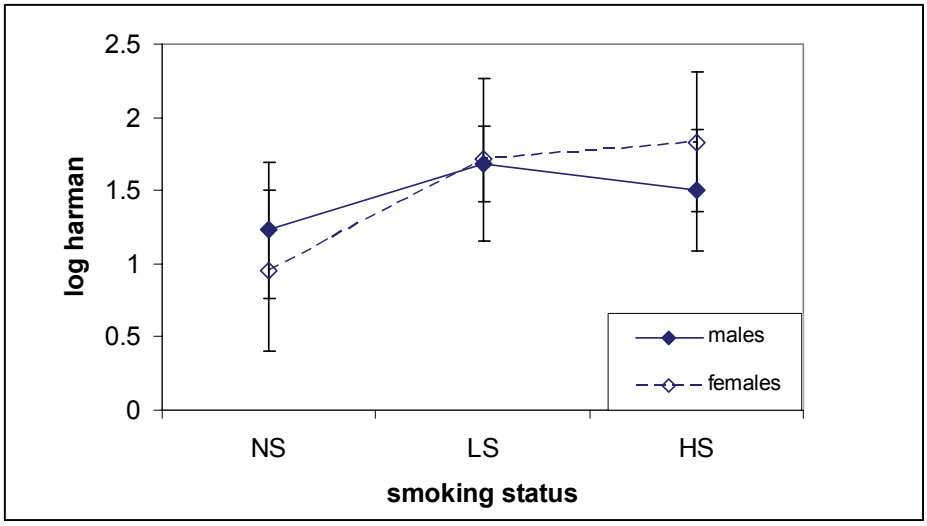


Figure 5-16: Log H as function of smoking status and gender (mean ± SD).

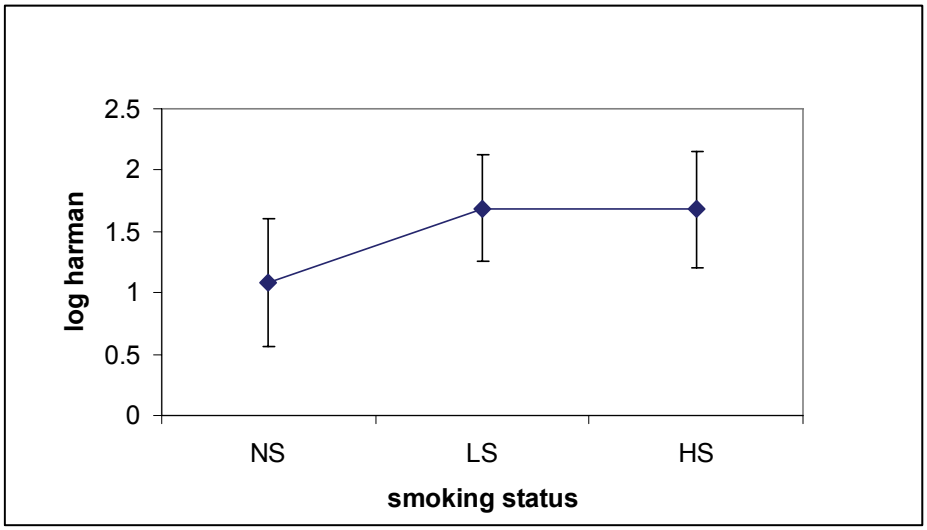


Figure 5-17: Log H as function of smoking status (mean ± SD). Statistically significant results observed between NS-LS and NS-HS groups.

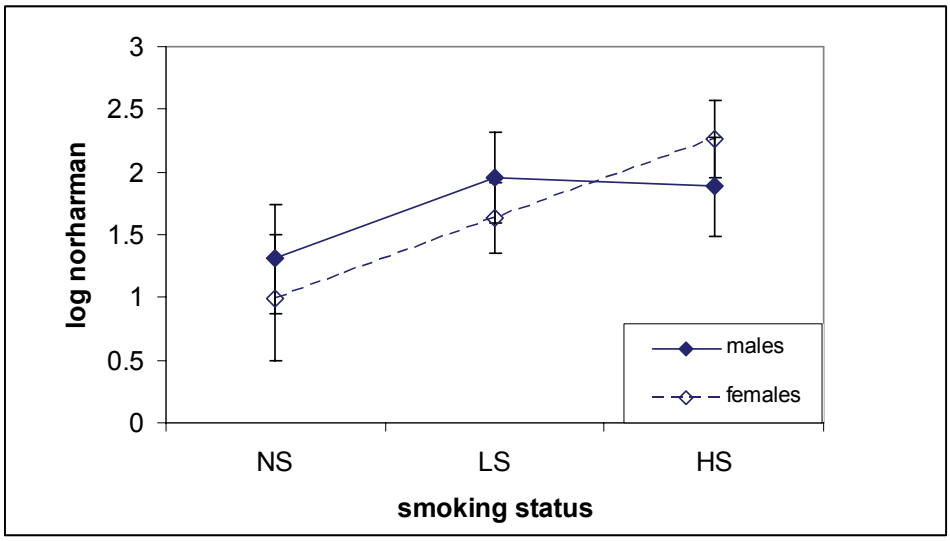


Figure 5-18: Log NH as function of smoking status and gender (mean ± SD).

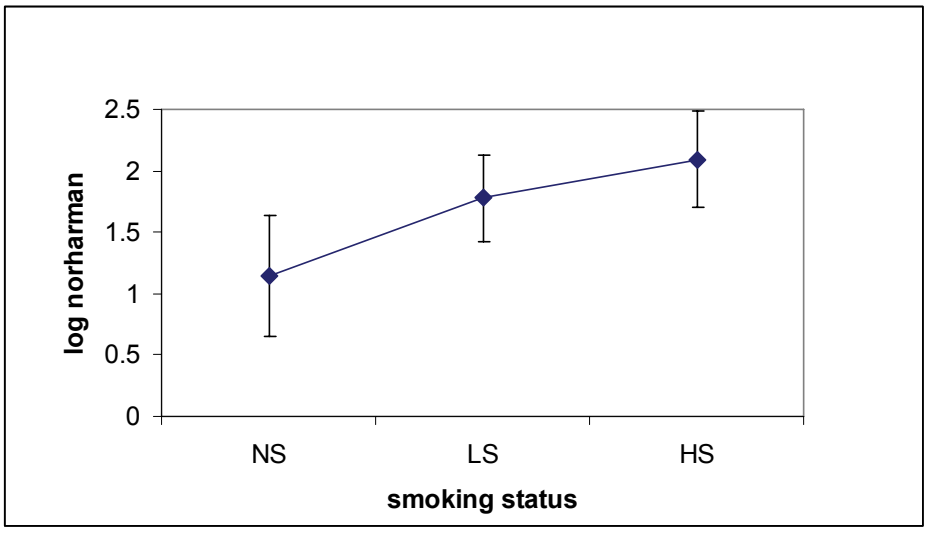


Figure 5-19: Log NH as function of smoking status (mean ± SD).
Statistically significant results observed between NS-LS and NS-HS groups.

5.4.2c β-carbolines – Exploratory Analyses

As observed from the primary analysis, a statistically significant effect of smoking status was observed on both log H and log NH plasma concentrations. In order to further explain the variability of H and NH exposure associated with smoking status, the criteria of FTND and number of cigarettes smoked per day were analyzed as continuous dependent variables to explain the individual subjects' H and NH exposures. FTND and the number of cigarettes smoked per day were replaced by the SS category for the ANOVA analysis. The individual concentrations for each subject were used to assess the relationship between FTND and number of cigarettes and the analyte concentrations in order to represent the intra-subject variability for the log H and log NH in the analysis.

The tables below summarize the regression analysis for log H and log NH using different explanatory variables for the dependent variable. For reference, results of the primary analysis using smoking status and gender for the model fit are supplied. As FTND and the number of cigarettes smoked per day are measures of nicotine dependence that were not expected to change between sampling periods, intra-subject average log H and log NH concentrations were also explored for an association.

Although the model fits were statistically significant on the individual log H concentrations, use of the FTND and number of cigarettes/day did not improve coefficient of determinations from the initial model. Of note, all model fits were

Table 5-8: Explanatory variable model comparison for Log H.

Log Harman				
Model	*Description*	R^2	*p-value*	*Slope (for continuous dependent variable) and notes*
SS + GEN (pre-specified primary factors)	Original 2-way ANOVA with SS and GEN as factors	0.3116	< 0.0001	No interaction
FTND + GEN	Linear regression with continuous FTND as dependent variable	0.2558	< 0.0001	0.1516, sig interaction observed between FTND and GEN
#cig/day+ GEN	Linear regression with continuous #cig/day as dependent variable	0.2757	< 0.0001	0.0342, no interaction, no effect of gender
Avg log H				
FTND + GEN	Linear regression on avg log H with continuous FTND as dependent variable	0.2123	0.003	0.0866, sig interaction observed between FTND and GEN
#cig/day + GEN	Linear regression on avg log H with continuous #cig/day as dependent variable	0.3306	< 0.0005	0.0303, no interaction No effect of gender.

statistically significant (all p-values < 0.003). Upon evaluation of the average log H concentrations, an improvement was observed when the number of cigarettes smoked per day was implemented into the model in place of smoking status. Average concentrations were expected to show a better model fit compared to the individual concentrations as the average masks the intra-subject variability associated with the observations. According to this model, gender or the gender:cigarettes/day interaction was not significant. Figure 5-20 below shows the final model regression for log H as a function of # of cigarettes smoked per day.

Smoking status was able to explain more of the variability associated with log H concentrations (31.6%) than the FTND (25.8%) and #cigarettes/day (27.5%) covariates.

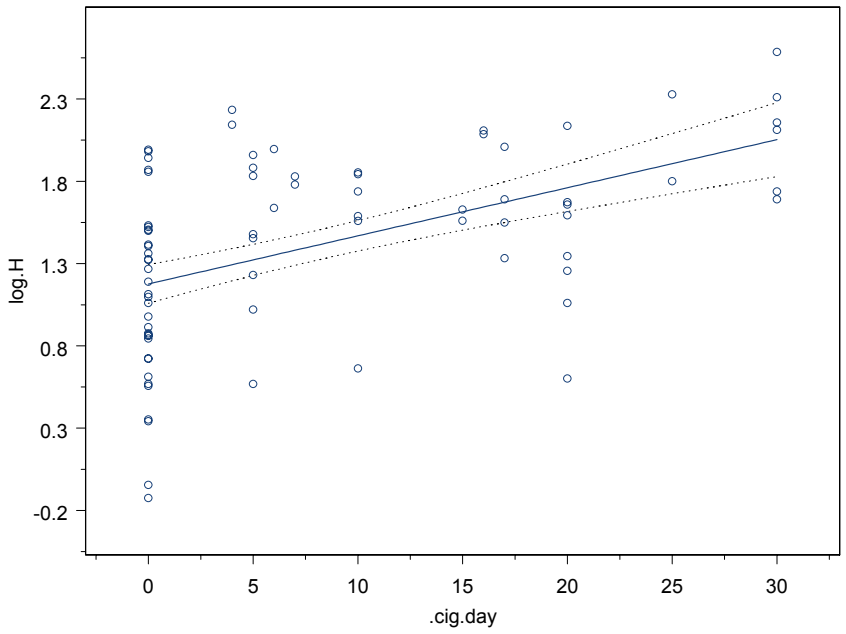


Figure 5-20: Linear regression of #of cigarettes smoked per day vs. average log H. Dotted lines represent 95% confidence bounds (R^2 = 0.3306, log H = 0.0303 #cig/day + 1.1).

According to this model, 33.6% of the variability associated with average log H plasma concentration can be explained by the number of cigarettes smoked per day. For every single cigarette smoked on average results in an increase in average log H concentration of 0.03.

With respect to the individual log NH, all model fits were statistically significant with use of the FTND and number of cigarettes/day (all p-values < 0.0001). The model fit is shown in the following table. In this case, the use of the # of cigarettes smoked per day was a slightly better predictor of individual log NH concentrations than smoking status possessing a R^2 of > 0.50. In other words, the # of cigarettes smoked per day was able to explain 50.2% of the variability associated with the log NH concentrations as opposed to the smoking status, which explained 49.7%. A plot showing the regression between the individual log NH concentrations as a function of the number of cigarettes smoked per day is shown below the table.

Table 5-9: Explanatory variable model comparison for Log NH.

Log norharman				
Model	*Description*	R^2	*p-value*	*Slope (for continuous dependent variable) and notes*
SS + GEN (pre-specified primary factors)	Original 2-way ANOVA with SS and GEN as factors	0.4976	< 0.0001	No interaction
FTND + GEN	Linear regression with continuous FTND as dependent variable	0.4417	< 0.0001	0.2006, sig interaction observed between FTND and GEN
#cig/day + GEN	Linear regression with continuous #cig/day as dependent variable	0.5016	< 0.0001	0.0443, no interaction
Avg log NH				
FTND + GEN	Linear regression on avg log NH with continuous FTND as dependent variable	0.5505	< 0.0001	0.0866, sig interaction observed between FTND and GEN
#cig/day + GEN	Linear regression on avg log NH with continuous #cig/day as dependent variable	0.5639	< 0.0001	0.0449, no interaction

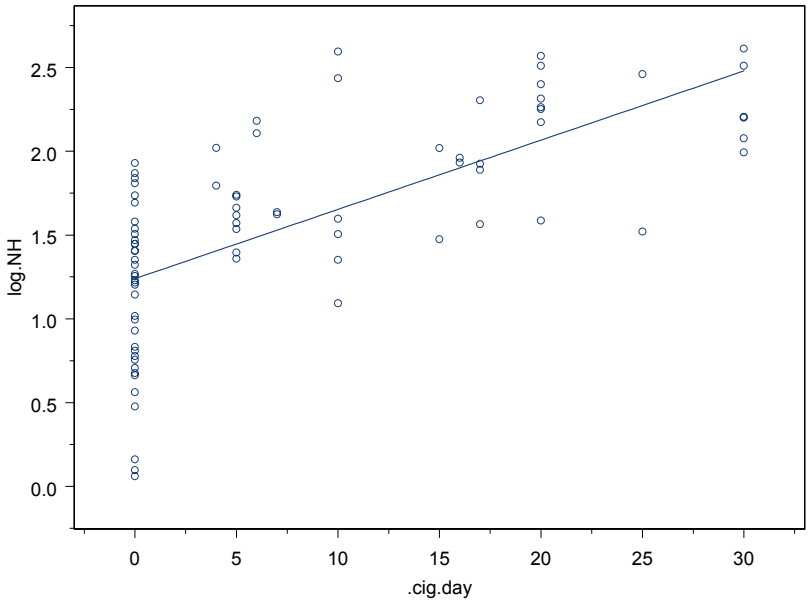


Figure 5-21: Linear regression of #of cigarettes smoked per day vs. individual log NH. Dotted lines represent 95% confidence bounds (R^2 = 0.5016, log NH = 0.0443 #cig/day + 1.2).

In this model, gender or the gender:cigarettes/day interaction was not significant and was not implemented into the model. Approximately 50.2% of the variability associated with individual log NH plasma concentration can be explained by the average number of cigarettes smoked per day. Every 1 cigarette smoked on average results in an increase in average log NH concentration of 0.044.

Upon comparison of all models for log NH, smoking status was able to and FTND were able to explain 49.7% and 44.2% of the variability associated with individual log NH concentrations, while the # of cigarettes smoked per day design variable was able to explain a slightly higher percentage of 50.2%.

Further evaluation of fitting the model to average log NH concentrations as a function of number of cigarettes smoked per day resulted in a superior fit (p-value < 0.0001, $R^2 = 0.5639$). Approximately 56.4% of the variability associated with average log NH concentrations could be explained by the average number of cigarettes smoked per day. This improvement of fit was expected as the factor of intra-subject variability is stabilized upon averaging, decreasing the variability associated with the linear regression fit.

According to the exploratory analyses, for both log H and log NH, the number of cigarettes smoked per day would be a better predictor of β-carboline exposure as opposed to smoking status. Further studies implementing the number of cigarette smoked per day as a design factor would be needed for further evaluation the association with log H and log NH. Smoking status, the FTND measurement and the average number of cigarettes smoked per day were better predictors for log NH measurement than log H. This may be due to the fact that norharman concentrations in dry weight of tobacco are two-fold higher than that of harman (Pfau and Skog, 2004).

5.4.2d β-carbolines – Covariate Analysis

Information including alcohol intake and weekly exposure to dietary total H and NH was recorded to evaluate for their effects on circulating levels of H and NH. As dietary intake of ethanol and/or β-carboline containing foods may affect the overall exposure, this covariate assessments may provide information into the variability associated with the plasma measurement log H and/or NH. If alcohol intake and/or dietary exposure were found to significantly correlate with SAL or β-carboline exposure, AAI or Food Intake was considered a significant covariate and implemented into the statistical model.

Linear regressions were performed on log H and log NH as a function of mg of annual absolute ethanol (AAI) or average weekly dietary H or NH (Food Inventory) intake. If the regression was significant (p-value < 0.05) with a coefficient of determination of > 0.2, the variable of interest was considered a significant covariate, in which it was implemented into the model. Of note the covariate analysis was incorporated into the model fit for the original factors smoking status and gender. Evaluation of the significance of the covariate was additionally assessed upon implementation into the final ANCOVA model. The p-value was evaluated for significance of the covariate. If considered significant, the fit of the entire model was evaluated for goodness of fit, with use of the covariate.

A table summarizing the covariate regression analysis for AAI and dietary intake is shown below.

Table 5-10: Covariate analysis results for log H and log NH

variable	Log harman		Log norharman	
AAI (mg ethanol/annum)	R^2	0.0091	R^2	0.1514
	p-value	0.3741	p-value	0.0003
	significance	NS	significance	YES
Weekly Dietary intake (ng/H or ng/NH)	R^2	0.0281	R^2	0.0215
	p-value	0.1330	p-value	0.1302
	significance	NS	significance	NS

The covariate analysis revealed that a poor association was observed between weekly dietary intake of H and NH to circulating concentrations, which was unexpected. Several reasons, including the use of a non-validated measure of dietary H and NH intake, may explain this discrepancy. As H and NH are present in various sources of foods in relatively large amounts, it was expected that influence of dietary consumption of H and NH would contribute to the circulating plasma concentrations. Formal assessment of pharmacokinetics has not been performed on the influence of circulating H and NH on acute exposure to a food source rich in β-carbolines. The results of this covariate analysis suggest that the weekly average intake of H and NH do not influence circulating levels. Implementation of the dietary H and NH intake was subsequently incorporated into the ANCOVA model and was found to be not significant for either analyte (H: p-value = 0.19, NH: p-value = 0.18).

Annual absolute alcohol did not have a significant effect on circulating H concentrations. The association between this alcohol intake measurement to log H was

inferior ($R^2 = 0.0091$) to that of the food inventory ($R^2 = 0.0281$). Upon incorporation of AAI into the ANCOVA statistical model for log H, significance of mg of ethanol per annum was not considered a significant covariate (p-value = 0.21). In the case of circulating NH, a statistically significant (p-value < 0.001) effect of AAI was apparent with the mg of ethanol consumed per annum explaining 15.1% of the variability associated with log NH concentrations. Upon incorporation of AAI into the ANCOVA statistical model for log NH, significance of mg of ethanol per annum was not considered a significant covariate (p-value = 0.38).

Use of the AAI or Food Intake as a covariate was not significant and was not implemented into the full model. Of note, AAI may be a confounding variable that shows a strong relationship with smoking status (see table 5-3). ANOVA analysis found a significant relation between AAI and smoking status (p-value < 0.01), suggesting that the smoking status factor and AAI are considered to be collinear. Therefore the AAI should not be considered a covariate because of its correlation with smoking status.

5.4.3 Primary Analysis for TIQ's and DA – Effects of Smoking and Gender

5.4.3a R/S-SAL and DA – Within Subject Variability

Before appropriate statistical comparison of R-SAL, S-SAL and DA for the smoking and gender factors, within subject variability of both TIQ's and DA for each individual was evaluated. This analysis was performed to evaluate the baseline concentration of R-SAL, S-SAL and DA over two observational time points. The intra-

subject variability (%COV and inter-quartile ranges) for R-SAL, S-SAL and the DA precursor may be seen in the tables 5-11, 5-12 and 5-13 below.

For R-SAL and S-SAL concentrations, it can be noted that the variability between sampling occasions ranged from a %COV 0.0 to 1745% for S-SAL and %COV 0.0 to 4473% for R-SAL, suggesting that baseline concentrations of the TIQ analytes vary considerably between occasions. The within-subject variability associated with the DA measurements was more consistent, as compared to the SAL analytes, with intra-subject %COV ranging from 3.8 – 159%.

For non-smokers %COV ranged from 0.0 – 1272% for S-SAL, 12.2 – 1352% for R-SAL and 2.5 – 159% for DA. Presumably, these persons have not been exogenously exposed to SAL enantiomers recently, inferring that other factors, other than smoking status or gender may contribute to the varying baseline between occasions. Of note, DA concentrations showed less intra-subject variability between occasions than that of the TIQ's. Results are suggestive that DA concentrations are less variable compared to the SAL enantiomers between sampling occasions.

Table 5-11: Within Subject Variability for Plasma S-SAL (pg/ml)

Sample #	GEN	S-SAL avg	stdev	%COV	median	25% IQR	75% IQR
Nonsmokers							
1	F	66	22	33	66	58	73
2	F	860	593	69	441	231	650
3	F	183	43	24	153	137	168
4	F	25	1	3	25	24	25
5	F	160	47	29	193	177	210
6	F	13	14	109	23	18	28
7	F	16	0	0	16	16	16
8	F	23	19	83	37	30	43
9	F	98	264	269	285	191	378
10	F	17	216	1273	170	94	247
11	M	359	240	67	190	105	274
12	M	75	36	48	50	37	62
13	M	56	8	14	51	48	53
14	M	54	8	16	48	45	51
15	M	57	13	24	48	43	52
16	M	42	17	40	30	24	36
17	M	91	43	47	61	45	76
18	M	67	182	272	196	132	261
19	M	50	14	28	60	55	65
Light Smokers							
21	F	18	14	79	28	23	33
22	F	306	194	64	169	100	237
23	F	25	16	65	37	31	42
24	F	47	14	30	37	32	42
25	F	36	51	141	72	54	90
26	F	808	281	35	609	510	709
31	M	448	209	47	300	226	374
32	M	236	98	42	306	271	340
33	M	134	49	36	100	82	117
34	M	1188	684	58	705	463	946
35	M	230	991	431	931	581	1282
Heavy Smokers							
41	F	66	16	24	77	72	83
42	F	1238	238	19	1070	986	1154
43	F	49	18	36	62	55	68
44	F	866	3690	426	3475	2171	4780
45	F	602	190	31	468	401	535
46	F	761	325	43	531	416	646
51	M	43	47	110	77	60	93
52	M	25	1	3	26	25	26
53	M	1210	752	62	679	413	944
54	M	2438	113	5	2518	2478	2558
55	M	90	1571	1746	1201	646	1757

Table 5-12: Within Subject Variability for Plasma R-SAL (pg/ml)

Sample #	GEN	R-SAL avg	stdev	%COV	median	25% IQR	75% IQR
Nonsmokers							
1	F	68	42	62	68	53	83
2	F	1210	837	69	619	323	914
3	F	222	48	22	188	171	205
4	F	21	5	24	18	16	19
5	F	286	75	26	339	313	366
6	F	17	22	129	33	25	40
7	F	13	6	49	9	6	11
8	F	32	20	62	46	39	53
9	F	100	384	384	372	236	507
10	F	24	325	1352	254	139	368
11	M	507	344	68	264	142	385
12	M	86	47	54	53	37	70
13	M	45	18	39	33	26	39
14	M	60	10	16	53	50	57
15	M	64	8	12	70	67	72
16	M	46	23	49	30	22	38
17	M	93	31	33	71	60	82
18	M	105	105	100	179	142	216
19	M	35	8	24	29	26	32
Light Smokers							
21	F	12	16	136	24	18	29
22	F	386	247	64	211	124	299
23	F	25	17	68	37	31	43
24	F	24	5	21	28	26	29
25	F	23	1029	4473	751	387	1114
26	F	1094	386	35	821	685	958
31	M	772	419	54	476	328	624
32	M	285	226	79	445	365	525
33	M	186	90	48	123	91	154
34	M	1304	716	55	798	544	1051
35	M	266	73	27	318	292	343
Heavy Smokers							
41	F	87	10	11	94	91	98
42	F	2864	1213	42	2006	1577	2435
43	F	50	15	30	61	55	66
44	F	1102	4675	424	4408	2755	6061
45	F	671	176	26	547	484	609
46	F	771	471	61	438	272	605
51	M	42	0	0	42	42	42
52	M	25	7	28	30	28	33
53	M	1450	865	60	838	532	1144
54	M	4308	186	4	4177	4111	4242
55	M	87	2337	2686	1740	913	2566

Table 5-13: Within Subject Variability for Plasma DA (ng/ml)

Sample #	GEN	DA avg	stdev	%COV	median	25% IQR	75% IQR
Nonsmokers							
1	F	10.3	6.4	62.1	10.3	8.0	12.5
2	F	9.4	3.6	37.8	6.9	5.6	8.2
3	F	13.7	1.2	8.6	12.8	12.4	13.3
4	F	5.7	0.5	8.3	6.1	5.9	6.2
5	F	6.6	5.4	82.5	10.4	8.5	12.3
6	F	2.3	0.6	25.9	1.9	1.7	2.1
7	F	4.6	0.8	17.3	5.1	4.8	5.4
8	F	3.2	0.7	21.9	2.7	2.4	2.9
9	F	3.7	1.2	31.2	2.9	2.5	3.3
10	F	5.1	0.4	8.5	4.8	4.6	4.9
11	M	9.1	2.3	25.5	7.5	6.6	8.3
12	M	13.7	1.1	8.1	14.5	14.1	14.9
13	M	6.3	0.8	12.9	6.9	6.6	7.2
14	M	8.7	2.1	24.7	7.2	6.4	7.9
15	M	8.4	0.2	2.5	8.5	8.4	8.6
16	M	6.0	1.0	17.4	5.2	4.9	5.6
17	M	3.6	3.5	98.0	6.1	4.8	7.3
18	M	5.1	8.1	158.6	10.8	8.0	13.7
19	M	4.0	5.0	124.9	7.5	5.8	9.3
Light Smokers							
21	F	11.3	14.7	130.3	21.7	16.5	26.9
22	F	12.9	0.6	4.8	12.4	12.2	12.6
23	F	8.6	1.7	20.2	9.8	9.2	10.4
24	F	4.0	0.9	22.4	4.6	4.3	4.9
25	F	6.8	0.5	7.3	6.5	6.3	6.6
26	F	14.2	4.7	32.7	10.9	9.3	12.6
31	M	9.8	1.5	15.4	8.8	8.2	9.3
32	M	22.7	4.5	19.9	19.5	17.9	21.1
33	M	11.7	9.5	81.0	18.4	15.1	21.8
34	M	9.9	6.8	68.6	5.1	2.7	7.5
35	M	7.0	1.7	23.9	5.9	5.3	6.4
Heavy Smokers							
41	F	10.2	5.2	51.3	13.9	12.1	15.8
42	F	22.4	10.8	48.4	30.1	26.2	33.9
43	F	17.8	0.7	3.8	18.2	18.0	18.5
44	F	30.7	2.1	6.7	32.2	31.4	32.9
45	F	27.8	9.8	35.3	20.9	17.4	24.4
46	F	9.8	4.2	42.5	12.7	11.3	14.2
51	M	17.6	2.1	12.0	16.1	15.3	16.8
52	M	9.1	1.4	15.1	8.1	7.6	8.6
53	M	7.4	0.6	8.3	6.9	6.7	7.1
54	M	14.3	6.8	47.7	9.5	7.1	11.9
55	M	9.3	3.7	39.6	11.9	10.6	13.2

For the LS population, %COV 30.1 – 430% for S-SAL, 20.6 – 4473% for R-SAL and 4.8 – 130.3% for DA were observed, while the HS population possessed a %COV range of 2.8 – 1745% for S-SAL, 0.0 – 2686% for R-SAL, and 3.8 – 48% for DA. The large variability between occasions observed within the smoking status populations are speculated to be due to the different times of sampling with respect to cigarette smoking.

Of important note, SAL enantiomers and DA are not found in tobacco. The exposure of SAL is hypothesized to occur from the endogenous biosynthesis of SAL from the acetaldehyde from cigarette smoke and endogenous circulating DA. *In-vivo* condensation reaction rates have not been characterized, but may contribute to the wide intra-subject variability observed within the smoking groups. Although each subject was sampled within 30 minutes of smoking, the sampling times varied considerably within this time frame for each subject. It is important to comment that DA concentrations within HS individuals were more stable than LS and NS.

Both genders possessed the similar amount of within subject variability with males possessing a %COV 2.8 – 1745% for S-SAL, 0.0 – 2686% for R-SAL and 2.5 – 158% for DA were observed, while in the female counterparts, a %COV 0.0 – 1272% for S-SAL, 11.4 – 4473% for R-SAL and 3.8 – 130% for DA were observed. As large within-subject variability was observed with majority of the subjects, individual subject concentrations were used, as opposed to average subject concentrations for providing descriptive statistics of the overall measure of central tendency and variability. This

was to ensure that the intra-subject variability was not masked during descriptive and inferential statistical analysis.

Tables 5-14, 5-15 and 5-16 show the descriptive statistics for the concentrations S-SAL, R-SAL and DA observed for each smoking status and gender. Measures of central tendency, such as mean and median, along with the variability including %COV and range is presented for each factor. Of note the distribution for the number of subjects are unbalanced with respect to smoking status and gender within each group. The distribution of gender and smoking status for subjects who completed the protocol is the same to that of the β-carbolines.

Table 5-14: Descriptive statistics for S-SAL concentrations (pg/ml) divided into smoking status and gender.

S-SAL

		Non-smoker	Light-smoker	Heavy-smoker	GRAND
Female	Mean	141	159	947	365
	Stdev	208	240	1665	935
	%COV	148	152	176	256
	Median	50	43	468	78
	MIN	13	18	49	13
	MAX	860	808	6084	6084
Male	Mean	81	468	900	399
	Stdev	97	519	1128	705
	%COV	119	111	125	177
	Median	52	233	129	83
	MIN	18	65	25	18
	MAX	359	1632	2598	2598
GRAND	Mean	113	299	926	381
	Stdev	166	413	1414	831
	%COV	147	138	153	218
	Median	50	143	318	78
	MIN	13	18	25	13
	MAX	860	1632	6084	6084

Table 5-15: Descriptive statistics for R-SAL concentrations (pg/ml) divided into smoking status and gender.

R-SAL

		Non-smoker	Light-smoker	Heavy-smoker	GRAND
Female	Mean	194	312	1259	517
	Stdev	299	493	2184	1239
	%COV	154	158	174	240
	Median	54	36	547	93
	MIN	4	12	50	4
	MAX	1210	1478	7714	7714
Male	Mean	87	432	1365	514
	Stdev	118	371	1824	1066
	%COV	136	86	134	207
	Median	48	288	157	87
	MIN	14	59	25	14
	MAX	507	1304	4308	4308
GRAND	Mean	143	366	1307	515
	Stdev	235	436	1982	1155
	%COV	164	119	152	224
	Median	49	226	324	87
	MIN	4	12	25	4
	MAX	1210	1478	7714	7714

Table 5-16: Descriptive statistics for DA concentrations (ng/ml) divided into smoking status and gender.

DA

		Non-smoker	Light-smoker	Heavy-smoker	GRAND
Female	Mean	6.4	11.0	21.3	11.7
	Stdev	4.2	7.4	9.2	9.1
	%COV	65.8	67.3	43.1	77.6
	Median	5.4	9.8	18.2	9.6
	MIN	1.5	4.0	9.8	1.5
	MAX	14.8	32.1	37.7	37.7
Male	Mean	8.2	11.5	10.5	9.7
	Stdev	3.8	7.8	4.4	5.3
	%COV	46.0	67.6	41.7	54.7
	Median	7.9	9.9	9.2	8.6
	MIN	3.6	0.3	4.7	0.3
	MAX	16.6	25.2	17.6	25.2
GRAND	Mean	7.3	11.2	16.4	10.8
	Stdev	4.1	7.4	9.1	7.6
	%COV	56.0	65.9	55.5	70.4
	Median	5.9	9.9	14.6	8.7
	MIN	1.5	0.3	4.7	0.3
	MAX	16.6	32.1	37.7	37.7

The overall average (mean and %COV), across all patients for S-SAL, R-SAL and DA were 380.8 (218.3%), 515.4 pg/ml (224.1%) and 10.8 ng/ml (70.4%) suggesting that the plasma SAL and DA concentrations are quite variable between all patients. The significant differences between the median and mean R/S-SAL and DA concentrations suggest that the data follow non-normal distribution. Upon comparison of the median concentrations, females were observed to have a median S-SAL concentration of 77.5 pg/ml, while the median R-SAL concentration was 92.5 pg/ml. Their male counterparts had similar median S-SAL concentration of 82.5 pg/ml and a median R-SAL concentration of 86.5 pg/ml. Median DA concentrations were similar between genders with similar ranges.

With respect to smoking status, the NS median S-SAL concentration was 50.0 pg/ml while the median R-SAL concentration was 48.5 pg/ml. LS possessed a higher median S-SAL concentration of 143 pg/ml while the median R-SAL concentration was 226.0 pg/ml. The HS group resulted in a median S-SAL concentration of 317.5 pg/ml while the median R-SAL concentration was 324.0 pg/ml. Median DA concentrations of female NS, LS and HS were 5.4, 9.8 and 18.2 ng/ml, respectively while males within NS, LS and HS groups had median DA concentrations of 7.9, 9.9, and 9.2 ng/ml.

5.4.3b TIQ's and DA – Primary factors of Smoking and Gender Analysis

The distribution of the data within each smoking group for R-SAL, S-SAL and DA followed a non-normal distribution upon assessment of quantile-quantile (Q-Q) plots. Divergence among the median and mean concentrations within each group also exemplified the non-normality of the distribution, with the Shapiro-Wilk test resulting in p-values all less than 0.048 for R/S-SAL and DA analytes across all groups, suggesting that the data was non-normally distributed. Moreover, unequal variance was present with the data upon visual inspection of the residuals. For these reasons, log-transformed data were used for the primary analysis.

Separate evaluation of the effects of smoking status and gender were performed on both log S-SAL and log R-SAL concentrations. Correlation analysis between the analytes suggest that the association between the two analytes is very strong ($r = 0.954$). Nevertheless, separate statistical analysis for the two analytes was conducted. Moreover, associations between DA and the SAL enantiomers was relatively weak with log DA vs. log S-SAL possessing a correlation coefficient of 0.410 and log DA vs. log R-SAL was 0.374. A matrix evaluating the correlation may be seen in figure 5-22 below.

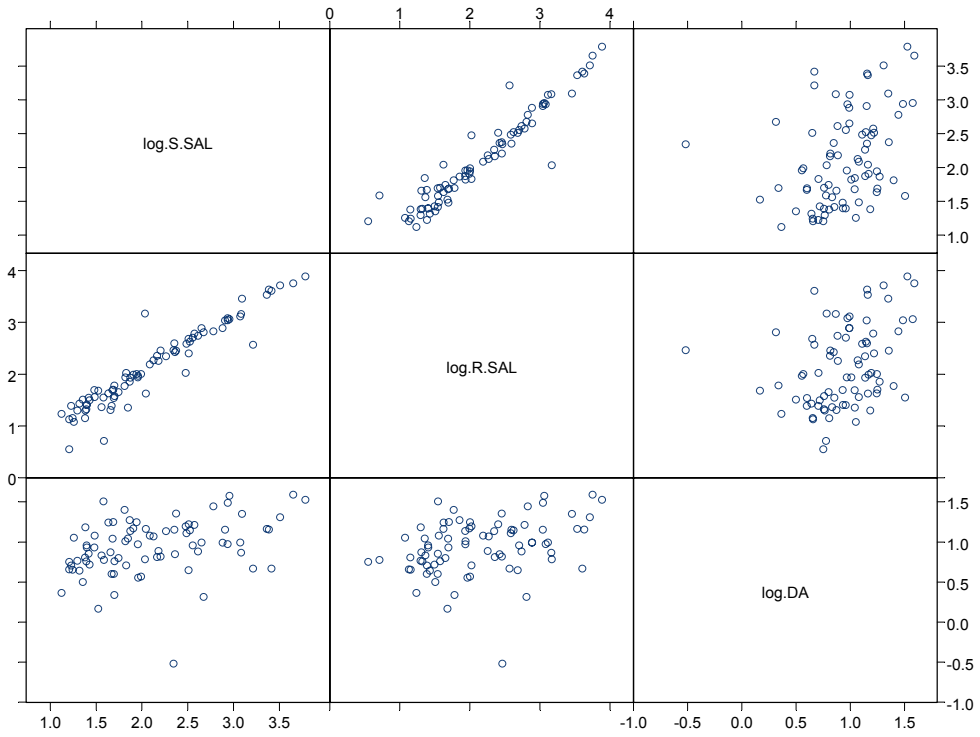


Figure 5-22: Correlation between log R-SAL vs. log S-SAL (r = 0.954), log DA vs. S-SAL (r = 0.410) and log DA vs. R-SAL (r =0.374).

Two-way ANOVA was performed on the log transformed R-SAL, S-SAL and DA, evaluating the factors of smoking status and gender. Box-plots exemplifying the median and distribution, including outliers for the effects of gender and smoking status are presented below for all analytes. Observation of the box-plots suggest that there is no significant difference between gender group while a trend is observed between smoking groups, with HS > LS > NS across all analytes.

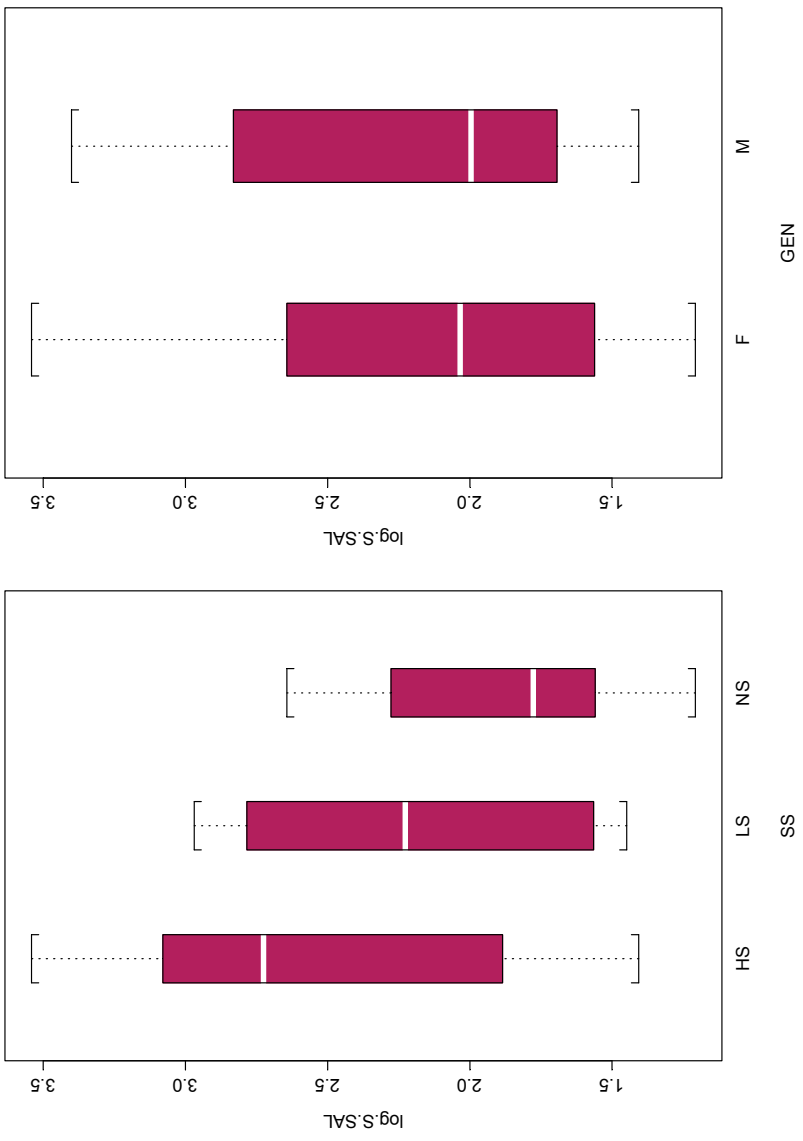


Figure 5-23: Boxplots of log S-SAL for smoking status (SS, left plot) and gender (GEN, right plot)

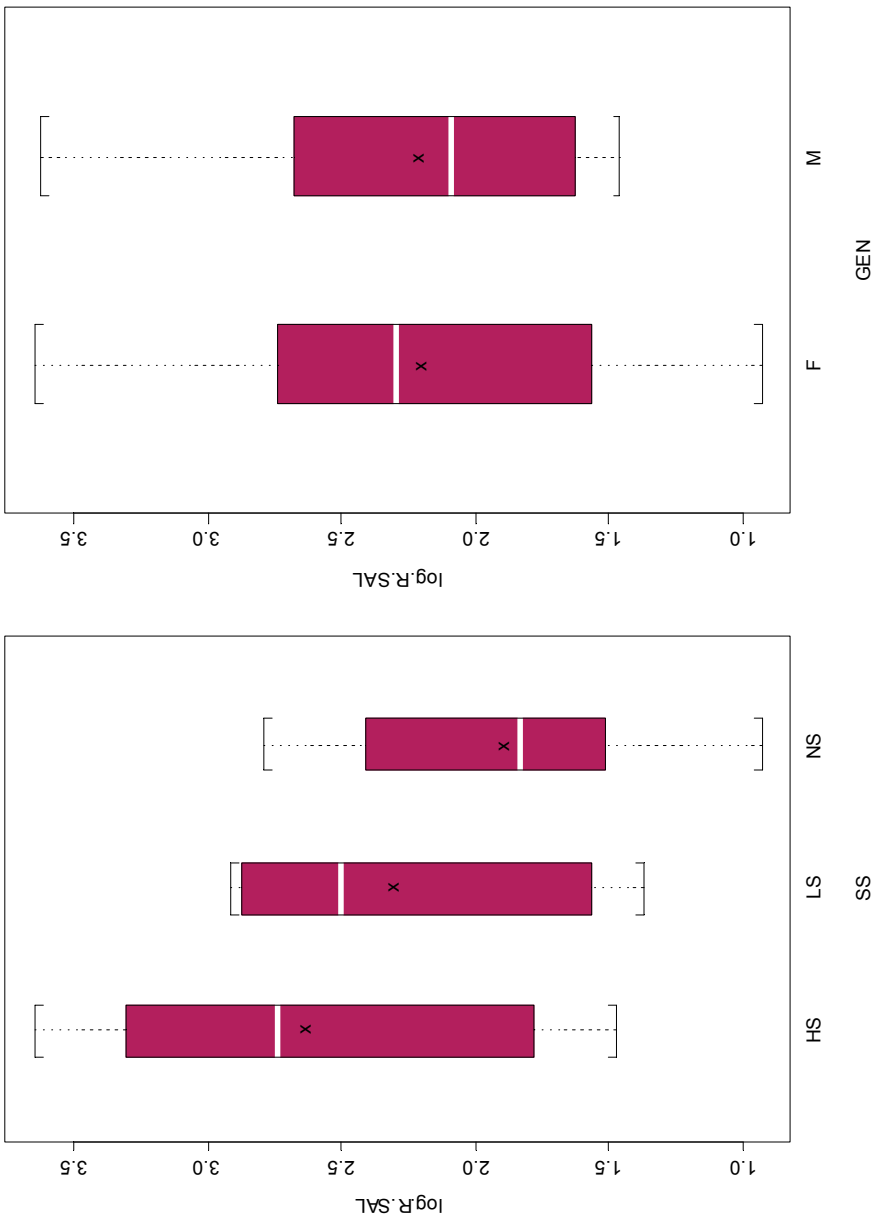


Figure 5-24: Boxplots of log R-SAL for smoking status (SS, left plot) and gender (GEN, right plot)

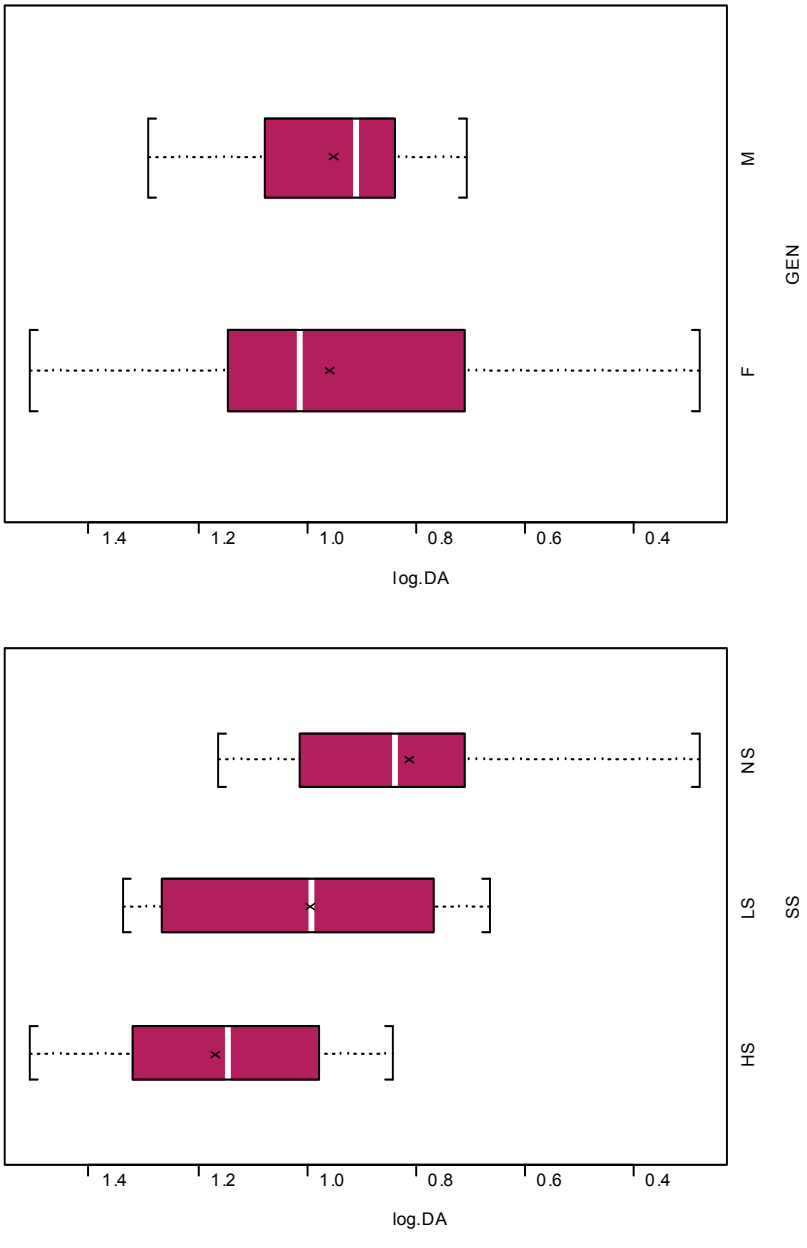


Figure 5-25: Boxplots of log DA for smoking status (SS, left plot) and gender (GEN, right plot)

Two-way ANOVA was performed to evaluate the effects of gender and smoking on log S-SAL concentrations. A significant effect of smoking status (SS) was observed with respect to log S-SAL concentrations with F (2, 75) = 15.5, p-value = 1.2×10^{-6}. The effect of gender (GEN) was not significant with the test statistic resulting in F (1, 75) = 0.57, p-value = 0.45. No interaction between the SS and GEN factors was observed (F (2, 75) = 2.99, p-value = 0.054). Therefore, linear contrasts were unnecessary. An effect of sampling visit was not observed (p-value = 0.89). The coefficient of determination (R^2) for the entire model was 0.192 (p-value = 0.008). The factors of SS and GEN account for approximately 19.2% of the variability associated with the log S-SAL concentration.

The Scheffé method for multiple comparisons found that the primary differences were observed between the HS and NS groups as well as the LS and NS groups. Via this comparison, a significant difference in S-SAL was not observed between the HS and LS smoking status groups. The full two-way ANOVA output for Log S-SAL may be seen in the figure below along with the residual plot of the model fit. Upon visual evaluation, the residuals show an even spread of the distribution of data suggesting the lack of unequal variance.

```
*** Analysis of Variance Model ***

Short Output:
Call:
   aov(formula = log.S.SAL ~ SS + GEN + SS:GEN, data =
       SAL.BC..Final.Bioanalysis.Data.VCU.Smoking.032608, na.action =
       na.exclude)


Response: LOG S-SAL

           Df Sum of Sq  Mean Sq  F Value     Pr(F)
       SS   2   7.89131 3.945656 15.52240 0.0000012
      GEN   1   0.14529 0.145293  0.57159 0.4512744
    visit   1   0.00779 0.007790 0.017859 0.8940485
   SS:GEN   2   1.52140 0.760699  2.99263 0.0543319
 Residuals 75  32.71512 0.436202


Multiple R-Squared: 0.1921
F-statistic: 3.402 on 5 and 76 degrees of freedom, the p-value is 0.007939


95 % simultaneous confidence intervals for specified
linear combinations, by the Scheffé method

critical point: 2.8393
response variable: log.S.SAL
rank used for Scheffé method: 3

intervals excluding 0 are flagged by '****'

      Estimate Std.Error Lower Bound Upper Bound
HS-LS    0.267     0.128     -0.0969       0.632
HS-NS    0.623     0.116      0.2930       0.952 ****
LS-NS    0.355     0.116      0.0252       0.685 ****
```

Figure 5-26: Two-way ANOVA output, with multiple comparisons, for the effects of SS and GEN on Log S-SAL plasma concentrations.

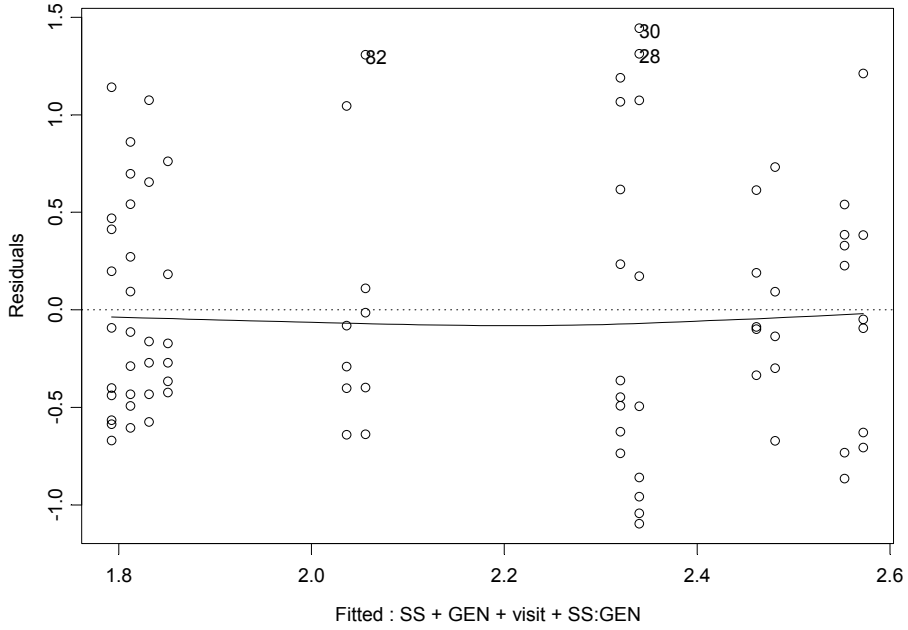


Figure 5-27: Residual plot for two-way ANOVA fit for Log S-SAL.

With respect to R-SAL, two-way ANOVA was performed to evaluate the effects of gender and smoking. A significant effect of smoking status was observed with respect to log R-SAL concentrations with $F(2, 75) = 13.9$, p-value = 0.4×10^{-6}. The effect of gender (GEN) was not significant with the test statistic resulting in $F(1, 75) = 0.052$, p-value = 0.819. No interaction between the SS and GEN factors was observed ($F(2, 75) = 2.05$, p-value = 0.113). The coefficient of determination (R^2) for the entire model was 0.271 (p-value = 1.8×10^{-5}). The factors of SS and GEN account for approximately 27.1% of the variability associated with the R-SAL concentration measurements.

The Scheffé method for multiple comparisons found that the primary differences were observed between the HS and NS groups as well as the LS and NS groups. Via this comparison, a significant difference in R-SAL was not observed between the HS and LS smoking status groups.

The full two-way ANOVA output for log R-SAL may be seen in the figure below along with the residual plot of the model fit. Upon visual evaluation, the residuals show an even spread of the distribution of data suggesting the lack of unequal variance.

```
*** Analysis of Variance Model ***

Short Output:
Call:
   aov(formula = log.R.SAL ~ SS + GEN + visit + SS:GEN, data =
       SAL.BC..Final.Bioanalysis.Data.VCU.Smoking.032908, na.action =
       na.exclude)



Response: LOG R-SAL

          Df Sum of Sq  Mean Sq  F Value     Pr(F)
       SS   2   9.62605 4.813024 13.97085 0.0000040
      GEN   1   0.01798 0.017981  0.05219 0.8197207
    visit   1   0.00047 0.000468 0.000777 0.9778414
   SS:GEN   2   1.41383 0.706913  2.05197 0.1334615
Residuals  75  37.20651 0.344505



Multiple R-Squared: 0.2571
F-statistic: 9.156 on 3 and 75 degrees of freedom, the p-value is 0.00001845


95 % simultaneous confidence intervals for specified
linear combinations, by the Scheffé method

critical point: 2.8401
response variable: log.R.SAL
rank used for Scheffé method: 3

intervals excluding 0 are flagged by '****'

      Estimate Std.Error Lower Bound Upper Bound
HS-LS    0.233     0.147     -0.1840       0.650
HS-NS    0.676     0.133      0.2990       1.050 ****
LS-NS    0.443     0.133      0.0656       0.821 ****
```

Figure 5-28: Two-way ANOVA output, with multiple comparisons, for the effects of SS and GEN on Log R-SAL plasma concentrations.

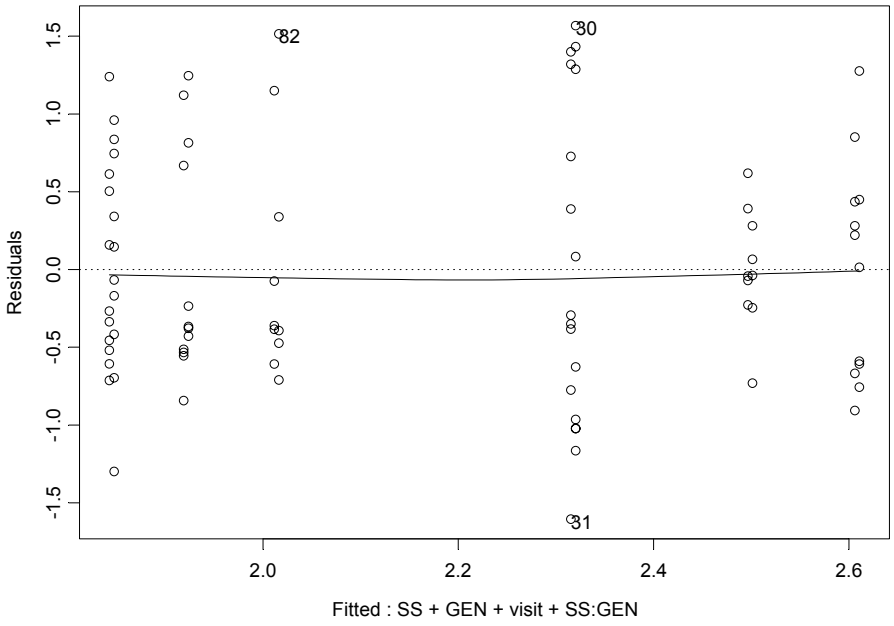


Figure 5-29: Residual plot for two-way ANOVA fit for Log R-SAL.

For DA, a significant effect of smoking status was observed with F (2, 75) = 5.89, p-value = 0.004. The effect of gender (GEN) was not significant with the test statistic resulting in F (1, 75) = 0.199, p-value = 0.656. A significant interaction between the SS and GEN factors was observed (F (2, 75) = 7.21, p-value = 0.021). Further linear contrasts with respect to the interaction were performed as the interpretability of the main effects were confounded by the interaction. Upon linear contrasts, it was observed that a significant effect for smoking status was observed between the NS females and HS females. For the most part, a significant effect of smoking status was not seen in the male groups. The coefficient of determination (R^2) for the entire model was 0.265 (p-value = 2.2×10^{-4}). The factors of SS and GEN, including the interaction, account for approximately 26.5% of the variability associated with the log DA concentration measurements. The Scheffé method for multiple comparisons across smoking status found that the primary differences were observed between the HS and NS groups.

The full two-way ANOVA output for log DA may be seen in the figure below along with the residual plot of the model fit. Upon visual evaluation, the residuals show an even spread of the distribution of data suggesting the lack of unequal variance.

```
*** Analysis of Variance Model ***

Short Output:
Call:
   aov(formula = log.DA ~ SS + GEN + visit + SS:GEN, data =
       SAL.BC..Final.Bioanalysis.Data.VCU.Smoking.032908, na.action =
       na.exclude)


           Df Sum of Sq   Mean Sq  F Value     Pr(F)
       SS   2  1.165686 0.5828430 5.897008 0.0041824
      GEN   1  0.019696 0.0196963 0.199280 0.6565887
    visit   1  0.000109 0.0001090 0.001103 0.9735986
   SS:GEN   2  1.426734 0.7133668 7.217604 0.0213590
Residuals 75  7.412780 0.0988371


R-Squared:  0.2656
F-statistic: 5.277 on 2 and 75 degrees of freedom, the p-value is 0.0002244

95 % simultaneous confidence intervals for specified
linear combinations, by the Scheffé method

critical point: 2.86
response variable: log.DA
rank used for Scheffé method: 3

intervals excluding 0 are flagged by '****'

      Estimate Std.Error Lower Bound Upper Bound
HS-LS   0.1980    0.0952     -0.0742       0.470
HS-NS   0.2650    0.0845      0.0238       0.507 ****
LS-NS   0.0674    0.0845     -0.1740       0.309
```

Figure 5-30: Two-way ANOVA output, with multiple comparisons, for the effects of SS and GEN on Log DA plasma concentrations.

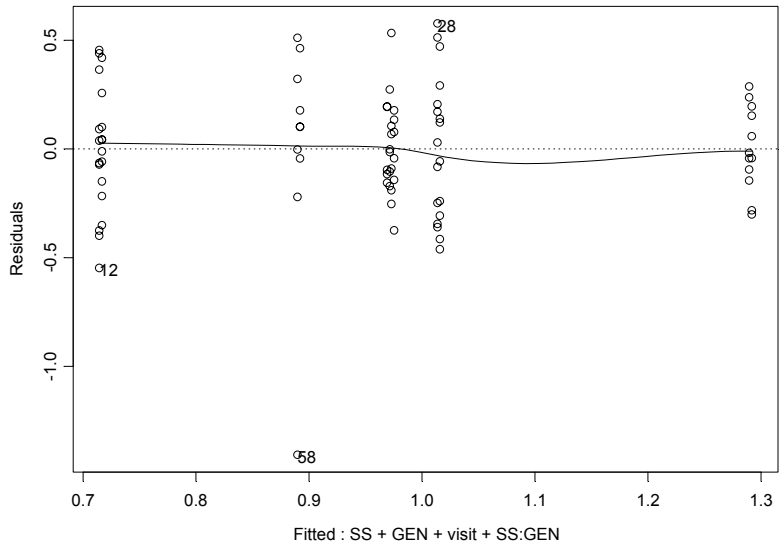


Figure 5-31: Residual plot for two-way ANOVA fit for Log DA.

A significant effect of smoking status was observed for log S-SAL, log R-SAL and log DA plasma concentrations. A statistically significant effect of gender was not observed in the exposure of log S- or R-SAL and an interaction between the factors was not observed for either analyte. A gender:smoking status interaction was observed with the log DA concentrations.

According to the two-way ANOVA, the following ranking was observed with plasma S-SAL concentrations (geometric means) across smoking groups and gender. NS F < NS M < LS F < LS M < HS M < HS F. The post-hoc multiple comparisons via Scheffé test showed that the major difference that was observed with the smoking groups were the HS – NS group and the LS – NS groups with no significant difference being observed between the two smoking groups of LS and HS.

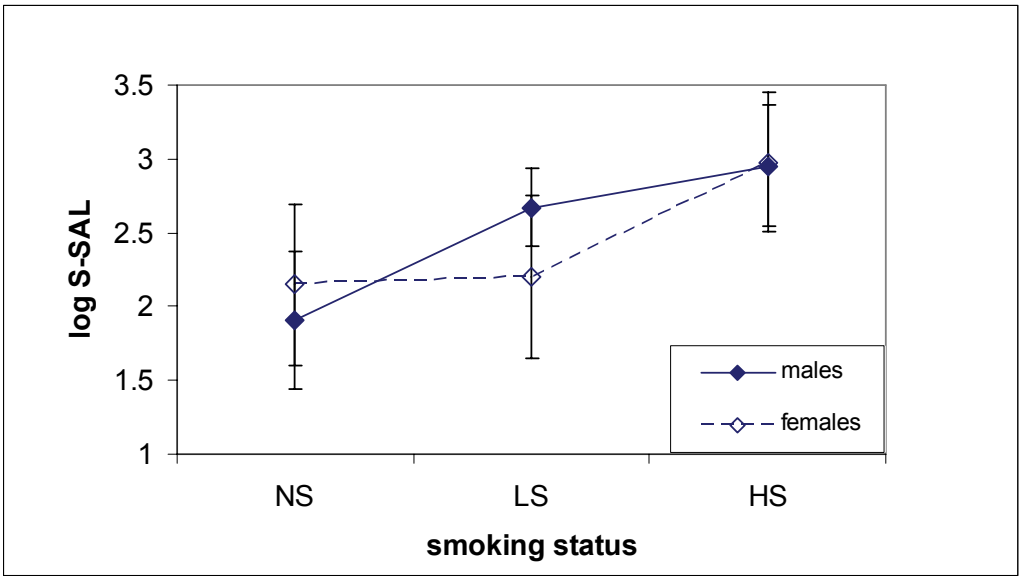


Figure 5-32: Log S-SAL as function of smoking status and gender (mean ± SD).

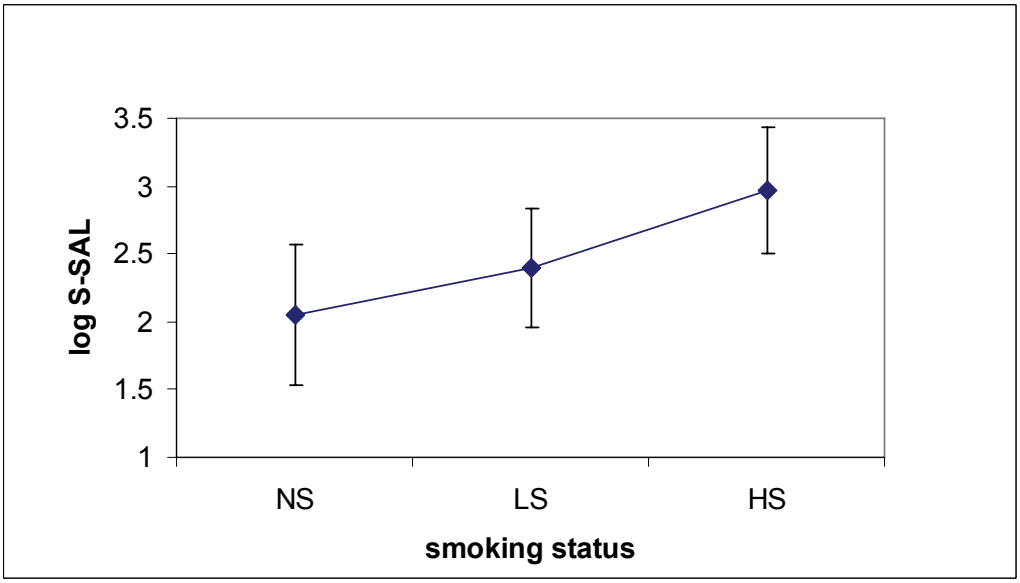


Figure 5-33: Log S-SAL as function of smoking status (mean ± SD). Statistically significant results observed between NS-LS and NS-HS groups.

Both HS and LS groups possessed larger means than NS. On average, the HS group had a larger mean than that of the LS groups for log S-SAL, but the difference was not statistically significant.

ANOVA results assigned the following rank with respect to observed plasma R-SAL concentrations across smoking groups and gender: NS M < NS F < LS F < LS M < HS F < HS M. The post-hoc multiple comparisons via Scheffé test showed that the major difference that was observed with the smoking groups were the HS – NS group and the LS – NS groups. A statistically significant difference was not observed between the two smoking groups of LS and HS. On average, across smoking groups, the HS group > LS group > NS group for log R-SAL. See figures below.

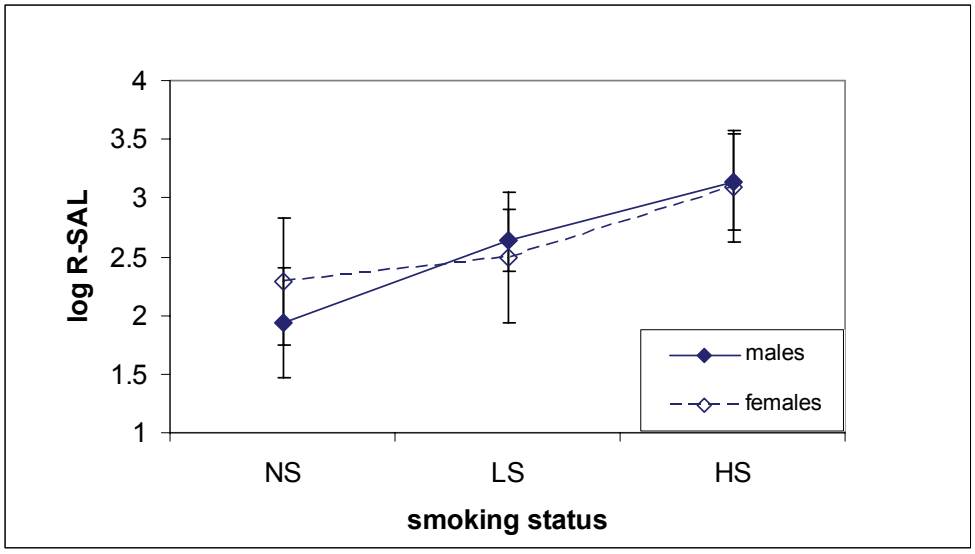


Figure 5-34: Log R-SAL as function of smoking status and gender (mean ± SD).

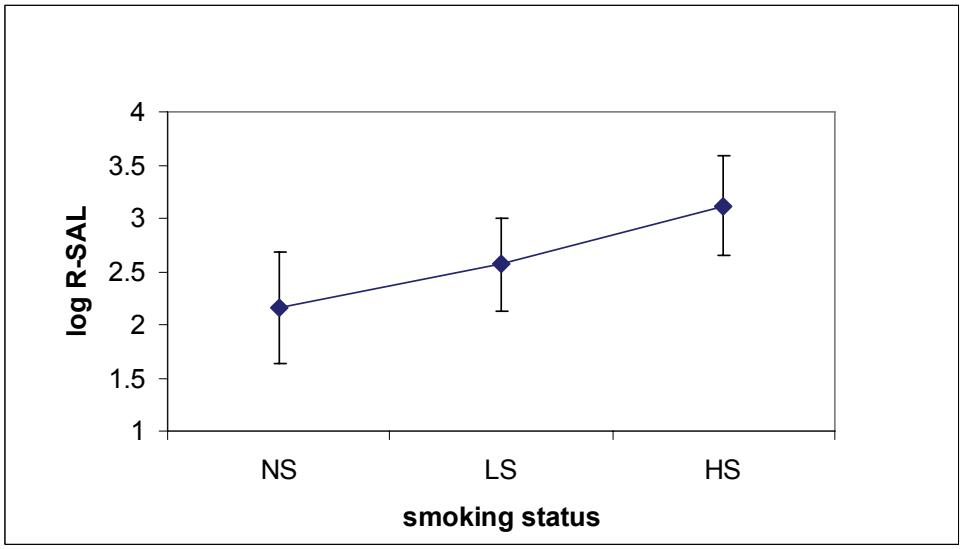


Figure 5-35: Log R-SAL as function of smoking status (mean ± SD). Statistically significant results observed between NS-LS and NS-HS groups.

ANOVA results assigned the following rank with respect to observed plasma log DA concentrations across smoking groups and gender: NS F < NS M < LS M < HS M < LS M < HS F. The post-hoc multiple comparisons via Scheffé test showed that the major difference that was observed with the smoking groups were the HS – NS group. A statistically significant difference was not observed between the NS and LS or the two smoking groups of LS and HS. On average, across smoking groups, the HS group > LS group > NS group for log DA. See figures below.

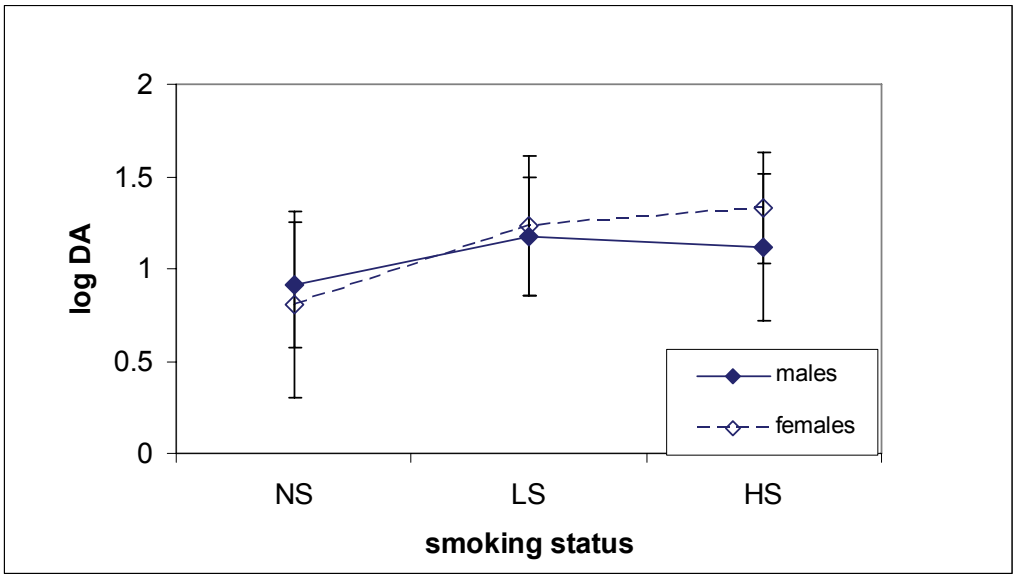


Figure 5-36: Log DA as function of smoking status and gender (mean ± SD).

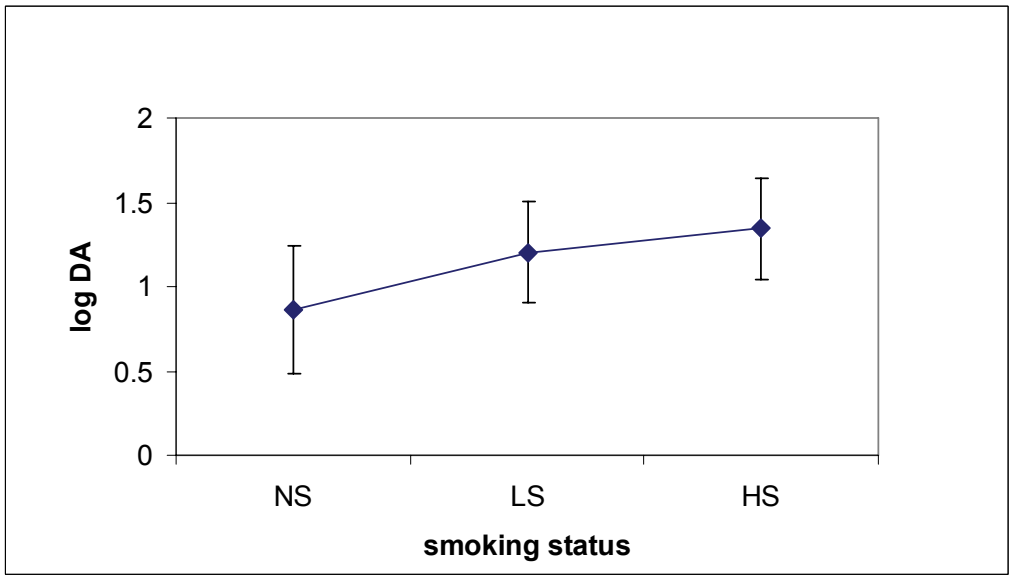


Figure 5-37: Log DA as function of smoking status (mean ± SD). Statistically significant results observed between NS-HS.

With respect to R and S-SAL, LS and HS smoking status groups were significantly different from NS.. Within the LS and HS groups, males possessed slightly higher R-SAL and S-SAL concentrations than females (not significant), but the incongruity was reversed in the case of the NS groups, with females resulting in larger SAL concentrations. Interestingly, a trend was observed with log DA, in which the trend in observed means between the smoking status groups was NS < LS < HS. DA concentrations were not statistically different from one another between genders.

For both SAL enantiomers an increase of log TIQ concentration was observed with the level of smoking status. This is presumed to be due to an acute effect of inhalation of aldehydes from the cigarette smoke. SAL enantiomers are not present in dry cigarette tobacco, but may form from pyrrolysis of tobacco and inhaled upon smoking. Acetaldehyde is a known component of cigarette tobacco that may be inhaled upon cigarette smoke exposure, thereby non-enzymatically condensing with endogenous circulating DA to form the SAL enantiomers. Of note, R and S-SAL enantiomers were present in similar concentrations within the plasma of nonsmokers and smokers. Therefore, mechanistic differences between the SAL enantiomers cannot be interpreted from the design of this clinical study

Of interesting note, a trend was observed with plasma DA concentrations in which smokers on average, possessed higher concentrations than nonsmokers. DA is not known to be present in tobacco or tobacco smoke. The trend observed in DA concentrations may be a physiological difference between smokers and non-smokers, due to different peripheral and central MAO activity between nonsmokers and smokers.

It is known that smoking a cigarette increases DA within the nucleus accumbens in the CNS, but the peripheral concentrations after acute cigarette smoke exposure have been less thoroughly evaluated. An investigation evaluated the combined impact of smoking and stress on catecholaminergic and cardiovascular reactivity in disease-free adult smokers (Robinson and Cinciripini, 2006). The authors propose that ad-lib smoking increases catecholamine and cardiovascular response to stress in smokers. All of these reasons may explain the trend of plasma DA observed within this study.

5.4.3c TIQ's and DA – Exploratory Analysis

A statistically significant effect of smoking status was observed on log R-SAL, log S-SAL and log DA plasma concentrations. To further elucidate the variability associated with R/S-SAL and DA exposure, the criteria of FTND and number of cigarettes smoked per day were analyzed as continuous dependent variables to explain the individual subjects' exposures. As in the case of the β-carbolines, FTND and the number of cigarettes smoked per day were replaced by the SS category for the ANOVA analysis for SAL enantiomers and DA. The individual concentrations for each subject were used to assess the association between FTND and number of cigarettes and the analyte concentrations in order to represent the intra-subject variability for the log R-SAL, log S-SAL, and log DA in the analysis.

The tables below summarize the regression analysis for all three analytes using different explanatory variables for the dependent variable. For reference, results of the primary analysis using smoking status and gender for the model fit are supplied. As

FTND and the number of cigarettes smoked per day are measures of nicotine dependence that were not expected to change between sampling periods, intra-subject average log R-SAL, S-SAL and DA concentrations were also explored for an association.

All model fits were statistically significant on the individual log S-SAL concentrations incorporating the use of the FTND and number of cigarettes/day. The use of FTND and number of cigarettes smoked per day in place of smoking status resulted in statistically significant fits but yielded an inferior coefficient of determinations from the initial model. In essence, the smoking status + gender factors was able to explain more to the variability associated with log S-SAL concentrations (19.2%) than its FTND (15.2%) and #cigarettes/day (11.4%) design variables.

Upon evaluation of the average log S-SAL concentrations, an improvement was observed when the number of cigarettes smoked per day was implemented into the model in place of smoking status. According to this model, gender or the gender:cigarettes/day interaction was not significant.

Table 5-17: Explanatory variable model comparison for Log S-SAL.

Log S-SAL				
Model	*Description*	R^2	*p-value*	*Slope (for continuous dependent variable) and notes*
SS + GEN (primary pre-specified factors)	Original 2-way ANOVA with SS and GEN as factors	0.1921	< 0.0001	No interaction
FTND + GEN	Linear regression with continuous FTND as dependent variable	0.1521	0.004	0.123, sig interaction observed between FTND and GEN
#cig/day + GEN	Linear regression with continuous #cig/day as dependent variable	0.1143	0.0113	0.024, no interaction, yes effect of gender
Avg log S-SAL				
FTND + GEN	Linear regression on avg log S-SAL with continuous FTND as dependent variable	0.1995	0.0257	0.1149, no sig interaction observed between FTND and GEN No effect of GEN
#cig/day + GEN	Linear regression on avg log S-SAL with continuous #cig/day as dependent variable	0.2544	< 0.01	0.0211, no interaction No effect of gender.

With respect to the individual log R-SAL, FTND model fits were not statistically significant (all p-values > 0.05). The results of the model fits are shown in the following table. In this case, the use of the # of cigarettes smoked per day was a more inferior predictor of individual log R-SAL concentrations than smoking status possessing a R^2 of > 0.061. In other words, the # of cigarettes smoked per day was only able to explain 6.1% of the variability associated with the log R-SAL concentrations as opposed to the smoking status, which explained 25.7%. Replacement of the smoking status factor with FTND did show statistically significant results (p-value= 0.01, R^2 = 0.101), suggesting that FTND is a poorer predictor of log R-SAL than the other smoking design variables.

Upon assessment of the *average* log R-SAL concentrations, a statistically significant effect of number of cigarettes smoked per day was observed. However, the variability that could be accounted for by this model was approximately 21%, which is inferior to the original smoking status model.

Table 5-18: Explanatory variable model comparison for Log R-SAL.

Log R-SAL				
Model	*Description*	R^2	*p-value*	*Slope (for continuous dependent variable) and notes*
SS + GEN (primary pre-specified factors)	Original 2-way ANOVA with SS and GEN as factors	0.2571	< 0.0001	No interaction
FTND + GEN	Linear regression with continuous FTND as dependent variable	0.1016	0.013	0.122, sig interaction observed between FTND and GEN
#cig/day + GEN	Linear regression with continuous #cig/day as dependent variable	0.0661	0.045	0.0215, no interaction
Avg log R-SAL				
FTND + GEN	Linear regression on avg log R-SAL with continuous FTND as dependent variable	NS	NS	NS, sig interaction observed between FTND and GEN
#cig/day + GEN	Linear regression on avg log R-SAL with continuous #cig/day as dependent variable	0.2191	< 0.026	0.0186, no interaction

For individual log DA, all model fits were statistically significant with use of the FTND and number of cigarettes/day (all p-values < 0.002). Information obtained from the model fits are shown in the following table. In this case, the use of FTND was a slightly better predictor of individual log DA concentrations than smoking status possessing a R^2 of > 0.288. In other words, FTND was able to explain 28.8% of the variability associated with the log DA concentrations as opposed to the smoking status, which explained 26.1%. Incorporating the number of cigarettes/day into the model resulted in an inferior fit compared to that of the smoking status group. Results of the model fits with respect to log DA should be interpreted with caution as significant interactions between the gender and FTND factors exist.

Upon assessment of the *average* log DA concentrations, a statistically significant effect of number of cigarettes smoked per day and FTND was observed upon incorporation into the model. Replacement of both FTND and number of cigarette per day design variables into the model resulted in fits that were able to explain 50.2% and 41.9% of the variability associated with log DA concentrations. Of note, in the case of FTND, a significant interaction was observed with GEN, hindering interpretability of the results.

Table 5-19: Explanatory variable model comparison for Log DA.

Log DA				
Model	*Description*	R^2	*p-value*	*Slope (for continuous dependent variable) and notes*
SS + GEN (primary pre-specified factors)	Original 2-way ANOVA with SS and GEN as factors	0.2606	< 0.0003	Interaction between GEN and SS
FTND + GEN	Linear regression with continuous FTND as dependent variable	0.2888	< 0.0001	0.105, sig interaction observed between FTND and GEN
#cig/day + GEN	Linear regression with continuous #cig/day as dependent variable	0.2311	< 0.002	0.0205, effect of GEN and interaction present
Avg log DA				
FTND + GEN	Linear regression on avg log DA with continuous FTND as dependent variable	0.5016	< 0.0001	0.1745, sig interaction observed between FTND and GEN
#cig/day + GEN	Linear regression on avg log DA with continuous #cig/day as dependent variable	0.4197	< 0.0002	0.0205, no interaction, no effect of gender

According to the exploratory analysis, for both individual log R-SAL and S-SAL, smoking status resulted in a superior model fit than that of the FTND and number of cigarettes smoked per day design variables. This is suggestive that variability associated with the log R- or S-SAL cannot be explained by the design factors themselves, but with the overall combination of both, which were used for stratification into the smoking status groups. Although a significant effect of smoking status was observed on log DA, similar results were observed upon replacement of the smoking status factor with the FTND and number of cigarettes smoked per day design variables. A significant interaction of gender was observed with all smoking design factors, hindering the statistical inference of the final model results.

5.4.3d TIQ's and DA – Covariate Analysis

As the case for the β-carbolines, information including alcohol intake and weekly exposure to dietary total SAL was recorded to evaluate for their effects on circulating levels of R-SAL, S-SAL and DA. As dietary intake of ethanol and/or SAL containing foods may affect the overall exposure, this covariate assessments may provide information into the variability associated with the plasma measurement log R-SAL and/or S-SAL. If alcohol intake and/or dietary exposure were found to significantly correlate with SAL exposure, AAI or Food Intake was considered a significant covariate and implemented into the statistical model.

Linear regressions were performed on log R-SAL, log S-SAL as a function of mg of annual absolute ethanol (AAI) or average weekly dietary total SAL (Food

Inventory) intake. Similar criteria aforementioned for the β-carboline covariate analysis were used for the SAL and DA analysis. Evaluation of the significance of the covariate was additionally assessed upon implementation into a final ANCOVA model. The p-value was evaluated for significance of the covariate. If considered significant, the fit of the entire model was evaluated for goodness of fit, with use of the covariate. Dietary DA intake was not captured from the study, therefore was not computed as a covariate of log DA concentrations.

A table summarizing the covariate regression analysis for AAI and dietary intake is shown below.

Table 5-20: Covariate analysis results for log R-SAL, log S-SAL and log DA

variable	AAI (mg ethanol/ annum)		Weekly Dietary intake (ng SAL)	
Log R-SAL	R^2	0.0743	R^2	0.001
	p-value	0.076	p-value	0.996
	significance	NS	significance	NS
Log S-SAL	R^2	0.0655	R^2	0.004
	p-value	0.203	p-value	0.882
	significance	NS	significance	NS
Log DA	R^2	0.0211	R^2	N/A
	p-value	0.1925	p-value	N/A
	significance	NS	significance	N/A

NS: not significant
N/A: dietary intake of DA was not available

The covariate analysis revealed that a poor association was observed between weekly dietary intake of total SAL to circulating concentrations. As SAL enantiomers are present in various sources of foods in substantial amounts, it was expected that influence of dietary consumption of SAL would contribute to the circulating plasma concentrations. Formal assessment of pharmacokinetics has not been performed on the influence of circulating SAL on acute exposure to a food source rich in these compounds. The results of this covariate analysis suggest that the weekly average intake of total SAL do not influence circulating levels of R-SAL or S-SAL. Implementation of the dietary SAL intake was subsequently incorporated into the ANCOVA model and was found to be not significant for either analyte (R-SAL: p-value = 0.99, S-SAL: p-value = 0.87).

Annual absolute alcohol intake did not have a significant effect on circulating R-SAL, S-SAL or DA concentrations. Upon incorporation of AAI into the ANCOVA statistical model for log R-SAL, mg of ethanol per annum was not considered a significant covariate (p-value = 0.06). In the case of circulating S-SAL, the AAI yielded an insignificant effect as a covariate (p-value = 0.164). Upon incorporation of AAI into the ANCOVA statistical model for log DA, significance of mg of ethanol per annum was not considered a significant covariate (p-value = 0.77). Use of the AAI or Food Intake as a covariate was not significant and was not implemented into the full statistical model. ANOVA analysis found a significant relation between AAI and smoking status (p-value < 0.01), suggesting that the smoking status factor and AAI are

considered to be collinear variables. Of note, AAI may be a confounding variable that shows a strong relationship with smoking status.

5.5 TIQ and β-carbolines – Acute Cigarette Exposure vs. Smoking Abstinence

All information presented thus far summarizes TIQ and β-carboline exposure information obtained from non-smokers, in addition to light-smokers and heavy smokers within thirty-minutes of smoking one complete cigarette. In essence, the presented information compares nonsmokers, who were not exposed to inhaled and environmental tobacco smoke to that of light-smokers and heavy smokers who just smoked a cigarette. The significant relationship of smoking status and TIQ and/or β-carboline exposure observed may be resultant of acute inhalation of tobacco smoke. To further clarify this relationship, a comparison was made to subjects who had abstained from smoking for 15 hours prior to plasma sampling of the analytes of interest.

In brief, the study design incorporated 18 healthy volunteers (6 NS, 6 LS, and 6 HS, in which there were 3 males and 3 females in each group). This study was designed to evaluate the effects of intranasal nicotine on neuroendocrine, cognitive and behavioral function in healthy, young, nonsmokers and smokers (Leu, 2002). Subjects were randomized into one of two sequences based on smoking status such that they received intranasal nicotine or placebo. Prior to treatment administrations during each of two visits, subjects provided plasma samples for the analysis of baseline TIQ's and β-carboline exposure. The volunteers were required to abstain from smoking for

15-hours prior to sampling, which was conducted pre-prandially during the inpatient visit.

Subjects were young and healthy, using the same inclusion/exclusion criteria, and more importantly, utilized the same smoking status classification as the study aforementioned. Plasma samples for the evaluation of TIQ's and β-carboline exposure were obtained on two-different visits. Similar procedures were used for the sampling, processing and ultimate analysis of the plasma. Demographic results of this study may be seen in the table 5-21 below. This study is referred to as abstained smokers (ABST SM). As a comparison was made to the primary investigation, a demographic summary of the study is also provided below. This study is referred to as the recently smoked (REC SM) keeping in mind that the NS group did not smoke a cigarette prior to biological sampling. Demographic variables such as age, FTND and number of cigarettes smoked per day are reported as mean ± SD.

Table 5-21: Demographic Results of ABST SM study (mean ± SD)

Smoking Status	Demographic variable	Males	Females	Overall
NS	N	3	3	6
	Age	28.0 (2.6)	25.7 (1.5)	26.8 (2.3)
	FTND	0 (0)	0 (0)	0 (0)
	# Cig/day	0 (0)	0 (0)	0 (0)
LS	N	3	3	6
	Age	24.3 (4.9)	23.3 (4.9)	23.8 (3.3)
	FTND	2.7 (1.5)	2.0 (1.0)	2.3 (1.0)
	# Cig/day	10.8 (3.8)	10.0 (6.1)	14.0 (3.6)
HS	N	3	3	6
	Age	26.7 (5.7)	24.0 (2.6)	25.3 (4.2)
	FTND	6.0 (2.6)	6.3 (2.5)	6.2 (2.3)
	# Cig/day	20.8 (1.4)	22.3 (4.5)	21.2 (4.8)

Table 5-22: Demographic Results of REC SM study (mean ± SD)

Smoking Status	Demographic variable	Males	Females	Overall
NS	N	9	10	19
	Age	24.9 (2.5)	26.0 (3.1)	25.5 (2.8)
	FTND	0 (0)	0 (0)	0 (0)
	# Cig/day	0 (0)	0 (0)	0 (0)
LS	N	5	6	11
	Age	25.4 (4.1)	23.3 (3.8)	24.3 (3.9)
	FTND	2.4 (2.3)	1.5 (1.4)	1.9 (1.8)
	# Cig/day	9.0 (4.8)	8.3 (4.6)	8.6 (4.5)
HS	N	5	6	11
	Age	25.6 (3.1)	24.7 (4.0)	25.1 (3.5)
	FTND	6.8 (0.8)	5.5 (1.4)	6.1 (1.3)
	# Cig/day	17.4 (2.5)	25.8 (4.9)	22.0 (5.8)

With the exception of the number of subjects within each smoking status and gender group, the demographics between studies are similar with respect to age, FTND and the number of cigarettes smoked per day.

As performed in the REC SM study, statistical analysis via two-way ANOVA was conducted on the individual concentrations of the β-carbolines, H and NH along with the TIQ's and DA. Log transformed values were compared evaluating the two primary factors of gender and smoking status.

Prior to formal analysis, within subject variability was assessed across the two sampling periods. Of note, the variability between occasions for smokers was much less in the ABST SM study compared to that of the REC SM study. For nonsmokers, the within-subject variability was similar between studies. Below is a chart of the ranges of within subject variability obtained from both studies reported in ranges of COV% across all subjects per study for each analyte.

Table 5-23: Ranges of Within-Subject Variability of Smokers between both studies

Analyte	ABST SM	REC SM
Harman	3 – 63 %COV	4 – 760% COV
Norharman	8 – 54% COV	2 – 112% COV
S-SAL	2 – 93% COV	3 – 1745% COV
R-SAL	8 – 82% COV	0 – 2686% COV
DA	0.5 – 28% COV	3.8 – 130% COV

The large range of the within-subject variability associated with the REC SM study suggests that the recent smoking of a cigarette, along with the lack of an inconsistent sampling schedule after smoking between sampling occasions, contributes to the overall variability associated with the analyte. The %COV observed in the ABST SM study is suggestive of a more stable baseline measurement and less inter-occasion variability.

Table 5-24 exemplifies the comparison of the two-way ANOVA fits, accounting for smoking status and gender between the study in which smokers abstained from smoking for 15 hours (ABST SM) and recently smoked smokers (REC SM). Multiple comparison tests were performed on both studies for each log transformed analyte concentration and the significant group differences are reported. Coefficients of determination are reported to evaluate the fit of the model between studies. To further support the observations of the model fits, median and ranges are reported in table 5-25 for both studies, accounting only for smoking status. An unpaired t-test was performed between studies (abstaining vs. smoking) on the average log transformed concentrations within each smoking status group. Note, the assumption equal variance was violated upon comparison of the smoking status groups between studies.

As in the case for the primary REC SM study, in which measurements were taken within 30 minutes of smoking, a smoking status effect was observed with respect to log S-SAL, log R-SAL, and log NH in the ABST SM study (table 5-24). A significant effect of smoking status was not observed for log H and log DA. A gender

effect or a gender – smoking status interaction was not observed with any of the analytes.

Contrary to the REC SM study, the only observed difference between smoking statuses resided between the NS-HS groups for R-SAL and S-SAL. No observed difference was apparent between the HS-LS and, as in the opposite case of the REC SM study, the LS-NS groups. For median R-SAL and S-SAL, a trend was observed in which HS > LS > NS. In the ANOVA model fit smoking status was able to account for approximately 33.7% of the variability associated with log S-SAL concentrations and 36.0% of the variability of log R-SAL concentrations. While in the REC SM study, only 18.3% and 25.5% of the variability was accounted for by smoking status for S-SAL and R-SAL respectively. This suggests that there exists an inherent constitutive difference in R-SAL and S-SAL concentrations between NS and HS, regardless of recent smoking. Upon consuming a cigarette, the difference in concentrations became more pronounced with LS-NS groups. In essence, R-SAL and S-SAL concentrations are intrinsically different between smoking status groups and additional smoking makes this discrepancy more pronounced. The incongruity in results between studies suggest that the exogenous contribution of these analytes from recent smoking provides additional exposure of R-SAL and S-SAL to the LS and HS groups, resulting in an overall observed difference between the smoking and nonsmoking groups.

With respect to log NH concentrations in the ABST SM study, the observed difference between smoking statuses resided both between the NS-HS and NS-LS groups. No observed difference was apparent between the HS-LS as already seen in the

REC SM study. For median NH a trend was observed in which HS > LS > NS. With the ANOVA model fit, smoking status in abstinent smokers was able to account for approximately 44.0% of the variability associated with log NH concentrations while after smoking 49.7% of the variability associated with log NH concentrations could be explained. This suggests that there exists an inherent constitutive difference in NH concentrations between NS-HS and NS-LS groups, regardless of recent smoking. Upon consuming a cigarette, the difference in concentrations became more pronounced between both sets of groups, especially the NS-HS difference. In essence, NH concentrations are fundamentally dissimilar between smoking status groups and additional smoking makes this discrepancy more pronounced. As in the case of SAL enantiomers, the disparity in observations between studies suggest that the exogenous contribution of these analytes from recent smoking provides additional exposure of NH to the LS and HS groups, resulting in an overall observed difference between the smoking and nonsmoking groups. This is expected, as NH is present in significant concentrations within tobacco.

It is of interest that H concentrations did not show a similar trend in the ABST SM study and the effect of smoking status was not observed. This suggests that harman differences between smoking status groups is due to the recent exposure of H after smoking, as seen in the REC SM study. For DA, it is apparent that a difference in median concentrations between smokers and nonsmokers is present. Recent smoking results in a greater and statistically different difference between HS-NS and LS-NS groups.

Table 5-24: Comparison of Two-way ANOVA model fits between ABST SM and REC SM study for all analytes.

Analyte	*Model Factor*	**ABST SM Study**	**REC SM Study**
log S-SAL	SS	sig (p-value = 0.017)	sig (p-value < 0.0001)
	GEN	NS	NS
	SS:GEN	NS	NS
	R^2	0.3371	0.1829
	difference	HS-NS	HS-NS, LS-NS
log R-SAL	SS	sig (p-value = 0.008)	sig (p-value < 0.0001)
	GEN	NS	NS
	SS:GEN	NS	NS
	R^2	0.3602	0.2556
	difference	HS-NS	HS-NS, LS-NS
log Harman	SS	NS	sig (p-value < 0.0001)
	GEN	NS	NS
	SS:GEN	NS	NS
	R^2	0.2254	0.2844
	difference	No difference	HS-NS, LS-NS
log Norharman	SS	sig (p-value = 0.001)	sig (p-value < 0.0001)
	GEN	NS	NS
	SS:GEN	NS	NS
	R^2	0.4440	0.4969
	difference	HS-NS, LS-NS	HS-NS, LS-NS
log DA	SS	NS	sig (p-value < 0.0002)
	GEN	NS	NS
	SS:GEN	NS	sig (p-value < 0.05)
	R^2	0.2421	0.2606
	difference	No difference	HS-NS, LS-NS

SS: Smoking status factor
GEN: Gender factor
SS:GEN: Smoking status : gender interaction
Sig: Significant
Difference: Observed difference via Scheffé multiple comparison

Table 5-25: Comparison of median (range) between ABST SM and REC SM study for all analytes.

Analyte	*Smoking status*	ABST SM Study ***median*** (range) NS, LS, HS = 6, 6, 6	REC SM Study ***median*** (range) NS, LS, HS = 19, 11, 11	significance unpaired t-test* (p-value)
S-SAL	NS	***77*** (51 – 437)	***50*** (13 – 860)	NS
	LS	***175*** (40 – 250)	***143*** (18 – 1632)	NS
	HS	***228*** (81 – 693)	***317*** (25 – 6084)	< 0.05
R-SAL	NS	***45*** (5 – 609)	***48*** (4 – 1210)	NS
	LS	***277*** (149 – 705)	***226*** (12 – 1478)	NS
	HS	***547***(76 – 1002)	***324*** (2 – 7714)	< 0.05
Harman	NS	***19*** (3 – 56)	***12*** (1 – 98)	NS
	LS	***31*** (12 – 54)	***71*** (4 – 172)	NS
	HS	***29*** (5 – 110)	***52*** (4 – 422)	NS
Norharman	NS	***12*** (2 – 65)	***17*** (2 – 850)	NS
	LS	***49*** (20 – 104)	***54*** (12 – 396)	< 0.05
	HS	***59*** (5 – 133)	***159*** (25 – 410)	< 0.05
DA	NS	***2.3*** (1.0 – 9.5)	***5.9*** (1.5 – 17)	NS
	LS	***5.5*** (3.6 – 7.5)	***9.9*** (0.3 – 32)	< 0.05
	HS	***5.1*** (3.4 – 8.2)	***14.6*** (4.7 – 37.7)	< 0.05

***** unpaired t-test performed on log-analyte concentration between two studies, assumption of unequal variance.

The measurements observed in the ABST SM study were presumably due to constitutive concentrations with no influence of acute exogenous exposure of cigarette smoke. Having abstained from smoking for 15 hours prior to sampling under fasting conditions, the significant effect of smoking status may also be due to accumulation of S-SAL, R-SAL and NH in which the analytes reside in the physiological system well after exposure to exogenous sources. In addition, the analytes that were significant with respect to smoking status may be an inherent discrepancy between smokers and nonsmokers that may be related to the dependence of tobacco smoking itself. Of importance, this study is not designed to test causality between these analytes to smoking dependence, but results obtained from the ABST SM study are suggestive of inherent differences in endogenous physiological concentrations of S-SAL, R-SAL and NH.

5.6 Summary of Clinical Study #1

This investigation was designed to test the effects of smoking status and gender on plasma concentrations of TIQ's and β-carbolines in a healthy population. The study utilized a nicotine dependence scale and the number of cigarettes smoked per day in order to stratify the subjects into smoking status groups. Besides the alleged nicotine dependence difference, all subjects were relatively homogeneous with respect to demographics.

Overall the variability observed between subjects was pronounced across all groups for all analytes tested. The effect of smoking status was significant within this

study with the primary difference being between nonsmokers and smokers. Although a trend was observed, a statistically significant difference was not noticed between the two LS and HS smoking groups. It is important to report that the smoking groups were sampled within thirty minutes of smoking an entire cigarette. Therefore, the exposures of the TIQ's and β-carbolines between smokers and nonsmokers may be resultant of the acute inhalation of β-carbolines from tobacco smoke or spontaneous condensation of the acetaldehyde and dopamine, as in the case of the SAL enantiomers. Moreover, the endogenous formation of the β-carbolines via condensation of acetaldehyde with 5-HT or tryptamine may also contribute to the overall exposure of H and NH from cigarette smoke. As β-carbolines are present in significant concentrations in tobacco, the rationale of inhalation of these analytes causing the "baseline" difference between nonsmoking and smoking groups is a plausible mechanistic reason. On the other hand, the SAL enantiomers and dopamine are not known to be constituents of tobacco smoke. It is presumed that the acute exposure of acetaldehyde from the tobacco smoke is responsible for the divergence of SAL concentrations between nonsmokers and smokers.

Dopamine differences between smoking status groups were also observed. This effect may be due to a few reasons. Acute smoking may induce a stress response thereby releasing dopamine from the medulla of the adrenal glands. The phasic responses of dopamine neurons are observed when a reward is presented, such as in smoking. A more indirect reason for the increase in peripheral dopamine concentrations observed pertained to the TIQ's and β-carbolines. As these analytes are

elevated upon acute cigarette exposure, their pharmacological actions upon inhibiting the enzymes responsible for degrading dopamine may cause the peripheral elevation of dopamine. In order to characterize this relationship, further studies are necessary for evaluating the enzymes responsible for the metabolism of dopamine and the inhibitory relationship with TIQ and β-carboline concentrations.

For all analytes, a large inter-occasion variability was observed between observational periods. Thorough characterization of the analyte pharmacokinetics is imperative in order to evaluate this variability. The within subject variability was relatively small for the nonsmokers as compared to smokers. The sampling schedule, with respect to inhalation of tobacco smoke, required more rigid control to minimize variability associated with the separate sampling occasion. This variability may contribute to the overall between group variability observed within the smoking subjects.

An attempt was made to characterize true baseline differences between smoking status groups with evaluation of a population of smokers who abstained from smoking for 15 hours. A significant baseline difference was observed for the SAL enantiomers and norharman within this study with the primary difference being between the heavy and nonsmokers. This is presumed to be a function of a true constitutive difference between smokers and nonsmokers or an additive accumulation of SAL enantiomer and norharman concentrations within the body. Of note, a formal conclusion with respect to this study cannot be deduced without full understanding of the pharmacokinetics. When compared to the study involving smoker's recent exposure to tobacco smoke, the

difference between nonsmokers and smokers was more pronounced. This suggests that, in addition to a supposed baseline difference, inhalation of tobacco smoke provides additional exposure to circulating TIQ and β-carbolines contributing to the incongruity of concentrations between smoking status groups. A major critique of this analysis is that the two separate populations were compared to assess the baseline differences, one study abstaining from smoking and one study not. Ideally, a study consisting of observations in the same population, sampling before and after smoking, with an adequate sampling schedule would be needed to further support the notion of "true" smoking status differences.

In addition, the primary study was not balanced with respect to gender and smoking status. This insufficiency confounds the results and variability associated between smoking status groups. Of note, a gender difference was not observed but the true difference may have been masked due to the unbalanced design for this factor.

Additional information was gathered from the volunteers that may have an effect on circulating TIQ and β-carboline concentrations. A non-validated food inventory was designed to evaluate average weekly intake of TIQ and β-carboline amounts. This assessment was used to review chronic dietary intake of the analytes and its speculated effects on circulating levels. Nevertheless, a significant difference was not observed between smoking groups with respect to dietary intake while smoking status had an effect on circulating TIQ and β-carbolines. A more robust manner to evaluate the effects is to have adequate dietary control throughout the observational period to sufficiently account for dietary intake. The exposure of circulating TIQ and β-

carbolines with respect to acute dietary intake of TIQ and β-carbolines needs to be further evaluated in order to properly assess its influence.

A well-known variable associated with TIQ and β-carboline exposure is alcohol intake. The AAI was used to evaluate the intake of absolute ethanol from alcoholic beverages over a year. It is important to account that a strong relationship between AAI and smoking status was present. In essence, the characteristics of smoking dependence, as deemed by the smoking status classification and the AAI showed a strong relation. Persons who had a higher degree of smoking dependence also imbibed more alcohol per annum. Using AAI as a sole predictor, alcohol consumption was only able to explain less than 10% of the variability associated with TIQ and β-carboline exposure. Further multiple regression techniques would be necessary to evaluate the relative contribution of AAI and smoking status on TIQ and β-carboline exposure in order to make an assessment. Nevertheless, smoking status as a sole predictor was able to explain more of the variability associated with the analyte concentrations compared to that of AAI.

It was found that, in a healthy population, a noteworthy trend was observed between smoking status and TIQ and β-carboline exposure. This trend is hypothesized to be a product of a combination of true endogenous differences between smoking status groups and exposure via the inhalation of the analytes themselves and/or inhalation of precursors required for endogenous synthesis, acetaldehyde. The concentrations of TIQ and β-carboline exposure are assumed to be reflective of central dopaminergic activity. The baseline level difference in the study involving smoking

abstinence suggests that smokers have higher concentrations. Smokers may require maintenance of these higher concentrations in order to experience feelings of pleasure, simultaneously circumventing negative symptoms of nicotine withdrawal. Behavioral studies do indicate that nicotine is an addictive drug that reinforces self-administration and the effects of nicotine on tests of reinforcement and behavioral sensitization are primarily mediated through the mesolimbic dopamine system. TIQ and β-carboline exposure within this "reward pathway" may play a synergistic role, along with the pharmacological actions of nicotine, in the reinforcing aspects of tobacco smoking.

TIQ and β-carboline exposure has been purported to be influenced by acute ethanol intake and chronic alcoholism (See tables 1-1 and 1-2). Significant variability within these studies have been reported, hampering the ability for these compounds to be an adequate marker for alcohol abuse. As the association of smoking and alcohol abuse is strong, it is suspected that the variability in TIQ and β-carboline exposure observed may be explained by tobacco smoking. As smoking status had a significant effect on TIQ and β-carboline exposure in a healthy population, it is expected that this effect would be observed in an alcoholic population. The next chapter attempts to investigate the effects of smoking status in an alcoholic population undergoing inpatient detoxification.

CHAPTER 6

CLINICAL STUDY #2 – TEMPORAL EFFECTS OF GENDER AND SMOKING ON R/S-SALSOLINOL DURING ALCOHOL DETOXIFICATION

6.1 Specific Aims

The key objective of this study was to evaluate the influence of time on R/S-SAL and DA concentrations in the plasma of alcohol-dependent patients undergoing alcohol-detoxification. This observational study was conducted as part of an ongoing NIH-IRB approved investigational protocol conducted at the National Institutes of Health – National Institute on Alcohol Abuse and Alcoholism (Herion, 2004), referred to here onward as the NIAAA-Natural History Protocol (NIAAA-NHP). Moreover, a formal comparison of R/S-SAL plasma concentrations between alcohol-dependent patients and the healthy population from Clinical Study #1 was conducted.

Thirty-six alcohol-dependent patients (18 M, 18 F) were stratified into non-, light-, and heavy smoker (NS, LS, and HS) subgroups to determine the influence of smoking on R/S-SAL and DA concentrations. In principle, observed differences of R/S-SAL levels could be interpreted as either a consequence of alcohol and/or tobacco

use (exogenous) or as a primitive condition of the brain that leads people to drink and smoke (endogenous). Specific aims of this study included:

1) Determine whether there are time-related changes in plasma TIQ's (R- and S- SAL) and DA in a cohort of alcohol-dependent subjects during early abstinence.
2) Assess if variations of R/S-SAL and DA exist between NS, LS and HS subgroups within the alcohol-dependent population; specifically, a comparison of the average concentrations and concentrations at predetermined time points during detoxification that may exist with R/S-SAL and DA levels between these subgroups.
3) Evaluate the Clinical Institute Withdrawal Assessment-Alcohol Revised (CIWA-AR) scores of the volunteers throughout the detoxification period and assess if differences are present between subgroups; appraise if an association exists with levels of R/S-SAL and DA with CIWA-AR scores.
4) Characterize severity of alcohol dependence of the volunteers via the Timeline Follow Back assessment and determine in an association exists with the exposure of alcohol and R/S-SAL concentrations.
5) Determine if a gender effect exists in R/S-SAL and DA concentrations and CIWA-AR scores within this special population.
6) Provide statistical measurement of inter- and intra-individual variability in the levels of R/S-SAL and DA concentrations along with CIWA-AR scores, which would permit formal sample size calculations in future studies.

7) Compare all pertinent endpoints and measurements between the abstaining, alcohol-dependent cohort and results from the study involving a healthy, non alcohol-dependent, healthy population of NS, LS, and HS (Clinical Study #1).

The design of the study allows characterization of plasma levels of R/S-SAL in order to evaluate these compounds as potential biomarkers alcohol dependence. It was additionally designed to evaluate the effects of smoking and gender on the observed R/S-SAL concentrations to allow comparison with clinical study #1. In essence, this study attempts to assess whether potential confounding factors, such as smoking status and gender, influence the temporal pattern of R/S-SAL concentrations in an alcoholic population.

A comparison between the alcohol dependent population and a healthy population will be made to assess influence of the effect of alcohol dependence on the R/S-SAL concentrations. The stratification of smoking status groups within both populations will permit formal assessment of the relative contribution of smoking or chronic alcoholism on R/S-SAL plasma concentrations.

6.2 Study Design

This clinical study was designed as an observational, longitudinal study in a cohort of alcohol-dependent patients that may or may not be dependent on cigarette smoking. The alcohol-dependent cohort participated in an inpatient detoxification program for duration of 4-weeks in which observations, such as plasma R/S-SAL and

DA concentrations, were assessed. The protocol involved the typical clinical course of events in people with alcohol dependence and abuse over a brief, intensive time period. During this time, the standard-of-care, as deemed by the clinical staff at NIH-NIAAA, was employed to all patients enrolled in the study. Informed consent was obtained before the start of the study after explaining the purpose, risks and benefits of the study to the prospective study subjects.

To determine the effects of smoking and gender on R/S-SAL and DA plasma concentrations, the study integrated a classification scheme to stratify the volunteers into nonsmoking (NS), light-smoking (LS) and heavy-smoking (HS) groups and with respect to gender, respectively. The stratification incorporated the same criteria incorporated in the healthy population from clinical study #1 (see table 5-1).

This investigation was not prospective in design, rather subjects were chosen from a database of 115 subjects that possessed already incurred samples and information with regards to health status, concurrent medications, demographics and smoking dependence information (FTND and number of cigarettes smoked per day). Thirty-six (36) alcohol-dependent male and female volunteers were chosen, including 12 NS, 12 LS, and 12 HS. The number of subjects in each group required to show a difference of 50% in SAL concentrations between smoking groups with an α risk of 0.05 and a power of $1 - \beta = 0.8$, was twelve (12), assuming a coefficient of variation (CV%) of 75%. To evaluate the effects of smoking, the FTND was administered to determine their dependence on smoking in order to classify the potential subjects into the three groups of NS, LS, and HS. To evaluate the effects of gender, an even

distribution was chosen within each group. The factorial design of this study was to be similar to the clinical study #1 design for comparison.

6.3 Experimental methods

6.3.1 Subject Selection

From a database consisting of 115 alcohol dependent patients that were recruited into the NIAAA-NHP protocol between December 2006 – December 2007, patients were chosen based on a few primary criteria. The NIAAA-NHP database consisted of information about each alcohol-dependent volunteer including: admission date, subject demographics (e.g., age, weight, height, gender, ethnicity, etc.), medications that were given as part of protocol and for other concurrent disease states, other drugs of dependence, and most importantly, smoking habit assessment. Each patient had been administered the FTND and a smoking history evaluation form during their stay in order to assess the severity of nicotine dependence. Moreover, information with respect to the sampling schedule that was conducted on each subject during the first tree weeks of inpatient detoxification was supplied.

The primary criteria that were used to choose subjects for the analysis included the following, in order of priority.

1) As the time-course of R/S-SAL and DA was of primary interest, subjects that obtained at least five-sampling time points throughout detoxification were included. Specifically, subjects that were sampled on day 1 (admission), day 2, day 3, day 8 and

day 15 were included in the analysis. The sampling schedule was restricted to only five-samples throughout the detoxification period. Moreover, the schedule involved observations that were hypothesized to characterize the effects of recent alcohol-detoxification on plasma R/S-SAL and DA concentrations.

2) Of the patients who possessed a complete time-course, subjects that met the criteria for inclusion into the smoking status groups were included in the analysis. Approximately 12 subjects for each smoking status group were required to evaluate the effects of smoking. Within each smoking status group, patients were selected to ensure that gender was evenly distributed. Therefore, the analysis required 6 HS males, 6 HS females, 6 LS males, 6 LS females, 6 NS males, and 6 LS females.

3) The subjects that were involved in the analysis were required to be relatively healthy and free of significant disease. As the physiological effects of alcohol detoxification are severe to life-threatening, benzodiazepines and/or acamprosate (Campral®) was used in subjects that required them for withdrawal symptoms. The use of these drugs did not preclude the subject from participation in the analysis. Further information, with respect to the protocol employed, is included in the section below.

6.3.2 NIH-NIAAA Natural History Protocol

As part of the NIAAA-NHP, participants were recruited through local media and professional avenues in the Washington, DC Metro area. They were evaluated by a

nurse and physician, among others, who determined the need for hospitalization, detoxification and to address other issues. For those participants who needed medically supervised detoxification, a standard program of monitoring and treatment with benzodiazepines and other medications was instituted.

The alcohol-dependent cohort participated in an inpatient detoxification program for duration of 4-weeks. Therefore, it consisted of a series of periods involving a pre-visit gathering of subject information, physical and psychiatric evaluation at NIH-NIAAA clinical research center, inpatient alcohol withdrawal and psychosocial management, and baseline observation. The procedures involved scheduled verbal and observational procedures typically used in detoxification, and minimally invasive procedures (e.g., phlebotomy and urine collection), vital signs evaluation, electrocardiogram, chest-X-ray to provide a comprehensive medical and psychiatric evaluation.

Volunteers who passed the initial telephone screening were invited to the NIH-NIAAA CRC for an outpatient visit including medical history (particularly personal or family history of psychiatric disorders and/or drug dependence), smoking history, physical and neurological exam, ECG, vital signs, blood chemistry, and complete blood count in order to ensure the health status of the subject prior to participation. This excluded the participation of subjects with significant concurrent disease.

6.3.2a Inclusion / Exclusion Criteria

In order to be enrolled in the study, subjects had to meet the following criteria for inclusion: 1) age of 21 years or older; 2) ability to give informed consent; 3) seeking help for alcohol drinking related problems; and 4) diagnosis of alcohol dependence by the Structures Clinical Interview for Diagnostics and Statistics Manual IV (DSM-IV, SCID-I) criteria.

Exclusion criteria for participation in the NIAAA-NHP included: 1) unstable or emergent medical or psychiatric conditions; 2) serious neuro-psychiatric conditions which impair judgment or cognitive function to an extent that precludes them from providing informed consent (incompetent individuals); 3) individuals with major depression, bipolar disorders, serious medical disorders, and those receiving psychotropic medications (with the exception of benzodiazepines used for withdrawal treatment); 4) people presenting with complicated medical problems such as, hypertensive emergency, serious GI bleeding, major organ or body system dysfunction such as decompensated liver disease, renal failure, myocardial ischemia, congestive heart failure or cerebrovascular disease, major endocrine problems such as uncontrolled diabetes, pancreatic or thyroid disease; and 5) people who are infected with the Human Immunodeficiency Virus (HIV).

Of note, current illicit drug misuse and a positive urine drug screen did not preclude any of the subjects for involvement in the protocol or the R/S-SAL analysis.

6.3.2b Procedures during detoxification period

The screening period included IRB-approved informed consent form discussion, comprehension and signing, a complete physical evaluation, blood and CHEM-20 panel, urinalysis, vital signs assessment, ECG, and chest X-ray. The DSM-IV/SCID-I was used to diagnose the volunteer as an alcohol-dependent patient. Throughout the study, participants underwent various verbal and observational evaluations. Based on these assessments, multidisciplinary treatment planning was undertaken by the staff with the active participation of the patient.

Therapies that were employed to the volunteer were recorded as drug name, strength, dose and time drug was given. Throughout the inpatient detoxification phase subjects were scheduled to be seen for clinic visits at admission, day 2, 3, 8 and day 15 for brief medical and psychiatric check-ups, selected blood and urine tests, as well as selected written and computer assessments. Following subject testing on discharge day (day 28), a complete physical exam was given.

6.3.2b-1 Biological measurements

Biological sampling for clinical visits took place at 7AM, prior to consumption of foodstuffs/beverages and smoking, with the exception of the admission date. Sampling time on the day of admission was sporadic, dependent on when the subject entered the clinic for detoxification. Breath alcohol (BrAC) was monitored at admission and throughout the inpatient detoxification period. Patient volunteers with positive (BrAC level of $\geq$ 0.01 g/210L breath) and negative ($<$ 0.01 g/210L breath)

results at admission were included in the analysis. Of note, subjects were required to have a negative BrAC throughout subsequent tests.

Throughout the detoxification period, a total of five (5) biological samples (e.g., plasma) were taken for R/S-SAL and DA concentration assessment on admission, day 2, 3, 8, and 15. At all sampling points, a 6-ml plasma sample was collected from the non-dominant forearm in a reclined, seated position during the study. The blood samples were centrifuged to obtain plasma. Briefly, blood samples were centrifuged at 2915 x g (3800 RPM) for 10 min, 4°C. An equivalent volume of anti-oxidant/aldehyde trapping solution containing 0.6 mol/L, 15 mg ascorbic acid, 6 mg semicarbazide HCl in distilled water was added to approximately 3-ml of plasma. Plasma samples were stored at -70°C until analysis for R-SAL, S-SAL and DA concentrations.

6.3.2b-2 Smoking and Alcohol Consumption Assessments

For the assessment of smoking dependence and alcohol consumption measures, the FTND with Smoking History form, and the Alcohol Timeline-Follow Back (TLFB) was administered once during the detoxification period, upon sobriety. The FTND and Smoking History Form were used to classify each subject into a smoking status subgroup. Further discussion may be reviewed in Chapter 5. The Alcohol TLFB is a drinking assessment method that obtains estimates of daily drinking. Using a calendar, subjects provided retrospective estimates of daily drinking over a specified time period (Sobell and Sobell, 1992a). This assessment has been shown to have good psychometric characteristics with a variety of drinker groups, and can produce variables

that provide a wide array of information about an individual's drinking. Trained clinical staff was responsible for the administration of the TLFB in which the subject recorded the total number and type of drinks consumed for the past 90-days into a computer.

6.3.2b-3 Clinical Endpoint Assessment: The CIWA-AR

A clinical endpoint characterizing the withdrawal severity during alcohol detoxification was administered to each subject. The Clinical Institute Withdrawal Assessment-Alcohol Revised (CIWA-AR) is a validated 10-item assessment tool that categorizes the severity of alcohol withdrawal based on symptoms and physical signs (Sullivan et al., 1989a). Scores of 8 points or fewer correspond to mild withdrawal, 9 to 15 points correspond to moderate withdrawal, and scores of > 15 points correspond to severe withdrawal symptoms (maximum score = 67). Initially, alcohol withdrawal assessment was done hourly or every 2-4 hours until the scores were consistently below a range of 5-7. It was also performed on an "as indicated" basis, at the discretion of the healthcare team. This assessment was used primarily to evaluate the withdrawal symptoms of the patient and to subsequently administer a benzodiazepine for alleviation of withdrawal symptoms. The CIWA-AR may be reviewed in Appendix I.

6.3.2b-4 Eligibility and Safety Procedures

Various safety procedures were conducted to evaluate the health status of the volunteer for qualification purposes and during sampling time points. The Structured Clinical Interview for Diagnostics and Statistics Manual-IV (DSM-IV, SCID-I) is a

standard clinical procedure to establish criteria for psychiatric diagnoses (First, 2002). It is a structured interview consisting of 11 modules with between 35-292 items/module that takes about 120-180 minutes. It was employed for the diagnosis of alcoholism.

Blood test panels (BTP) were used throughout the detoxification period to assess physiological functions and screen for organ damage, as well as assessment of the extent of alcohol and drug exposure, including toxicology and biomarkers. The blood tests included Complete blood count with differential, CHEM-20 Panel, thyroid screen, Lipid panel, Viral Markers Protocol Screen and Trace Mineral panel. They were performed once in the alcohol-dependent population during screening. Of importance, evaluation of liver function was performed by investigation of markers of liver function such as blood aspartate aminotransferase (AST), alanine aminotransferase (ALT), albumin, γ-glutamyl transferase (GGT) and total bilirubin. These measures were performed at admission and throughout the detoxification period.

Urine drug screens including the qualitative and Drug Profile #1 tests were administered to evaluate whether the subject was using other drugs of abuse such as benzodiazepines, barbiturates, cocaine, LSD, methamphetamines, opiates, phencyclidine, and tetrahydrocannabinol (THC). It was performed at baseline and throughout inpatient detoxification for subject characterization and to appraise whether the subject required additional treatment for other drugs of abuse besides alcohol. Subjects who had tested positive for a drug of abuse, at any time other than admission, was not included in the primary analysis.

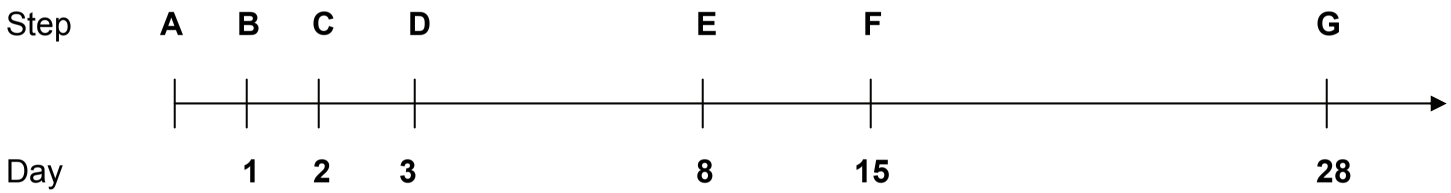
Other procedures used to screen for medical diseases and abnormalities were incorporated at baseline and throughout the study to test for abnormalities that may prevent study participation.

6.3.2b-5 Diet and Smoking

Dietary intake for each of the subjects undergoing alcohol detoxification was not based on a specific restrictive diet (e.g., low monoamine diet). The TIQ/BC food and beverage inventory was administered to assess average weekly intake of dietary SAL (Appendix D). Further discussion about the food inventory may be viewed in the preceding chapter.

Cigarette use of the subjects was carefully monitored to assess any influences of these factors on R/S-SAL levels, at baseline and throughout the study. Subject participants who were in the LS and HS groups were permitted to smoke ad-libitum throughout the inpatient period. Of importance, biological sampling was conducted pre-prandially and prior to smoking the initial cigarette of the day, with the exception of on admission day.

For all tests and procedures, a timeline and study flow chart for the NIAAA-NHP is presented below in figure 6-1 and table 6-1, respectively.



Step A: Potential subject contact with NIAAA; Interview and assess whether patient would like to participate. Informed Consent Form discussion, comprehension, and signing; Physical and psychiatric examination including DSM-IV/SCID-I and Drugs of Abuse testing; Inclusion and exclusion criteria evaluation of patient.

Step B: Admission for detoxification and stabilization at NIAAA/CRC. BrAC evaluation; Baseline sampling will be performed including: Blood biological sampling, CIWA-AR, and vital signs, urine Drugs of Abuse testing.

Step C: Day 2 after admission. BrAC evaluation; Baseline sampling will be performed including: blood biological sampling, CIWA-AR, and vital signs, urine Drugs of Abuse testing; Biological testing for biomarkers performed in the AM, *before* smoking and eating. FTND administration to volunteer for categorization into subgroup (NS, LS, HS).

Steps D-F: Days 3, 8 and 15 after admission. Same as step C. Alcohol TLFB, Food Inventory administration only once any time after sobriety.

Step H: Day 28 after admission. Stop point, end of study. Complete health examination. Option to pursue outpatient treatment.

Figure 6-1: Timeline for Subject Participation in NIAAA-NHP Study

Table 6-1: Study Flow Chart for NIAAA-NHP Observational Study

Screening	**Admission**	**Clinical Visit**	**Clinical Visit**	**Clinical Visit**	**Clinical Visit**	**Discharge**
Outpatient STEP A	Inpatient STEP B (d1)	Inpatient STEP C (d2)	Inpatient STEP D (d3)	Inpatient STEP E (d8)	Inpatient STEP F (d15)	Inpatient STEP G (d28)
ICF PE BTP, UA ECG, VS MHx / SH Drug Screen PT* FTND	BrAC VS Drug Screen CIWA-AR Blood Sample	BrAC VS Drug Screen CIWA-AR Blood sample	→	Same procedure FTND SH TLFB	→	BrAC Blood sample CIWA-AR Drug Screen Exit PE ECG, VS BTP

*For female subjects only

BrAC:	Breath Alcohol Test
BTP:	Blood Test Panel
CIWA-AR:	Clinical Institute Withdrawal Assessment-Alcohol Revised
ECG:	Electrocardiogram
FTND:	Fagerström Test for Nicotine Dependence
MHx:	Medical history
PE:	Physical Examination
SH:	Smoking History
TLFB:	Timeline Follow Back
UA:	Urinalysis
VS:	Vital Signs
ICF:	Informed Consent Form
PT:	Pregnancy Test

6.3.3 Blood Sample Analysis

The samples containing TIQ's were kept at -70°C until analysis. TIQ samples were analyzed and assayed for S-SAL, R-SAL and DA by the investigator using resources and equipment with generous permission granted from the Laboratory of Molecular Signaling, National Institute on Alcohol Abuse and Alcoholism-NIH, Rockville, MD. The assay procedure incorporated the use of 1-ml of patient plasma sample in addition to the equivalent volume of 1-ml antioxidant solution (total volume of 2-ml). Details have been described earlier (see Chapter 4).

6.3.4 Statistical Analysis

6.3.4a Descriptive statistics

Descriptive statistics, including mean, standard deviation, and coefficient of variation were calculated for each endpoint. In addition, intra-individual variability (e.g., COV%) was calculated for all measured endpoints, i.e., R/S-SAL and DA concentrations along with and CIWA-AR, for each volunteer.

In the case that assumptions of normal distribution and equal variance was not met, raw data were log-transformed to comply with the parametric assumptions of equal variance across groups and normal distribution of the residuals. Appropriate summary statistics, using the log-transformed data, such as, median, COV%, percentiles and ranges were computed.

6.3.4b Inferential statistics

The data obtained from this investigation were used to compare prespecified factors of smoking status and gender responses to detoxification on the pertinent endpoints, including exposure levels of R/S-SAL, DA and CIWA-AR scores. To evaluate the effects of time on R/S-SAL and DA concentrations and the clinical endpoint, CIWA-AR, regression analysis was conducted across all subjects. In the case that assumptions of normal distribution and equal variance was not met, raw data was log-transformed to comply with the parametric assumptions and the analysis was conducted on the log-transformed concentrations.

Inter-individual variability (i.e., COV% and range) was calculated for the above endpoints as well as all the rating scale scores and CIWA-AR for each of the three groups and across all groups via two-way ANOVA, incorporating comparison of smoking history and gender factors. Specifically, R/S-SAL individual concentrations were evaluated using the model descried in section 5.3.4b. The two-way ANOVA was performed on the grand total mean (GTM) concentrations, and concentrations obtained from admission (day 1) and day 15. GTM concentrations consisted of the mean of the individual subjects concentrations obtained across time.

Moreover, R/S-SAL and DA concentrations were correlated with the measures dietary intake (TIQ/BC Food Inventory) and alcohol consumption (TLFB) using Pearson's product-moment correlation and linear regression to evaluate if these are significant covariates that need to be implemented into the full model. Associations were considered significant with a p-value of <0.05 and a coefficient of determination >

0.2**.** Multiple covariate analysis was performed on SAL and DA exposure if we have more than one significant covariate. Exploratory analyses using these procedures were performed on clinical variables, such as liver function, to evaluate their effects on the analyte concentrations on the GTM, day 1, or day 15. The clinical endpoint, CIWA-AR, was assessed for a relationship with SAL concentrations during the first three days of patient observation.

To evaluate differences between the healthy (Clinical study #1) and alcoholic populations, an unpaired t-test was employed for all log-transformed biological endpoints between smoking status groups in each cohort. The baseline control from Clinical study #1 will be compared to the admission and discharge levels of the alcoholic population.

All endpoints were tested and compared across all groups in S-PLUS 8.0 (Insightful Corporation, Seattle, WA). The full statistical model was implemented incorporating covariates, if required. The residuals were tested for normality using Quantile-Quantile (Q-Q) plots and further tested using the Shapiro-Wilk test, where α was set to 0.05 such that any p-values > 0.05 indicated that the data was normally distributed. If the data were not normally distributed, the data were log-transformed and the full model was repeated. The level of significance was set *a-priori* at 0.05. Any statistically significant differences found via ANCOVA were further investigated via Scheffé test to isolate factor differences. All ANCOVAS performed are included in Appendix O.

6.4 Results

6.4.1 Clinical Results

6.4.1a Subject Demographics

Out of 115 subjects who were enrolled and completed the detoxification protocol, a total of thirty-six (36) subjects were chosen for the SAL investigation using the aforementioned criteria. Upon plasma concentration analysis, one subject disqualified for the study due to a positive HIV testing (subject #13). Therefore, a total of thirty-five subjects were included in the analysis. Into each smoking status group, 12 NS, 11 LS and, 12 HS were included, with seventeen females and eighteen males comprising the gender distribution within the entire population. The distribution of gender and smoking status for subjects who completed the protocol and met the selection criteria is as follows: 6 NS females, 6 NS males, 5 LS females, 6 LS males, 6 HS females and 6 HS males. Final subject demographics are included in Table 6-2. The subjects incorporated in the analysis were of a mean age of 41.1 years old, ranging from 28-58 years. No statistically significant differences in weight and age were found between smoking groups. Twenty-four Caucasians, seven African-Americans, two Hispanics and two subjects of unknown origin completed the study.

Table 6-2: Clinical Study #2 Demographics

Nonsmokers

Subject #	Race	Gender	Age (yrs)	Weight (kg)	FTND	# Cig/day
1	White	F	56	74.1	0	0
2	White	F	53	80.2	0	0
3	White	F	32	54.1	0	0
4	White	F	32	63.7	0	0
5	White	F	46	47.2	0	0
6	Black	F	40	69.8	0	0
7	Hispanic	M	36	78.7	0	0
8	Hispanic	M	31	98.9	0	0
9	White	M	41	98.8	0	0
10	Black	M	38	72.5	0	0
11	Unknown	M	46	71.8	0	0
12	White	M	48	103	0	0
Total Mean			41.6	76.1	0.0	0.0
Total SD			8.3	17.4	0.0	0.0
Mean Female			43.2	64.9	0.0	0.0
SD			10.3	12.4	0.0	0.0
Mean Male			40.0	87.3	0.0	0.0
SD			6.4	14.5	0.0	0.0

Light-smokers

Subject #	Race	Gender	Age (yrs)	Weight (kg)	FTND	# Cig/day
14	White	F	34	63.4	5	10
15	White	F	45	75.2	1	10
16	White	F	31	70.0	4	10
17	Black	F	28	99.2	2	5
18	Black	F	50	76.6	6	12.5
19	Unknown	M	41	89.5	4	10
20	White	M	58	87.9	6	10
21	White	M	45	91.2	4	6
22	White	M	29	116.1	3	10
23	Black	M	43	100.2	6	12
24	Black	M	30	75.2	5	13
Total Mean			39.5	85.9	4.2	9.9
Total SD			9.8	15.5	1.7	2.5
Mean Female			37.6	76.9	3.6	9.5
SD			9.4	13.5	2.1	2.7
Mean Male			41.0	93.4	4.7	10.2
SD			10.7	13.7	1.2	2.4

Table 6-2: Clinical Study #2 Demographics (continued)

Heavy-smokers

Subject #	Race	Gender	Age (yrs)	Weight (kg)	FTND	# Cig/day
25	White	F	33	48.8	8	20
26	White	F	57	44.1	8	20
27	White	F	40	63.1	10	20
28	White	F	40	69.6	10	50
29	White	F	35	70.0	8	20
30	Black	F	40	52.1	9	31
31	White	M	33	87.2	8	35
32	White	M	52	85.3	7	40
33	White	M	56	105.0	10	40
34	White	M	53	92.0	8	30
35	White	M	28	89.4	5	28
36	White	M	39	78.0	6	20
Total Mean			42.2	73.7	8.1	29.5
Total SD			9.9	19.1	1.6	10.1
Mean Female			40.8	58.0	8.8	26.8
SD			8.5	11.1	1.0	12.2
Mean Male			43.5	89.5	7.3	32.1
SD			11.7	9.0	1.8	7.8

Additional clinical variables were obtained from each of the subjects during admission such as information regarding liver function (AST, ALT, albumin, GGT, and total bilirubin). Information about clinical variables is supplied in table 6-3 below and divided according to smoking status (NS, LS, and HS) and gender (male or female). Clinically significant anomalies were not present amongst the subjects chosen for the analysis. Liver function tests such as AST, ALT, and GGT were all less than three-times normal values of healthy liver function. The majority of subjects possessed albumin, and total bilirubin within normal ranges.

Information regarding therapies utilized during detoxification and other drugs of abuse present in drug screen at admission was obtained is supplied in table 6-3.

Table 6-3: Admission clinical variables and other therapies incorporated throughout detoxification

Subject #	SS	GEN	ALB (g/dL)	ALT (U/L)	AST (U/L)	GGT (U/L)	T Bil (mg/dL)	ADM Drug Screen	ACAMP	other therapies
1	NS	F	4.1	21	23	28	1.4	none	N	alum hydrox/mag, pseuephedrine, NK1 antag
2	NS	F	3.9	41	42	86	0.5	none	N	dyphenhydramine
3	NS	F	3.9	17	23	16	0.9	none	N	diazepam, trazodone
4	NS	F	4.1	32	43	30	1.2	none	N	dyclonine lozenge, pseudoephedrine
5	NS	F	5.0	53	80	291	1.1	AMPHET	N	diazepam, trazodone
6	NS	F	3.6	26	31	44	0.5	none	N	HCTZ, sertraline, diazepam, diphenhydramine
7	NS	M	4.2	32	29	51	0.8	none	N	thiamine, ranitidine
8	NS	M	3.7	49	44	134	0.4	none	N	thiamine
9	NS	M	4.5	19	22	38	0.8	BZD	N	pseudoephedrine, naltraxone,
10	NS	M	4.3	122	169	188	1.9	BZD	N	diazepam, thiamine, fluoxetine, quetiapine
11	NS	M	4.0	66	272	1195	3.0	none	N	naltrexone, omeprazole
12	NS	M	3.9	40	54	113	0.9	BZD	N	diazepam, atenolol, HCTZ
14	LS	F	4.6	31	50	36	0.8	none	N	nicotine, expt drug (NK1 antag)
15	LS	F	4.5	38	30	55	0.6	none	N	venlfaxine,
16	LS	F	4.1	13	15	17	0.7	none	N	sertraline, oxycodone, nicotine
17	LS	F	4.0	101	98	148	0.8	THC	Y	diazepam, dexamethasone
18	LS	F	2.5	44	104	149	0.3	none	N	amoxicillin, HCTZ, levoflxacin
19	LS	M	4.3	40	36	14	1.0	COCAINE	N	ketoconazole, diphenhydramine,
20	LS	M	5.4	25	32	24	1.1	none	N	clonidine, enalapril
21	LS	M	3.9	29	25	49	0.6	BZD	N	thiamine
22	LS	M	4.1	131	86	119	0.6	none	Y	diazepam, cholecalciferol, diphenhydramine
23	LS	M	3.3	50	80	321	0.9	none	N	thiamine, cephalexin
24	LS	M	4.2	22	24	29	0.3	THC	Y	naltrexone, dexamethasone, cholecalciferol
25	HS	F	3.9	11	16	11	0.6	COCAINE	Y	dexamethasone
26	HS	F	3.3	51	72	216	0.9	BZD	N	folic acid, cetirizine
27	HS	F	3.9	14	21	19	0.7	COCAINE	N	fluoxetine, naltrexone, diazepam, cyclobenazeprine
28	HS	F	4.0	28	34	32	0.5	none	Y	diazepam, mag sulfate, dexamethasone, naltrexone
29	HS	F	4.8	96	108	70	0.4	none	Y	dexamethasone
30	HS	F	3.8	30	32	14	0.5	COCAINE	Y	folic acid, clonidine, HCTZ, diltiazem, fluconazole
31	HS	M	4.6	46	40	70	0.8	none	Y	dexamethasone, nicotine
32	HS	M	4.2	26	30	30	1.1	none	N	thiamine
33	HS	M	3.8	42	35	55	0.7	BZD	N	varenicline, diazepam, thiamine, HCTZ
34	HS	M	3.9	11	22	27	0.6	none	N	thiamine
35	HS	M	4.8	173	185	120	1.2	none	Y	lorazepam
36	HS	M	4.3	41	38	45	0.7	none	Y	dexamethasone, ranitidine, sertraline, trazodone, naltrexone

ACAMP: acamprosate
AMPHET: amphetamines
BZD: benzodiazepines
THC: tetrahydrocannabinol (marijuana)
HCTZ: hydrochlorothiazide
NK1 antag: neurokinin antagonist

It is important to note that 13 of 35 subjects (37%) that were evaluated showed a positive urine drug screen at admission for various drugs of abuse (ADM drug screen). Moreover, acamprosate (ACAMP) was administered to 9 of the 35 subjects (26%) accrued, with all of the subjects being smokers. All patients were administered various therapies throughout the course of detoxification, including benzodiazepines. Consideration of these clinical and therapeutic observations is necessary, as their effects on R/S-SAL concentrations have not been established.

6.4.1b Smoking, alcohol and dietary exposure variables

With respect to smoking status and frequency of cigarette smoking, figures 6-2 and 6-3 report FTND scores and mean cigarette consumption per day, respectively. Both NS males and females scored a mean of 0 (± 0 SD) on the FTND and self-reported mean number of cigarettes smoked per day. LS females scored a mean FTND of 3.6 (± 2.1 SD) while LS males scored a slightly higher value of 4.2 (± 1.7 SD). HS females scored 8.8 (± 1.0 SD) compared to HS males who scored on average 7.3 (± 1.8 SD). Of note, the FTND difference between the genders was not significantly different (two-tailed unpaired t-test, p-values > 0.073 for LS and HS status). For the number of cigarettes smoked per day, LS females and LS males had similar results with averages of both groups being 9.5 (± 2.7 SD) and 10.2 (± 2.4 SD) cigarettes smoked per day, respectively. HS subjects smoked on average a three-fold more number of cigarettes per day than the LS counterparts. In this group, male smokers smoked more cigarettes

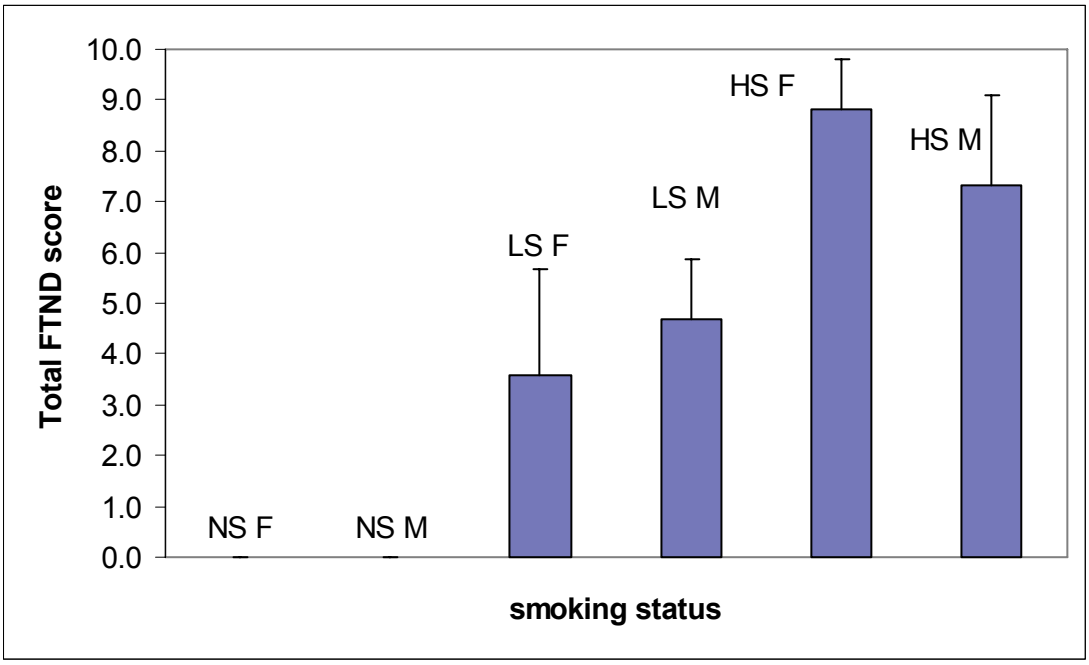


Figure 6-2: Mean (± SD) Fagerström Test for Nicotine Dependence (FTND) Score vs. Smoking Status (F: female, M: male)

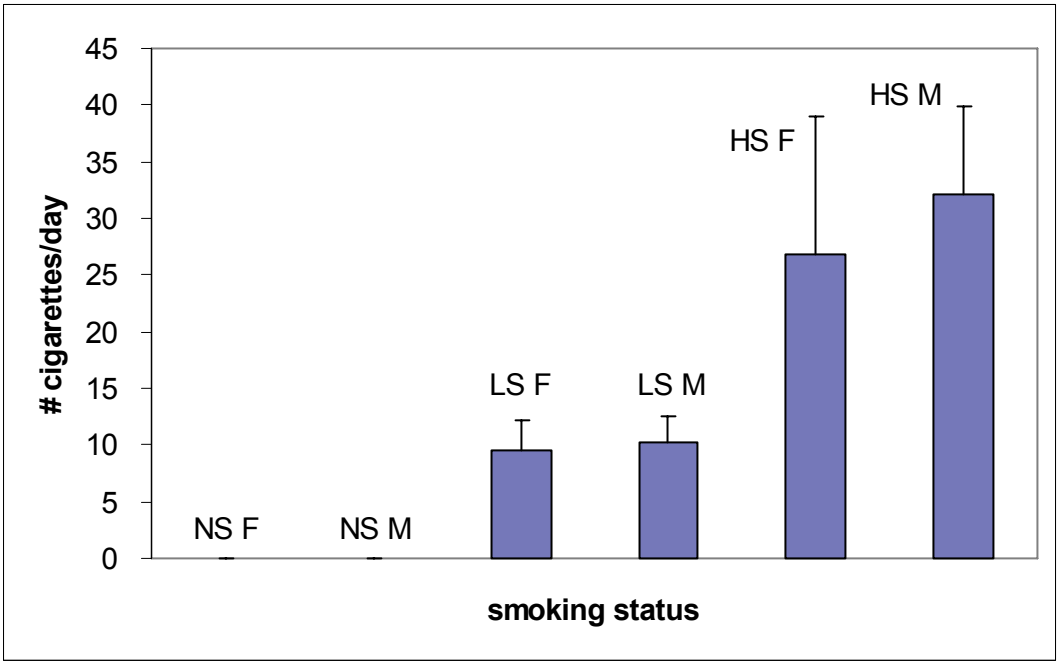


Figure 6-3: Mean (±SD) Cigarettes smoked per day vs. Smoking Status (F: female, M: male)

per day on average than that of females with an average number of cigarettes smoked per day of 32.1 (± 7.8 SD) for males and 26.8 (± 12.2 SD) for females. Although a significant difference was observed with the number of cigarettes smoked per day between smoking statuses, a gender difference within each smoking status was not detected.

In addition to the demographic and R/S-SAL and DA plasma measurements, additional information including alcohol intake and weekly exposure to dietary total SAL, was recorded via the TLFB and TIQ/BC Food Inventory. As intake of ethanol has been reported to influence the exposure of SAL enantiomers, a measurement of the past ninety (90) days of alcohol intake was reported by a validated measure of alcohol consumption, the TLFB. The TLFB assessment was administered to each subject on a single occasion during the detoxification period. The total amount of alcohol consumed, in terms number of drinks in the past 90 days, was calculated upon self-report on the number of alcoholic beverages consumed. Evaluation of the daily frequencies all different types of alcoholic beverages including, wine, beer, wine coolers, and spirits were reported by the subject and tallied to calculate the total number of drinks within the time period, prior to detoxification. Of primary note, this is a self-report measure of overall estimate of total ethanol consumption for the past three months. The raw data for the TLFB can be viewed in table 6-4 while the descriptive results can be viewed in the table 6-5 reported as total number of ethanol containing beverages consumed in the past ninety days prior to inpatient abstinence.

Breath alcohol (BrAC) was measured for each subject upon admission to the clinical center. Subjects who possessed no measureable BrAC were classified as negative (neg) while persons who yielded a positive BrAC, as defined by > 0.01 g/210L breath, were classified as positive (pos). If positive, the level of BrAC was reported for each subject. The information with respect to BrAC may be seen in the table 6-4 below

Ethanol exposure estimated by the TLFB resulted in substantial differences between genders within smoking groups and between smoking groups. Results here are reported as mean, median and range. NS males and females showed a similar median number of drinks of 657 drinks/90 days and 621 drinks/90 days, respectively. Conversely, LS and HS possessed significantly higher TLFB scores. The median number of alcoholic drinks estimated in LS males and females was 1381 drinks/90 days and 666 drinks/90 days while in HS males and females resulted in 1504 drinks/90 days (range: 610 – 2445) and 629 drinks/90 days (range: 75 – 1890), respectively. In both smoking groups, males drank more alcoholic beverages in the past ninety days compared to females. Nonsmokers had a median of 617 drinks/90 days (range: 105 – 1819) while the estimated LS and HS alcoholic beverage intake was 1112 drinks/90 days (range: 167 – 2191) and 1134 (range: 75 – 2445).

A systematic trend was observed between smoking groups and gender for the total amount of alcoholic beverages consumed per last 90 days. On average, females had a lower estimated intake compared to males as a whole and across smoking groups. Smoking status showed a significant trend with a resultant increase in estimated alcoholic beverage consumption with increasing level of smoking status. Formal

Table 6-4: Individual reports for TLFB and % BrAC on admission

Subject #	SS	GEN	TLFB (90 days)	BrAC	BrAC level (%)
1	NS	F	655.4	neg	0.00
2	NS	F	579	neg	0.00
3	NS	F	105	neg	0.00
4	NS	F	361	neg	0.00
5	NS	F	731	pos	0.29
6	NS	F	1295	pos	0.08
7	NS	M	385.3	neg	0.00
8	NS	M	337.7	neg	0.00
9	NS	M	327	neg	0.00
10	NS	M	1819	neg	0.00
11	NS	M	930	neg	0.00
12	NS	M	1268	neg	0.00
14	LS	F	666.6	neg	0.00
15	LS	F	167	neg	0.00
16	LS	F	242	neg	0.00
17	LS	F	837	pos	0.11
18	LS	F	2191	pos	0.31
19	LS	M	1272	neg	0.00
20	LS	M	1122	neg	0.00
21	LS	M	683	neg	0.00
22	LS	M	1818.5	pos	0.30
23	LS	M	2011	pos	0.10
24	LS	M	1491	pos	0.10
25	HS	F	75	neg	0.00
26	HS	F	366	neg	0.00
27	HS	F	354	neg	0.00
28	HS	F	1560	pos	0.48
29	HS	F	891	pos	0.15
30	HS	F	1890	pos	0.07
31	HS	M	1104.8	neg	0.00
32	HS	M	1164	neg	0.00
33	HS	M	1705	neg	0.00
34	HS	M	610	pos	0.05
35	HS	M	1999	pos	0.18
36	HS	M	2445	pos	0.06

pos : positive BrAC
neg : negative BrAC
% : g/210L of breath

evaluation of TLFB as a significant covariate will be explored further as the history alcohol intake prior to detoxification may affect the exposure of plasma SAL.

With respect to admission BrAC, 13 out of 35 subjects (37%) showed a positive measureable breath alcohol. While two of these subjects were part of the NS smoking status group, 5 subjects were part of LS and 6 subjects out of HS groups. If a significant effect of positive BrAC was present with respect to admission R/S-SAL concentrations, further analysis was employed for correlation between the numeric value obtained vs. SAL concentration. Formal evaluation of BrAC as a significant covariate was also explored.

Table 6-5: Descriptive results for the TLFB measurement by SS and GEN

			Non-smoker	Light-smoker	Heavy-smoker	GRAND
TLFB (# drinks in past 90 days)	Male	Mean	844	1399	1434	1249
		Median	657	1381	1504	1216
		Range	327 - 1819	683 - 2011	610 - 2445	327 - 2445
	Female	Mean	617	820	856	762
		Median	621	666	629	655
		Range	105 - 1295	167 - 2191	75 - 1890	75 - 2191
	Overall	Mean	732	1136	1180	1013
		Median	617	1122	1134	891
		Range	105 - 1819	167 - 2191	75 - 2445	75 - 2445
		%COV	69.5	60.4	63.4	66.2

In regard to dietary exposure of SAL, the food survey incorporated measurements of type of food, average frequency and average portion size of each known food source to have significant quantities of total SAL. Similar calculations used in the Clinical study #1 were used to assess the average nanograms of weekly total

SAL consumed from dietary intake (see section 5.4.1b). The amount of dietary intake of these SAL containing food and beverages were not reported for the foods consumed during the detoxification period. The descriptive results of the TIQ/BC Food Inventory can be viewed table 6-6 below in average nanograms of average weekly total SAL consumed.

Table 6-6: Descriptive results for the dietary total SAL by SS and GEN

			Non-smoker	Light-smoker	Heavy-smoker	GRAND
Dietary total SAL (ng/week)	Male	Mean	3112	2977	3769	3286
		Median	2871	3099	2563	2844
		Range	1134 - 4520	1996 - 4005	1004 - 4296	1004 - 4520
	Female	Mean	3098	1872	2444	2471
		Median	4562	2031	2671	3088
		Range	2091 - 5029	1032 - 5136	1562 - 4010	1032 - 5136
	Overall	Mean	3291	2544	2736	2857
		Median	3455	2781	2620	2952
		Range	1134 - 5209	1032 - 5136	1004 - 4296	1004 - 5209
		%COV	75.2	85.4	56.7	71.8

With the exception of the NS status group, a gender difference was not observed within smoking status groups. Overall, the NS group possessed a higher median intake of dietary SAL (3455 ng/week) than that of the LS (2781 ng/week) and HS (2620 ng/week) groups. The variability (%COV) within each smoking status group was > 56%. As dietary intake of SAL containing foods may affect the overall exposure, the food inventory measurement may provide clues into the variability associated with the plasma measurement of enantiomeric SAL. If dietary exposure was found to significantly correlate with SAL exposure, average nanograms of total dietary SAL per

week was considered as a significant covariate and implemented into the statistical model.

6.4.2 Primary Analysis for R/S-SAL and DA – Effects of Smoking and Gender

6.4.2a R/S-SAL and DA – Within Subject Variability

Preceding the statistical assessment of the effects of time, and the factors of smoking status and gender on R/S-SAL and DA concentrations, within subject variability was evaluated. This analysis was conducted to assess the fluctuation of concentrations over fifteen days of early alcohol abstinence. The intra-subject variability (median, inter-quartile ranges, %COV) for S-SAL, R-SAL and DA can be seen in the tables 6-7, 6-8 and 6-9 below.

Table 6-7: Within Subject Variability for Plasma S-SAL (pg/ml), n =5 timepoints.

Subject #	GEN	S-SAL avg	stdev	%COV	median	25% IQR	75% IQR
Nonsmokers							
1	F	236	146	62	263	113	321
2	F	290	451	156	145	31	164
3	F	181	196	108	93	74	199
4	F	77	40	53	61	52	105
5	F	117	78	67	95	79	181
6	F	207	178	86	153	134	211
7	M	30	11	37	30	20	38
8	M	136	237	174	36	26	38
9	M	146	90	61	189	61	216
10	M	86	120	139	8	5	141
11	M	20	28	143	9	7	11
12	M	75	34	46	72	48	105
Light Smokers							
14	F	108	122	113	92	17	104
15	F	35	18	52	26	22	50
16	F	217	196	90	144	114	154
17	F	40	49	120	33	6	40
18	F	31	43	136	14	5	26
19	M	68	29	43	55	51	72
20	M	71	102	144	24	18	46
21	M	22	16	72	24	8	31
22	M	136	145	107	85	26	179
23	M	31	13	41	28	26	30
24	M	90	82	91	79	26	106
Heavy Smokers							
25	F	38	21	55	31	30	41
26	F	73	53	73	40	39	124
27	F	189	222	117	102	82	147
28	F	313	443	142	113	71	217
29	F	40	18	44	38	34	43
30	F	50	27	54	41	39	43
31	M	135	191	141	65	29	91
32	M	111	87	79	61	48	169
33	M	58	7	12	61	51	61
34	M	287	368	128	175	14	337
35	M	128	203	158	32	18	90
36	M	92	74	80	59	37	125

Table 6-8: Within Subject Variability for Plasma R-SAL (pg/ml), n =5 timepoints.

Subject #	GEN	R-SAL avg	stdev	%COV	median	25% IQR	75% IQR
Nonsmokers							
1	F	322	266	83	303	155	322
2	F	594	1007	169	243	45	274
3	F	185	257	139	70	40	216
4	F	112	81	72	76	74	198
5	F	181	137	76	133	103	322
6	F	285	258	90	242	174	297
7	M	27	16	58	30	15	31
8	M	200	382	191	30	26	47
9	M	218	141	65	293	96	311
10	M	123	169	137	12	5	217
11	M	27	51	189	4	3	10
12	M	129	67	52	125	71	170
Light Smokers							
14	F	423	589	139	146	21	524
15	F	42	25	60	29	22	66
16	F	313	309	99	166	158	238
17	F	69	76	111	65	17	67
18	F	57	74	129	27	10	57
19	M	95	48	51	77	69	103
20	M	105	150	142	25	22	92
21	M	28	25	87	27	10	35
22	M	241	268	111	150	50	313
23	M	16	15	89	11	9	18
24	M	113	119	105	88	23	119
Heavy Smokers							
25	F	77	49	64	64	48	96
26	F	69	55	80	51	30	99
27	F	226	281	125	120	96	157
28	F	567	919	162	168	85	300
29	F	42	27	64	33	24	46
30	F	49	33	68	36	35	42
31	M	187	284	152	95	36	107
32	M	143	128	89	77	50	268
33	M	67	27	41	60	45	78
34	M	452	641	142	240	11	456
35	M	178	318	179	32	9	98
36	M	98	102	105	45	30	150

Table 6-9: Within Subject Variability for Plasma DA (ng/ml), n =5 timepoints.

Subject #	GEN	DA avg	stdev	%COV	median	25% IQR	75% IQR
Nonsmokers							
1	F	8.3	1.5	19	8.3	8.2	9.3
2	F	10.3	6.4	61	8.1	6.1	12.7
3	F	6.9	2.0	29	6.5	5.5	7.9
4	F	6.7	0.9	14	6.7	5.7	7.4
5	F	3.7	1.7	46	3.0	2.9	3.7
6	F	9.8	2.8	28	10.6	8.4	11.5
7	M	3.0	0.5	18	2.8	2.6	3.5
8	M	2.8	1.5	53	3.1	2.8	3.3
9	M	5.5	0.9	17	5.3	5.2	6.2
10	M	3.2	1.3	42	2.8	2.6	3.5
11	M	3.4	0.9	27	3.5	2.5	4.0
12	M	7.0	1.2	18	6.3	6.3	7.8
Light Smokers							
14	F	4.8	2.2	46	4.2	3.6	4.4
15	F	5.5	1.3	24	6.2	4.2	6.4
16	F	4.9	1.0	21	4.6	4.3	5.1
17	F	2.4	1.2	52	2.1	1.4	3.3
18	F	3.2	0.9	29	2.8	2.6	3.8
19	M	3.2	0.5	15	3.2	2.8	3.6
20	M	4.2	1.2	29	3.7	3.6	4.4
21	M	5.4	1.1	21	5.2	5.1	5.4
22	M	5.6	3.4	60	4.5	3.7	5.7
23	M	3.8	1.3	35	3.4	2.9	4.2
24	M	8.2	2.2	26	8.4	6.4	9.1
Heavy Smokers							
25	F	5.2	0.8	16	5.0	5.0	5.8
26	F	5.4	2.0	38	4.2	4.1	5.8
27	F	5.4	2.7	49	4.4	3.3	6.5
28	F	20.1	24.6	122	7.9	7.5	14.6
29	F	5.2	1.8	35	5.4	4.4	5.8
30	F	7.3	1.0	14	7.4	6.7	8.1
31	M	4.3	2.3	52	3.7	3.4	4.1
32	M	7.8	4.6	59	5.7	5.1	8.4
33	M	5.2	2.0	37	5.3	3.7	6.5
34	M	15.9	12.6	79	16.6	4.9	27.8
35	M	11.9	13.7	115	5.9	4.9	8.0
36	M	5.7	3.2	56	4.5	4.3	5.7

For S-SAL and R-SAL concentrations, it is apparent that the within-subject variability was large ranging from a %COV 12 to 174% for S-SAL and %COV 41 to 191% for R-SAL, suggesting that concentrations of these analytes fluctuate throughout detoxification. For non-smokers, %COV ranged from 37 – 174% for S-SAL and 52 – 191% for R-SAL. For the LS population, a range of %COV's of 41 – 144% for S-SAL and 51 – 142% for R-SAL were observed, while the HS population possessed %COV 12 – 158% and %COV 41 – 179% for S-SAL and R-SAL, respectively. As the subjects who were smokers were allowed to smoke ad-libitum throughout the detoxification period, it was expected that the intra-individual variability within the LS and HS groups would be larger than that of NS. However, this observation was not apparent from the analysis, as the intra-subject variability was not affected by smoking status or gender. In other words, the variability associated with the measurements was large, regardless of smoking status or gender.

In the case of DA concentrations within-subject variability was relatively smaller compared to that of the SAL enantiomers. DA concentrations ranged from %COV 14 to 122%, suggesting that concentrations of DA are more stable throughout detoxification. For non-smokers %COV ranged from 14 – 53%, while for the LS and HS populations, a range of %COV's of 15 – 60% and 14 – 122% were observed, respectively. An interesting note is that the HS populations possessed larger intra-subject variability than that of the LS and NS smoking status groups. An effect of gender was not observed with DA concentrations.

As biological sampling of the analytes were pre-prandial and before the initial cigarette of the day, it is hypothesized that the intra-subject variability is not due to consumption of SAL containing foods or from acute inhalation of SAL precursors. Rather, the large fluctuation in R/S-SAL concentrations may be a consequence of the physiological effects of alcohol detoxification upon early abstinence. Moreover, several factors such as pharmacological therapies and alcohol exposure and/or other drugs of abuse used prior to admission may contribute to the variability. These variables will be formally addressed in the subsequent analyses within this chapter. An interesting observation was that the SAL precursor, DA, fluctuated less over sampling periods for each subject, relative to R/S-SAL. This is suggestive that, regardless of the DA precursor, the R/S-SAL concentrations between sampling occasions vary and may be a consequence of different endogenous sources/precursors or from exogenous contribution to overall exposure. Moreover, the biosynthetic pathway of SAL may be affected via pathophysiological effects during alcohol detoxification.

6.4.2b Effect of time on R/S-SAL and DA concentrations

Prior to evaluation of time and the major study design factors of smoking status and gender, correlation analysis was performed between S-SAL, R-SAL and DA. Of note, in order to meet the assumptions of normal distribution and equal variance across smoking groups, all data were log-transformed. Separate evaluation of the effects of time, smoking-status and gender were performed on both SAL enantiomers and DA. Although the correlation analysis between the analytes suggest that the association

between the two SAL enantiomers is very strong (r = 0.933), separate statistical analyses for the two enantiomers was conducted. In addition, associations between DA and SAL enantiomers were relatively weak with DA vs. S-SAL possessing a correlation coefficient of 0.390 and DA vs. R-SAL was 0.352. Correlation results from this study were similar to those seen in Clinical study #1. A matrix evaluating the correlation can be seen in Figure 6-4 below.

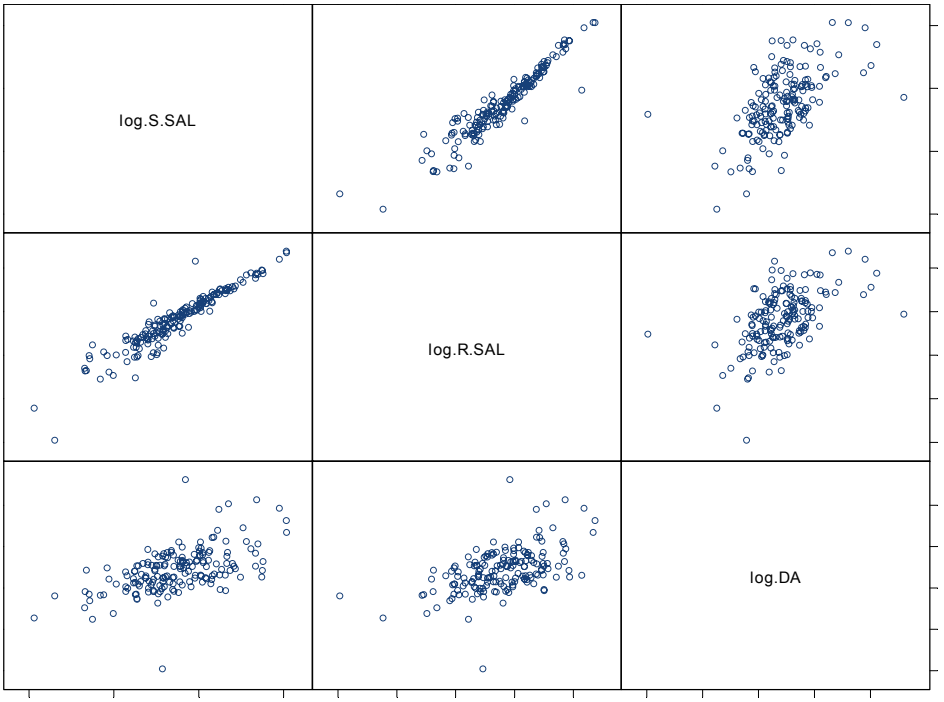


Figure 6-4: Correlation between log S-SAL vs. log R-SAL (r = 0.933), log DA vs. log S-SAL (r = 0.390) and log DA vs. log R-SAL (r = 0.352).

As it was expected that time would be a major influence on the concentration of the SAL enantiomers, statistical evaluation was conducted on the analytes over the detoxification period. In figures 6-5, 6-6, and 6-7, spaghetti plots of concentration vs.

time for S-SAL, R-SAL, and DA, respectively, are presented for all subjects. Plots are depicted on a logarithmic scale with average concentrations, for each time point, denoted by the solid line. Individual plots of concentration vs. time may be viewed in Appendix M.

Upon visual inspection of the plots on logarithmic scale, the concentrations of S-SAL and R-SAL within and between subjects were quite variable. Baseline admission concentrations varied approximately 1000-fold between patients for both SAL enantiomers. A well defined, systematic trend in concentrations is not observed within subjects with concentrations fluctuating throughout the detoxification period. Moreover, the trend of concentration vs. time profiles for both enantiomers reveals that subjects mat either increase or decrease in concentration over the sampling period. At each time point, at least a 100-fold variability in R/S-SAL concentration is observed between subjects.

In the case of the DA precursor, concentrations within- and between-subjects was less variable. Moreover, the concentration time profiles are relatively stable throughout the detoxification period. The majority of the inter-individual variability is observed during the baseline and approximately a 10-fold difference is seen between subjects at all time points.

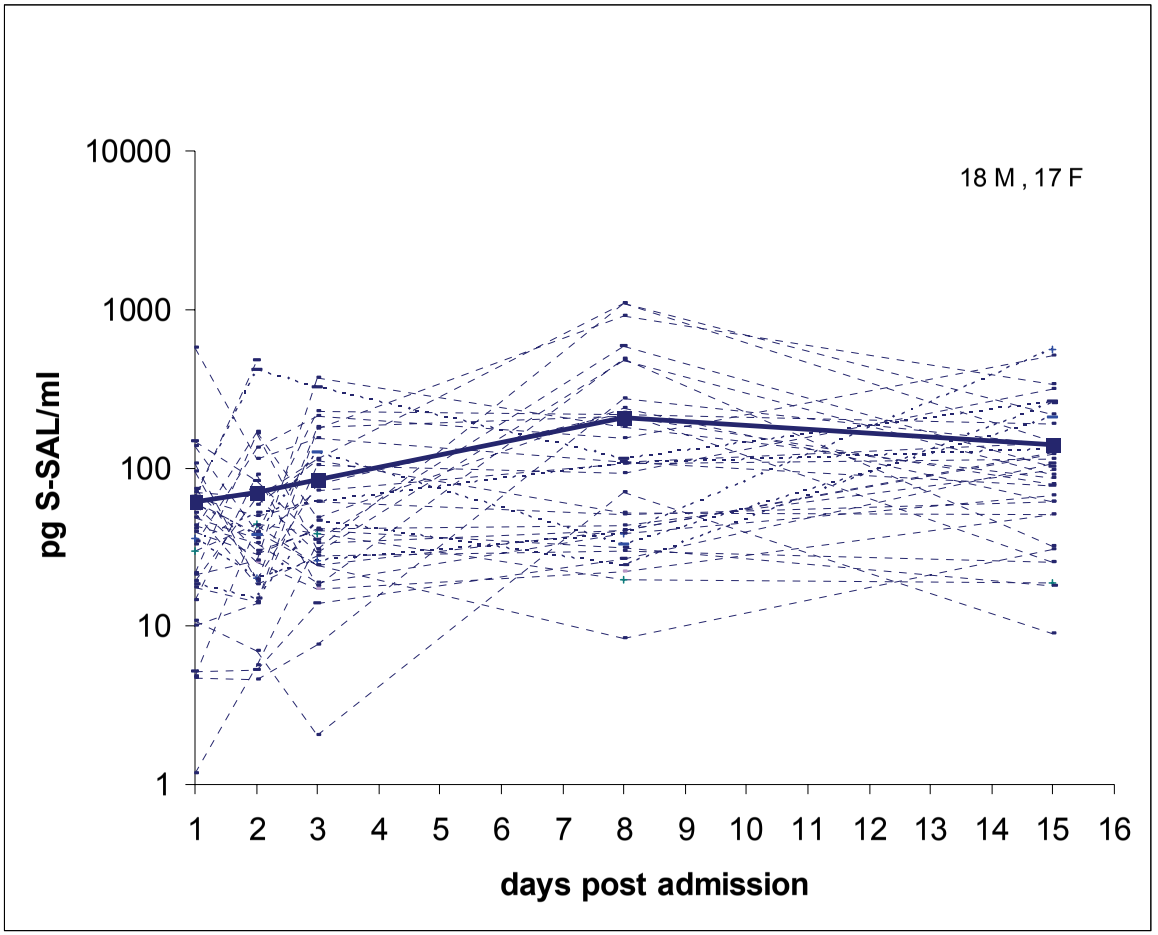


Figure 6-5: Plot of S-SAL over time on logarithmic scale for all subjects (pg/ml). Dotted lines represent individual profiles, while the solid line represents average across subjects for the time-point.

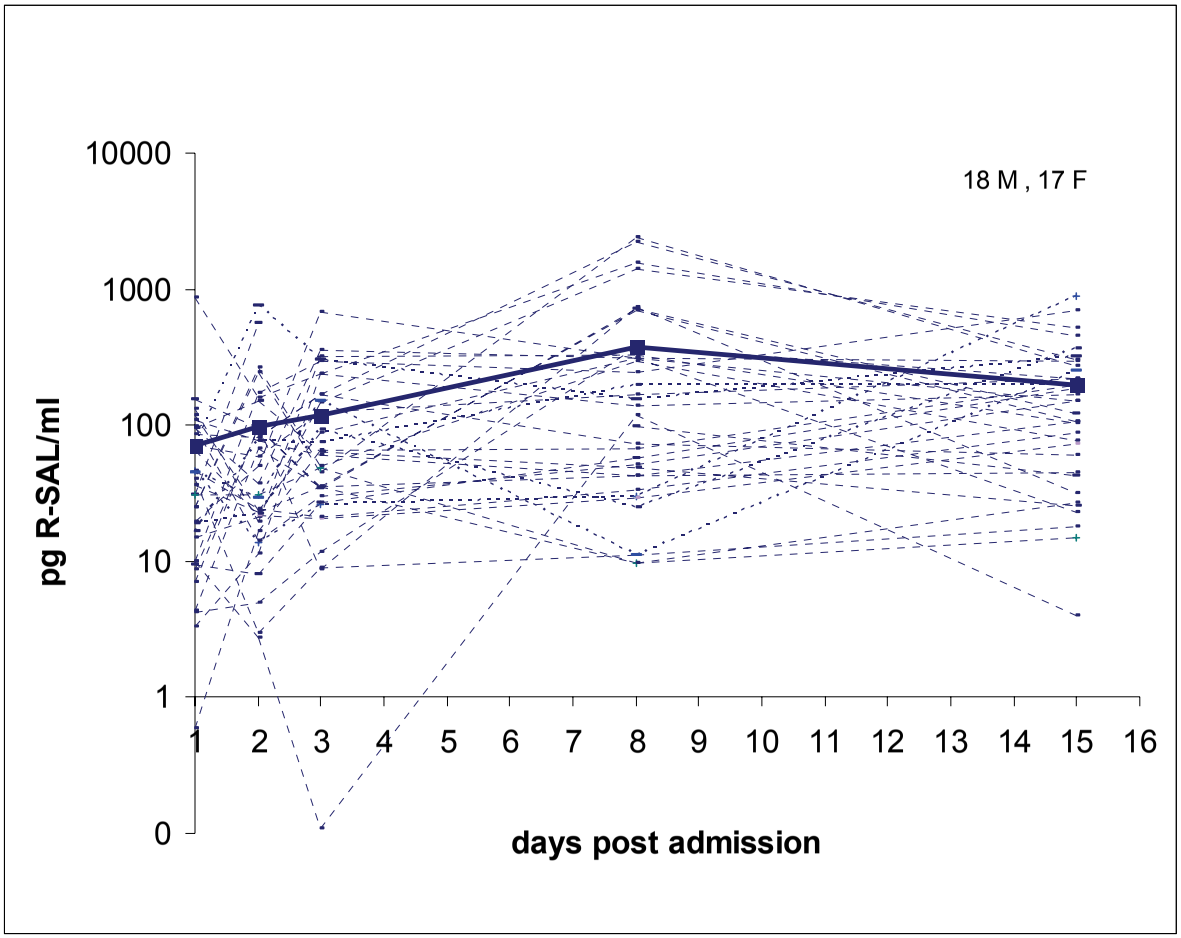


Figure 6-6: Plot of R-SAL over time on logarithmic scale for all subjects (pg/ml). Dotted lines represent individual profiles, while the solid line represents average across subjects for the time-point.

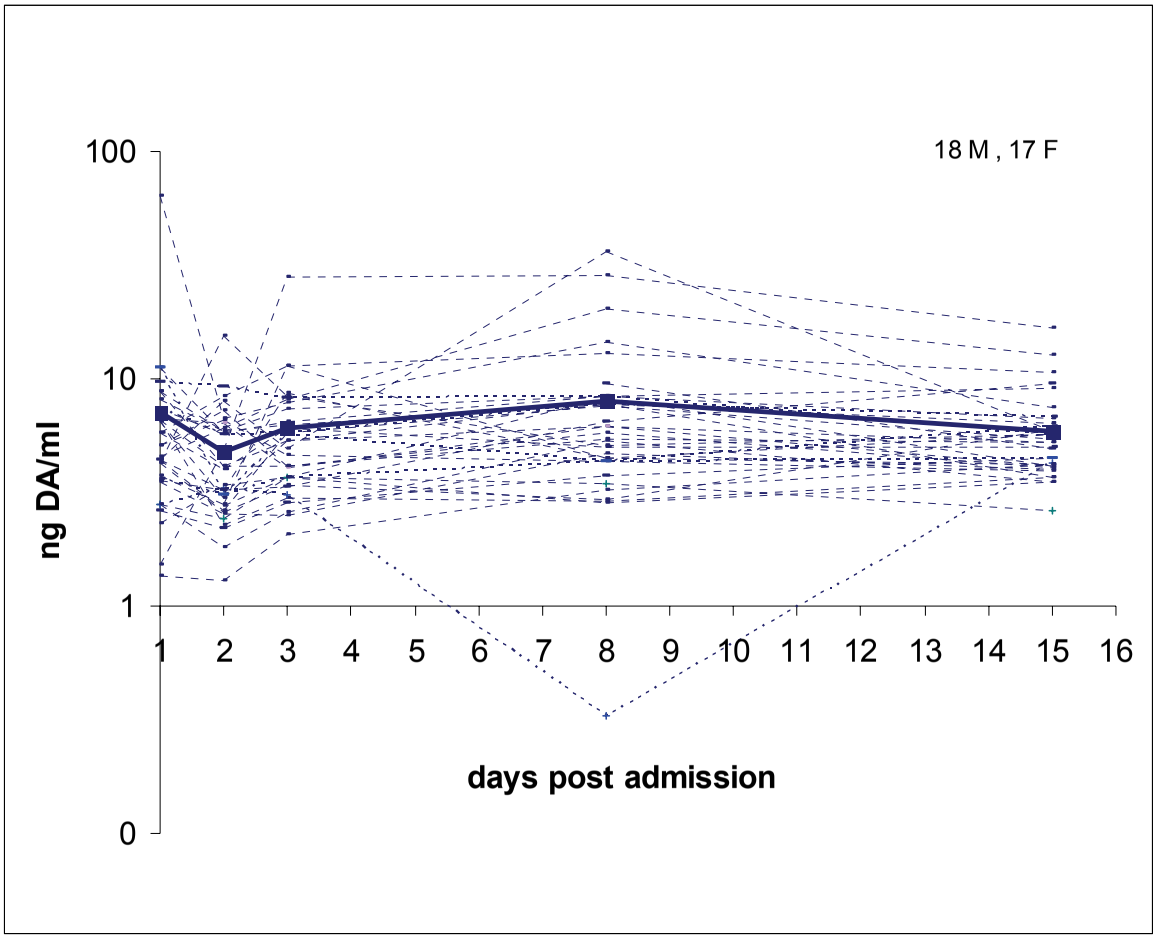


Figure 6-7: Plot of DA over time on logarithmic scale for all subjects (ng/ml). Dotted lines represent individual profiles, while the solid line represents average across subjects for the time-point.

Regression analysis was performed on all subjects to evaluate the effects of time on log S-SAL, log R-SAL and log DA concentrations over the detoxification period. Furthermore, the factors of SS and GEN were incorporated in the full model to assess whether the design variables have an influence on the time course of the log analyte concentrations. If the effects were considered insignificant, exploratory analyses involving individual time points, including admission day, day 15 and GTM, were assessed for design factor effects. Statistical data are presented as F test for the regression (*df* for factors, *df* for residuals = F-statistic, p-value).

A statistically significant effect of time was observed with respect to log S-SAL concentrations with F (1, 170) = 21.7, p-value = 6.3×10^{-6}. The coefficient of determination (R^2) for the entire model was 0.113. Thus, the factor of time accounts for approximately 11.3% of the variability associated with the log S-SAL concentration measurements. On average, a slight increase in log S-SAL concentrations was observed throughout the detoxification period across all subjects. For every day spent abstinent from alcohol in the clinical center, an average increase of 0.033 log S-SAL concentration was observed from admission day to day 15.

The full linear model output for log S-SAL can be seen in figure 6-8 below along with the residual plot of the model fit. Upon visual evaluation, the residuals show an even spread of the distribution of data suggesting the lack of unequal variance.

```
*** Linear Model ***

Call: lm(formula = log.S.SAL ~ Day, data =
NIAAA.d.14.SAL.DA.time.assess.041108,
       na.action = na.exclude)

Coefficients:
              Value Std. Error t value Pr(>|t|)
(Intercept)  1.5513  0.0563    27.5628  0.0000
        Day  0.0334  0.0072     4.6606  0.0000

Multiple R-Squared: 0.1133
```

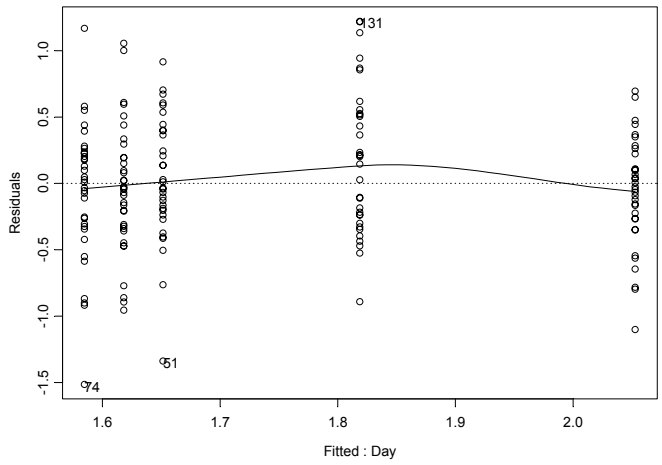


Figure 6-8: Regression output for log S-SAL concentrations for all subjects as a factor of time along with the residual plot of the model fit.

With regard to R-SAL concentrations, a statistically significant effect of time was observed with the model fit resulting in F (1, 170) = 19.2, p-value = 2.1 x 10^{-5}. The R^2 for the entire model was 0.101. Thus, the factor of time accounts for approximately 10.1% of the variability associated with the log R-SAL concentration measurements. On average, a slight increase in log R-SAL concentrations was observed throughout the detoxification period across all subjects. For every day spent abstinent from alcohol in the clinical center, an average increase of 0.04 log R-SAL

concentration was observed from admission day to day 15. The full linear model output for Log R-SAL can be seen in figure 6-9 below along with the residual plot of the model fit. As in the case of log S-SAL, the residuals for log R-SAL show an even spread of the distribution of data suggesting the lack of unequal variance.

```
*** Linear Model ***

Call: lm(formula = log.R.SAL ~ Day, data =
NIAAA.d.14.SAL.DA.time.assess.041108,
       na.action = na.exclude)

Coefficients:
              Value Std. Error t value Pr(>|t|)
(Intercept)  1.5674  0.0717    21.8645  0.0000
        Day  0.0400  0.0091     4.3792  0.0000

Multiple R-Squared: 0.1014
```

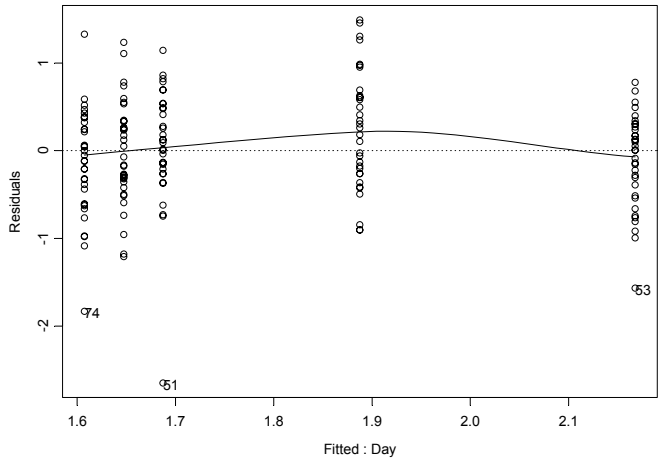


Figure 6-9: Regression output for log R-SAL concentrations for all subjects as a factor of time along with the residual plot of the model fit.

Converse to log S- and R-SAL concentrations, a significant effect of time was not observed with log DA concentrations across all subjects throughout the detoxification period (F (1, 170) = 1.6, p-value = 0.208). The R^2 for the entire model was 0.009. For every day spent abstinent from alcohol in the clinical center, an average of 0.68 log DA concentration was observed from admission day to day 15. The full linear model output for log DA can be seen in figure 6-10 below along with the residual plot of the model fit.

```
*** Linear Model ***

Call: lm(formula = log.DA ~ Day, data = NIAAA.d.14.SAL.DA.time.assess.041108,
       na.action = na.exclude)

Coefficients:
             Value Std. Error t value Pr(>|t|)
(Intercept)  0.6834  0.0309    22.0835  0.0000
        Day  0.0050  0.0039     1.2625  0.2085

Multiple R-Squared: 0.009289
```

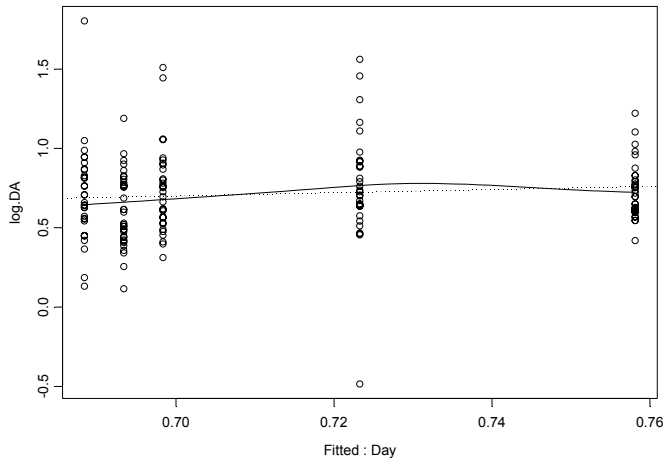


Figure 6-10: Regression output for log DA concentrations for all subjects as a factor of time along with the residual plot of the model fit.

In essence, a significant effect of time was observed for the R- and S-SAL enantiomers while DA concentrations were stable throughout the detoxification period. On average across all subjects, a slight increase in concentrations was observed from day 1 to day 15, although time was only able to explain 11.3% and 10.1% of the variability associated with log S-SAL and log R-SAL concentrations, respectively. Of note, regression analysis on the individual concentration–time profiles for the log R-SAL and S-SAL analytes yielded significant effects of time with p-values for all subjects < 0.046. The direction of the trend-in-time effect varied between subjects with increase and decreases being seen in concentrations over the detoxification period. Further assessment of the variability associated with time was further scrutinized upon evaluation of concentrations between different smoking status and gender groups.

6.4.2c Effect of Smoking Status and Gender on R/S-SAL and DA concentrations

The average concentration vs. time plots for S-SAL, R-SAL and DA for smoking status and gender are shown in Figures 6-11 through 6-16 below. The plots are depicted as average concentrations within each factor group (smoking status or gender) with corresponding standard deviation for each time point. Individual concentration-time profiles may be viewed in Appendix M.

With respect to S-SAL and R-SAL concentrations, a systematic trend (i.e., increase or decrease) in concentrations was not observed within and between each smoking status group. Regardless of smoking status, concentrations of S-SAL fluctuate with all subjects over the detoxification period.

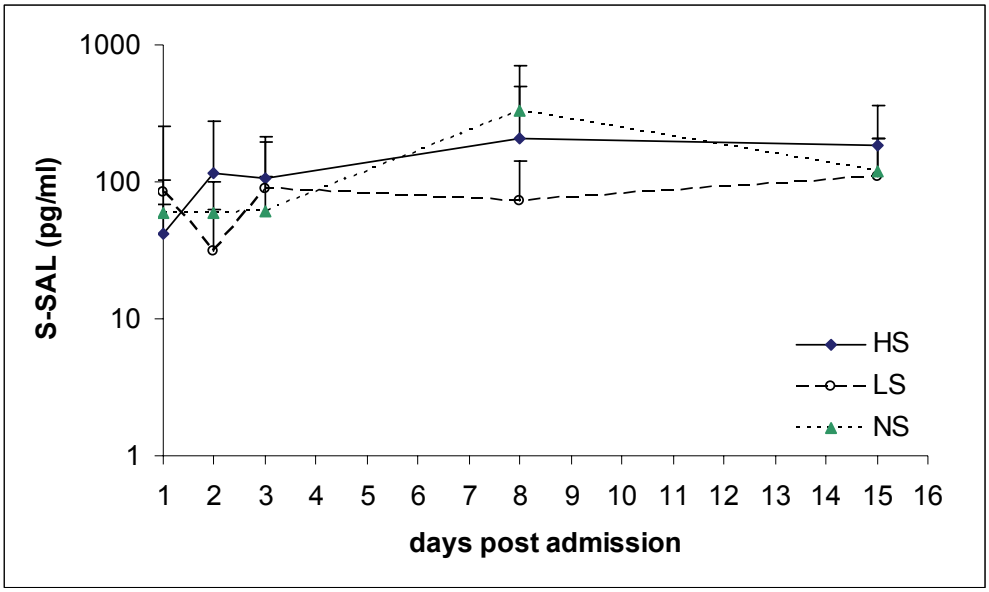


Figure 6-11: Plot of S-SAL over time on logarithmic scale for subjects within each smoking status group (pg/ml). Each time point represents an average concentration within each group with corresponding + SD.

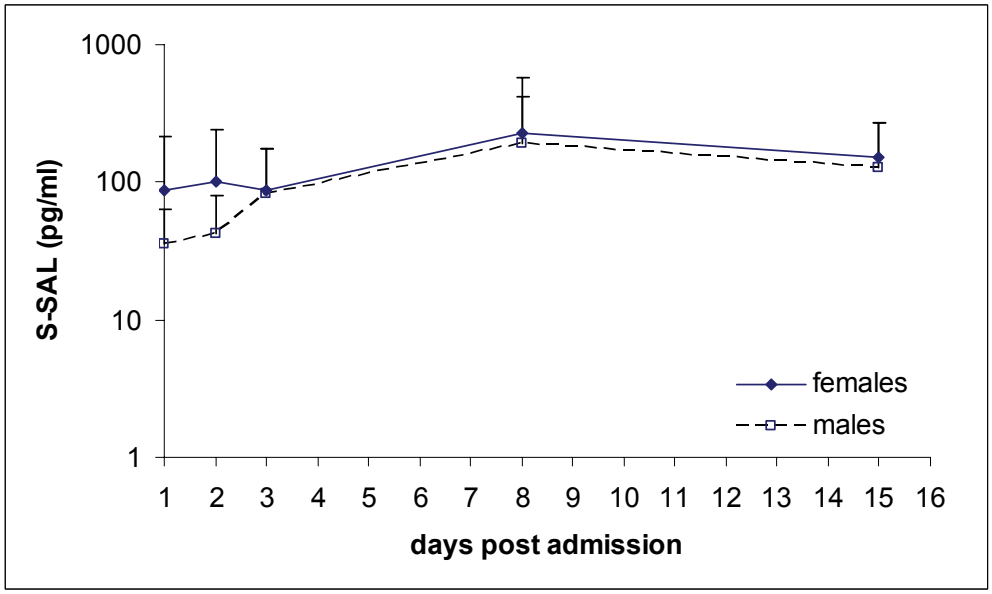


Figure 6-12: Plot of S-SAL over time on logarithmic scale for subjects within each gender group (pg/ml). Each time point represents an average concentration within each group with corresponding + SD.

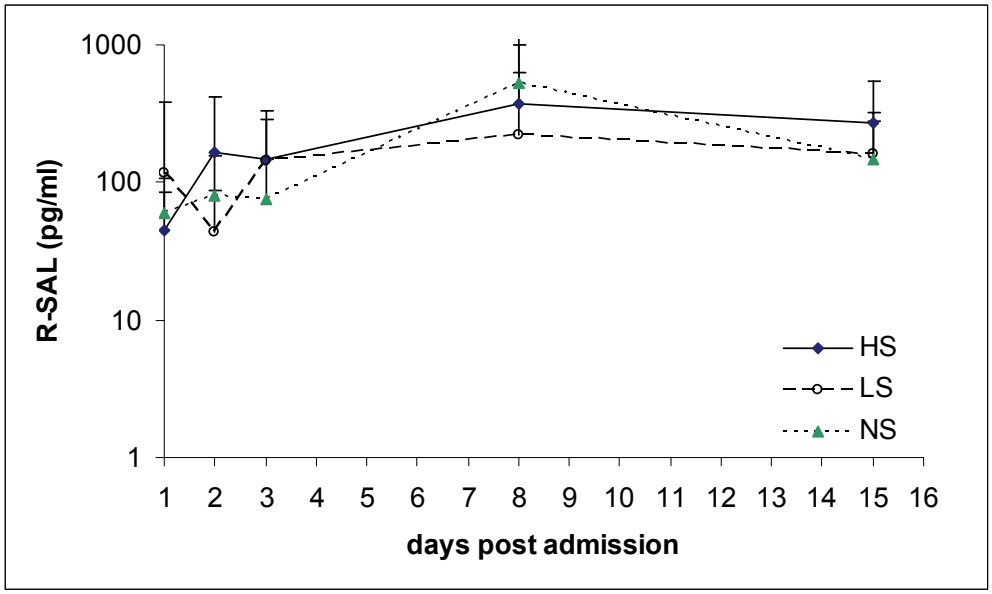


Figure 6-13: Plot of R-SAL over time on logarithmic scale for subjects within each smoking status group (pg/ml). Each time point represents an average concentration within each group with corresponding + SD.

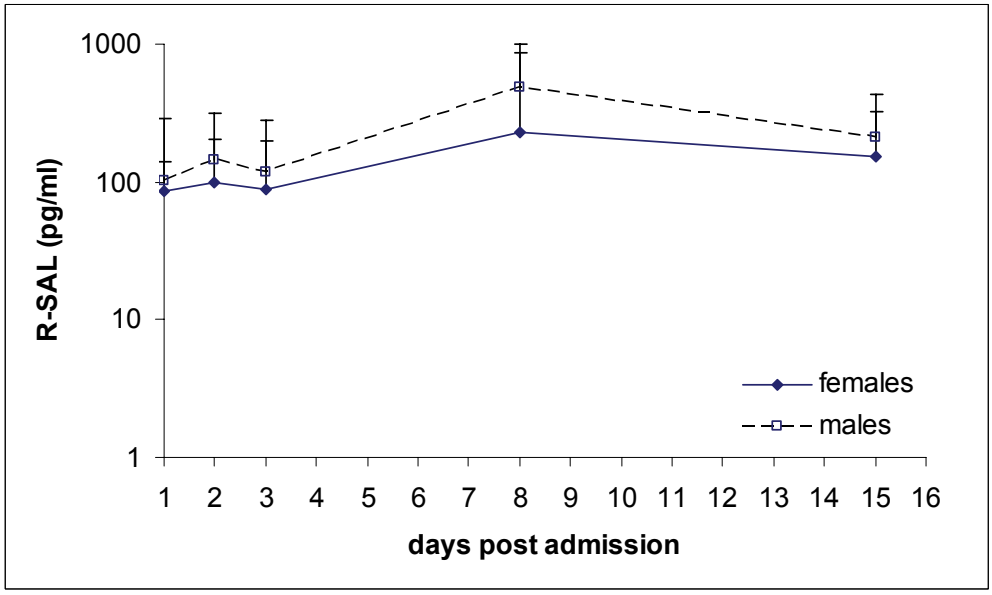


Figure 6-14: Plot of R-SAL over time on logarithmic scale for subjects within each gender group (pg/ml). Each time point represents an average concentration within each group with corresponding + SD.

Incorporation of the factors of SS and GEN into the full statistical model found insignificant effects of both factors on the time course of log S-SAL. Although a significant effect of time was observed, neither SS nor GEN had an influence on the time-course of log S-SAL. Moreover an interaction was not observed between time and SS or GEN. The full statistical model output for the regression analysis may be shown in figure 6-15 below. The factors of time, SS and GEN were able to account for 13.1% of the variability associated with log S-SAL concentrations over the detoxification period.

```
Call:
   lm(formula = log.S.SAL ~ Day + SS + GEN + Day:SS + Day:GEN, data =
       NIAAA.d.14.SAL.DA.time.assess.041108, na.action = na.exclude)

  Coefficients:
               Value Std. Error  t value Pr(>|t|)
(Intercept)   1.5718   0.1571    10.0037   0.0000
        Day   0.0241   0.0202    21.1908   0.0000
         SS  -0.0478   0.0680    -0.7039   0.4825
        GEN   0.1535   0.1129     1.3604   0.1756
     Day:SS   0.0060   0.0087     0.6909   0.4906
    Day:GEN  -0.0050   0.0144    -0.3477   0.7285

Residual standard error: 0.4915 on 166 degrees of freedom
Multiple R-Squared: 0.1312
F-statistic: 5.015 on 5 and 166 degrees of freedom, the p-value is 0.0002637
```

Figure 6-15: Regression output for the factors of SS, GEN and time on log S-SAL concentrations throughout detoxification.

The rank order of average concentrations between each smoking status group are not consistent throughout time. For instance, at day 2 of detoxification the rank of HS > NS > LS was observed for the average S-SAL concentrations while the rank changes the next day (day 3) to HS > LS > NS. Upon observation of the average profiles of both SAL enantiomers for each smoking status group, the variability associated with each time point appears to make the effect of smoking status indistinguishable.

Interestingly, a difference of gender was observed upon visual inspection of the profiles (figure 6-12). For S-SAL, females had on average a higher concentration throughout the detoxification period. The difference was more pronounced on admission day and day 2 of detoxification where a 20-fold difference was observed for the average S-SAL concentrations. Including and subsequent to day 3, average S-SAL concentrations were similar between genders. Both genders showed a parallel concentration time profile during the detoxification period, with the exception of the first two days.

Conversely, the concentration disparity for average R-SAL concentrations was observed in an opposite manner with respect to gender (figure 6-14). Throughout detoxification, males showed, on average, higher R-SAL concentrations compared to their female counterparts. At each time point, males had at least a five-fold higher average R-SAL concentration than females. The concentration-time profiles between genders showed analogous trends for average R-SAL concentrations.

Incorporation of the factors of SS and GEN into the full statistical model found insignificant effects of SS and GEN on the time course of log R-SAL. A significant effect of time was observed with the test statistic for the factor of time yielding F (1, 166) = 19.4, p-value = 1.8×10^{-5}. An interaction was not observed between time and SS or GEN. The full statistical model output for the regression analysis may be shown in figure 6-16 below. The factors of time, SS and GEN were able to account for 13.4% of the variability associated with log R-SAL concentrations over the detoxification period.

```
Call:
   lm(formula = log.R.SAL ~ Day + SS + GEN + SS:Day + GEN:Day, data =
       NIAAA.d.14.SAL.DA.time.assess.041108, na.action = na.exclude)


Coefficients:
              Value Std. Error t value Pr(>|t|)
(Intercept)  1.5275  0.1986     7.6934  0.0000
        Day  0.0293  0.0255    19.1470  0.0000
         SS -0.0411  0.0859    -0.4786  0.6328
        GEN  0.2501  0.1426     1.7536  0.0813
     Day:SS  0.0065  0.0110     0.5905  0.5557
    Day:GEN -0.0038  0.0182    -0.2078  0.8356

Multiple R-Squared: 0.1335
F-statistic: 5.113 on 5 and 166 degrees of freedom, the p-value is 0.0002182
```

Figure 6-16: Regression output for the factors of SS, GEN and time on log R-SAL concentrations throughout detoxification.

Average DA concentration profiles for smoking status and gender are shown in figures 6-17 and 6-18, respectively. Across all time-points, the rank of average DA

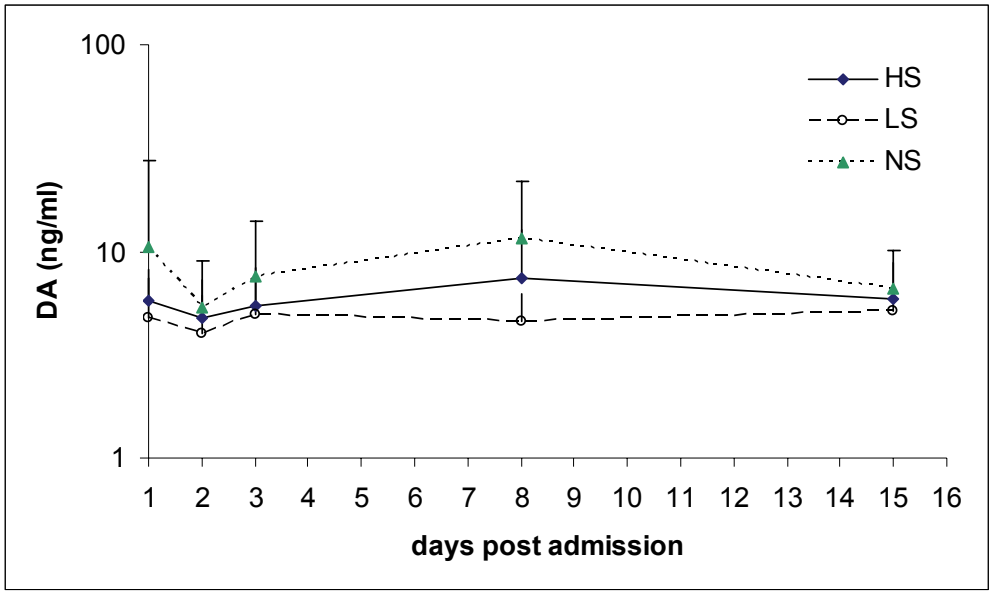


Figure 6-17: Plot of DA over time on logarithmic scale for subjects within each smoking status group (ng/ml). Each time point represents an average concentration within each group with corresponding + SD.

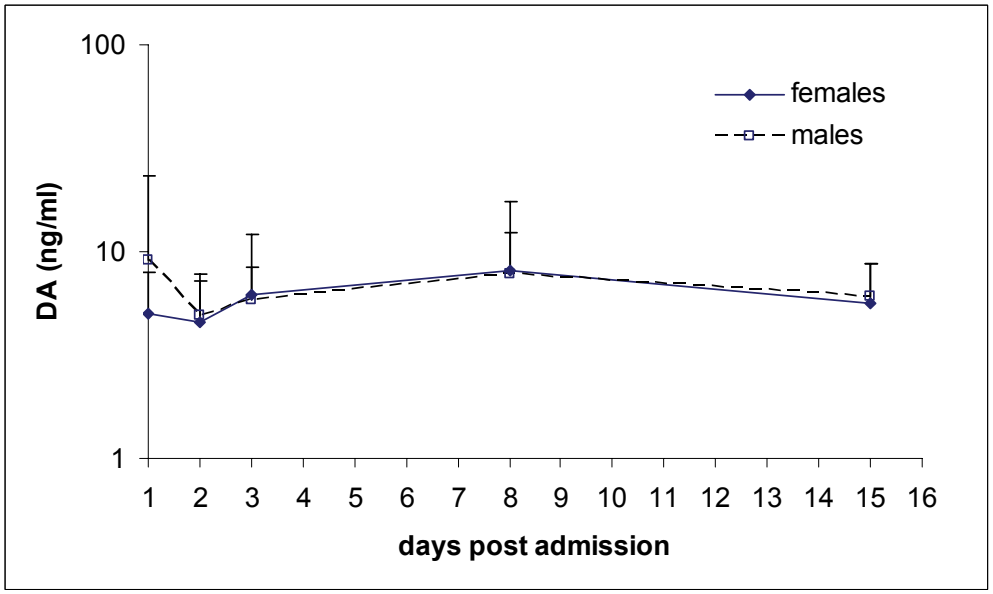


Figure 6-18: Plot of DA over time on logarithmic scale for subjects within each gender group (ng/ml). Each time point represents an average concentration within each group with corresponding + SD.

concentrations resulted in NS > HS > LS. On average, NS possessed higher DA concentrations than both smoking groups. Throughout the detoxification period, no more than a five-fold difference was observed between smoking status groups. Although the within-group variability at each time point was large, the concentration time profile for DA was relatively stable as compared to the SAL enantiomers. With respect to gender, the concentration-time profiles were parallel with the exception of admission day, in which males possessed a three-fold higher DA concentration than females on average.

Incorporation of the factors of SS and GEN into the full statistical model found insignificant effects of both factors on the time course of log DA. An insignificant effect of time was observed, with neither SS nor GEN having an influence on the time-course of log DA. The full statistical model output for the regression analysis may be shown in figure 6-19 below.

```
Call:
   lm(formula = log.DA ~ Day + SS + GEN + SS:Day + GEN:Day, data =
       NIAAA.d.14.SAL.DA.time.assess.041108, na.action = na.exclude)

     Coefficients:
                 Value Std. Error t value Pr(>|t|)
(Intercept)  0.7241  0.0860     8.4211  0.0000
        Day  0.0089  0.0111     0.8024  0.4235
         SS -0.0383  0.0372    -1.0292  0.3049
        GEN  0.0724  0.0618     1.1722  0.2428
     Day:SS -0.0010  0.0047    -0.2087  0.8349
    Day:GEN -0.0038  0.0079    -0.4783  0.6331

Multiple R-Squared: 0.03846
F-statistic: 1.328 on 5 and 166 degrees of freedom, the p-value is 0.2548
```

Figure 6-19: Regression output for the factors of SS, GEN and time on log DA concentrations throughout detoxification.

For all analytes, an effect of SS was not observed with respect to the time course of the R/S-SAL and DA analytes over the detoxification period. In order to further characterize the variability associated with the R/S-SAL enantiomers and DA concentrations, further evaluation of smoking status and gender was evaluated on the GTM of concentrations over the detoxification period, on the day of admission (day 1) and day 15. Separate analyses for each analyte were performed for each of these time-periods in order to assess for the effects of smoking status and gender.

6.4.2c-1 Grand total mean of R/S-SAL and DA

Tables 6-10, 6-11 and 6-12 show the descriptive statistics for the concentrations S-SAL, R-SAL and DA observed for each smoking status and gender, respectively. Measures of central tendency, such as mean and median, along with the variability including %COV and range is presented for each factor. With the exception of the LS female group, the gender distribution was similar for each smoking status group with

Table 6-10: Descriptive statistics for GTM S-SAL concentrations (pg/ml) divided into smoking status and gender.

S-SAL		Non-smoker	Light-smoker	Heavy-smoker	GRAND
Female	Mean	117	86	184	132
	Stdev	111	80	78	96
	%COV	95	92	42	72
	Median	61	40	194	108
	MIN	38	31	77	31
	MAX	313	217	290	313
Male	Mean	135	70	82	97
	Stdev	79	41	52	65
	%COV	59	59	64	67
	Median	120	70	81	90
	MIN	58	22	20	20
	MAX	287	136	146	287
GRAND	Mean	126	77	133	113
	Stdev	93	59	83	81
	%COV	73	76	62	72
	Median	102	68	126	90
	MIN	38	22	20	20
	MAX	313	217	290	313

Table 6-11: Descriptive statistics for GTM R-SAL concentrations (pg/ml) divided into smoking status and gender.

R-SAL		Non-smoker	Light-smoker	Heavy-smoker	GRAND
Female	Mean	172	181	280	213
	Stdev	205	175	172	181
	%COV	120	97	61	85
	Median	73	69	235	181
	MIN	42	42	112	42
	MAX	567	423	594	594
Male	Mean	187	100	121	136
	Stdev	138	80	82	104
	%COV	73	81	68	77
	Median	160	100	126	118
	MIN	67	16	27	16
	MAX	452	241	218	452
GRAND	Mean	179	137	200	173
	Stdev	167	132	153	150
	%COV	93	96	76	86
	Median	120	95	183	123
	MIN	42	16	27	16
	MAX	567	423	594	594

Table 6-12: Descriptive statistics for GTM DA concentrations (ng/ml) divided into smoking status and gender.

DA		Non-smoker	Light-smoker	Heavy-smoker	GRAND
Female	Mean	7.3	4.2	7.6	6.5
	Stdev	4.0	1.3	2.4	3.1
	%COV	55	31	32	48
	Median	5.4	4.8	7.6	5.4
	MIN	5.2	2.4	3.7	2.4
	MAX	15.2	5.5	10.3	15.2
Male	Mean	8.5	5.1	4.2	6.1
	Stdev	4.5	1.8	1.7	3.4
	%COV	53	35	41	57
	Median	6.8	4.8	3.3	5.4
	MIN	4.3	3.2	2.8	2.8
	MAX	15.9	8.2	7.0	15.9
GRAND	Mean	7.9	4.7	5.9	6.2
	Stdev	4.1	1.6	2.7	3.2
	%COV	52	34	46	52
	Median	5.6	4.8	6.1	5.4
	MIN	4.3	2.4	2.8	2.4
	MAX	15.9	8.2	10.3	15.9

each group of 6 males and 6 females. In the LS group, 6 males and 5 females were analyzed.

The overall averages (mean and %COV), across all patients for S-SAL, R-SAL and DA were 113 (72%), 173 pg/ml (86%) and 6.2 ng/ml (52%), suggesting that the GTM plasma SAL and DA concentrations are quite variable between all patients. The large differences between the median and mean R/S-SAL and DA concentrations suggest that the data follow non-normal distribution. Upon comparison of the median concentrations, females were observed to have a slightly higher median S- and R-SAL concentrations compared to males. Median DA concentrations and ranges were similar between genders for the GTM throughout detoxification.

With respect to smoking status, the ranking of median S-SAL concentration was HS > NS > LS with similar within-group variability observed. The same ranking was observed with median R-SAL concentrations with a higher amount of within-group variability. Within each smoking status group a gender difference in the ranking was observed with S-SAL. Females had the same overall smoking status group ranking while males had the ranking of NS > LS > HS for median S-SAL GTM concentrations. A discrepancy was also observed with R-SAL GTM concentrations, in which females had the similar overall smoking status group ranking, while in males the following ranking was observed of the median concentrations: NS > HS > LS. For DA, the HS > NS > LS ranking was observed. The inconsistency of ranking between genders was also observed with males possessing a rank for median concentration of NS > LS > HS, while the female counterparts shared the same ranking as the smoking status group.

The distribution of the GTM data within each smoking group for R-SAL, S-SAL and DA followed a non-normal distribution upon assessment of quantile-quantile (Q-Q) plots. Moreover, unequal variance was present with the data upon visual inspection of the residuals. For these reasons, log-transformed data were used for the primary analysis. Two-way ANOVA was performed on the log transformed R-SAL, S-SAL and DA GTM concentrations, evaluating the factors of smoking status and gender. Box-plots exemplifying the median and distribution, including outliers for the effects of gender and smoking status are presented below for the GTM log S-SAL (figure 6-20) and GTM log R-SAL (figure 6-21).

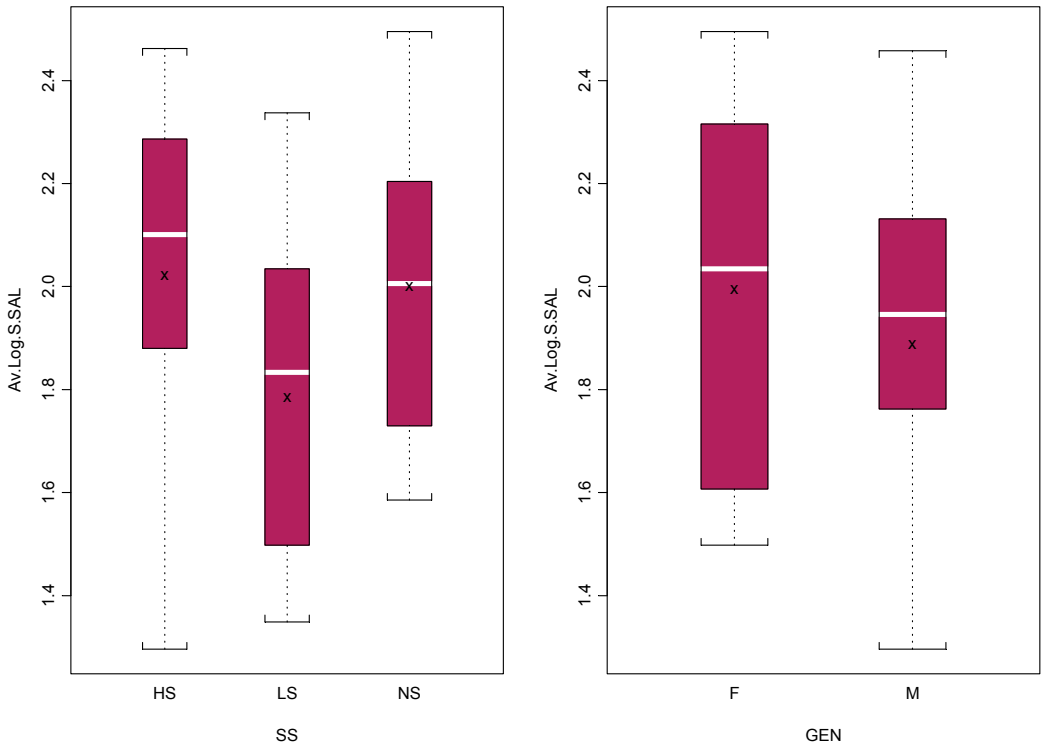


Figure 6-20: Boxplots of the GTM of log S-SAL for smoking status (SS, left plot) and gender (GEN, right plot).

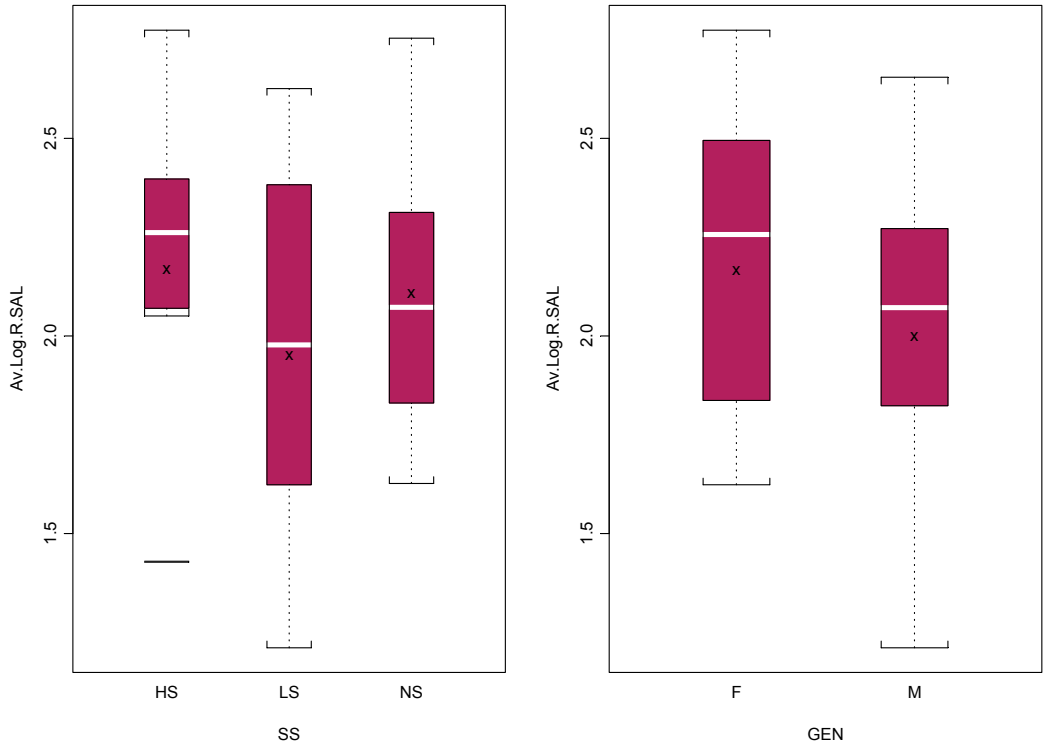


Figure 6-21: Boxplots of the GTM of log R-SAL for smoking status (SS, left plot) and gender (GEN, right plot).

Observation of the S-SAL and R-SAL box-plots suggests that there was no significant difference between gender groups and a lack of a significant trend was observed between smoking groups. Two-way ANOVA was performed to evaluate the effects of gender and smoking on GTM log S-SAL and log R-SAL concentrations. A significant effect of smoking status (SS) and gender (GEN) was not observed with respect to GTM log S-SAL and R-SAL concentrations. For both enantiomers, the smoking status factor was insignificant with the test statistic resulting in $F(2, 29) = 2.0$, p-value = 0.15 for S-SAL and $F(2, 29) = 0.97$, p-value = 0.39 for R-SAL. The effect of gender (GEN) was not significant with the test statistic resulting in p-values > 0.22 for both enantiomers. No interaction between the SS and GEN factors was observed, therefore, linear contrasts were unnecessary. The coefficient of determination (R^2) for the entire model was 0.257 (p-value = 0.107) for S-SAL and 0.095 (p-value = 0.368). Abbreviated ANOVA tables may be viewed in figure 6-22 below. Full two-way ANOVA outputs may be viewed in Appendix O along with the residual plots of the model fit. Upon visual evaluation, the residuals show an even spread of the distribution of data suggesting the lack of unequal variance. As significance was not found with the main effects, further multiple comparisons were not performed.

```
*** log S_SAL GTM Analysis of Variance Model ***

Short Output:
Call:
   aov(formula = Av.Log.S.SAL ~ SS + GEN + SS:GEN, data =
       LOG.SAL.Data.NIAAA.Detox.d1.d15.AVG.041108, na.action = na.exclude)


          Df Sum of Sq   Mean Sq  F Value     Pr(F)
       SS  2  0.386512 0.1932561 1.993205 0.1544943
      GEN  1  0.084351 0.0843511 0.869980 0.3586619
   SS:GEN  2  0.504868 0.2524341 2.603555 0.0912186
Residuals 29  2.811766 0.0969575


Multiple R-Squared: 0.2576
F-statistic: 2.013 on 5 and 29 degrees of freedom, the p-value is 0.1065




*** log R_SAL GTM Analysis of Variance Model ***

Short Output:
Call:
   aov(formula = Av.Log.R.SAL ~ SS + GEN + SS:GEN, data =
       LOG.SAL.Data.NIAAA.Detox.d1.d15.AVG.041108, na.action = na.exclude)

          Df Sum of Sq   Mean Sq  F Value     Pr(F)
       SS  2  0.288904 0.1444520 0.969432 0.3912530
      GEN  1  0.225614 0.2256137 1.514116 0.2284000
   SS:GEN  2  0.557630 0.2788149 1.871155 0.1720623
Residuals 29  4.321198 0.1490068


Multiple R-Squared: 0.0954
F-statistic: 1.09 on 3 and 31 degrees of freedom, the p-value is 0.368
```

Figure 6-22: Two–way ANOVA output for the effects of SS and GEN on GTM log S-SAL (top) and GTM log R-SAL (bottom) concentrations.

Contrary to the SAL enantiomers results, GTM log DA box-plots suggest that there was a trend observed between smoking groups. (see figure 6-23 below). Two-way ANOVA was performed to evaluate the effects of gender and smoking on GTM log DA concentrations. While a significant effect of GEN was not observed, the factor of SS was significant. The SS factor was significant with the test statistic resulting in F (2, 29) = 4.1, p-value = 0.026. The effect of gender (GEN) was not significant with the test statistic resulting in a p-value = 0.403. No interaction between the SS and GEN factors was observed, therefore, linear contrasts were unnecessary. The coefficient of determination (R^2) for the entire model was 0.346 (p-value = 0.024). The factor of SS and GEN account for approximately 34.6% of the variability associated with the GTM log DA concentration.

Multiple comparisons via the Scheffé method found the difference to be between the NS and LS groups while differences were not observed between the NS-HS and LS-HS groups. The two-way ANOVA output may be viewed below in figure 6-24 along with the results of the multiple comparison tests. The residuals of the model fit showed a lack of unequal variance.

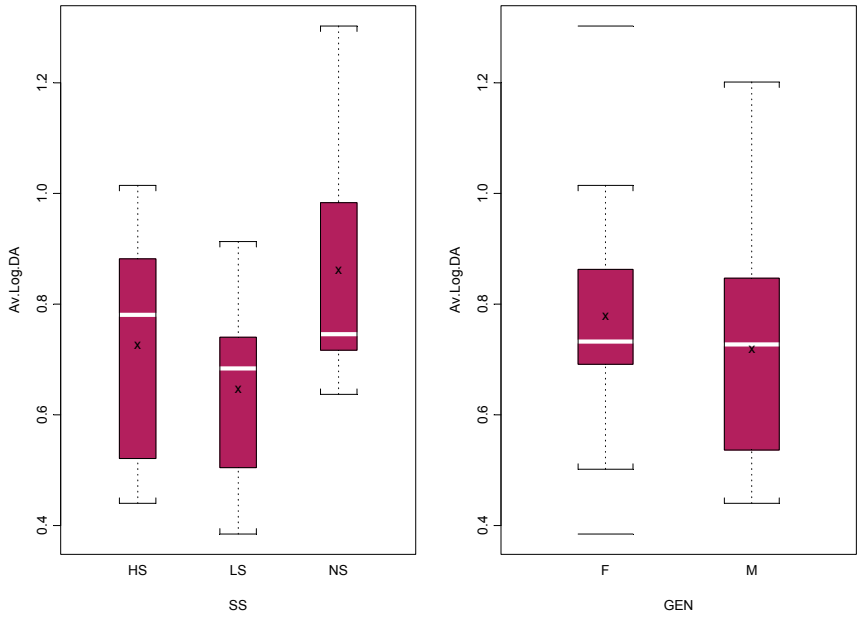


Figure 6-23: Boxplots of the GTM of log DA for smoking status (SS, left plot) and gender (GEN, right plot).

```
Call:
   aov(formula = Av.Log.DA ~ SS + GEN + SS:GEN, data =
       LOG.SAL.Data.NIAAA.Detox.d1.d15.AVG.041108, na.action = na.exclude)

             Df Sum of Sq   Mean Sq  F Value     Pr(F)
          SS  2 0.2755988 0.1377994 4.106723 0.0268920
         GEN  1 0.0241072 0.0241072 0.718446 0.4035956
      SS:GEN  2 0.2139152 0.1069576 3.187569 0.0560593
Residuals 29 0.9730832 0.0335546

Multiple R-Squared: 0.3455
F-statistic: 3.061 on 5 and 29 degrees of freedom, the p-value is 0.0244

95 % simultaneous confidence intervals for specified
linear combinations, by the Scheffé method

intervals excluding 0 are flagged by '****'

      Estimate Std.Error Lower Bound Upper Bound
HS-LS   0.0825    0.0766      -0.145       0.310
HS-NS  -0.1370    0.0748      -0.359       0.085
LS-NS  -0.2190    0.0766      -0.447      -0.008 ****
```

Figure 6-24: Two–way ANOVA output for effects of SS and GEN on GTM log DA.

6.4.2c-2 Exploratory and Covariate analysis for GTM

A statistically significant effect of smoking status was not observed on log R-SAL and log S-SAL plasma GTM concentrations. Further evaluation of the variability associated with GTM R/S-SAL and DA exposure was performed by using the FTND score and number of cigarettes smoked per day as continuous dependent variables to explain the individual subjects' exposures.

As in the case for Study #1, the analyses incorporated FTND and the number of cigarettes smoked per day in lieu of the SS category for the ANOVA analysis of GTM concentrations. For GTM log S-SAL, neither the FTND nor the number of cigarettes smoked per day had a significant influence on the ANOVA fit with resultant p-values being 0.591 and 0.844, respectively. The same observation was seen for GTM log R-SAL with the FTND fit having a p-value of 0.552 and the number of cigarettes having a p-value of 0.774. Moreover, use of these smoking variables yielded inferior fits as compared to the smoking status factor. Full linear model results may be viewed in Appendix O. Results of the primary analysis, using smoking status as a factor and the exploratory analysis, using FTND and the number of cigarettes per day, suggest that smoking does not have a significant influence on GTM log S-SAL and log R-SAL concentrations.

Information including alcohol intake and weekly exposure to dietary total SAL and whether or not acamprosate was used in the subject was recorded to evaluate for their effects on the GTM of R-SAL, S-SAL and DA. As dietary intake of ethanol and/or SAL containing foods may affect the overall exposure, this covariate

assessments may provide information into the variability associated with the plasma measurement of GTM log R-SAL and/or S-SAL. Moreover, it is unknown whether acamprosate has an effect on circulating R/S-SAL or DA, therefore it was prudent to evaluate whether this drug may have a significant effect. If alcohol intake, dietary exposure, or acamprosate administration were found to significantly correlate with SAL exposure, the TLFB, Food Intake and whether or not the subject was taking acamprosate was considered a significant covariate and implemented into the statistical model.

Linear regressions were performed on GTM log R-SAL, log S-SAL as a function of number of drinks in the past ninety days (TLFB) or average weekly dietary total SAL (Food Inventory) intake. Similar criteria aforementioned for covariate analysis were used for the SAL and DA analysis. Evaluation of the significance of the covariate was additionally assessed upon implementation into a final ANCOVA model. The p-value was evaluated for significance of the covariate. If considered significant, the fit of the entire model was evaluated for goodness of fit, with use of the covariate. A table summarizing the covariate regression analysis for the TLFB, dietary SAL intake, and acamprosate administration is shown below (table 6-13).

Table 6-13: Covariate analysis results for GTM log R-SAL, log S-SAL and log DA

variable		TLFB (# of drinks in past 90 days)	Weekly Dietary Intake (ng SAL)	Acamprosate administration (yes or no)
Log R-SAL	R^2	0.0551	0.0382	0.002
	p-value	0.175	0.324	0.803
	significance	NS	NS	NS
Log S-SAL	R^2	0.069	0.102	0.006
	p-value	0.128	0.546	0.657
	significance	NS	NS	NS
Log DA	R^2	0.008	N/A	0.006
	p-value	0.611	N/A	0.646
	significance	NS	N/A	NS

NS: not significant
N/A: dietary intake of DA was not available

The covariate analysis revealed a poor association between the TLFB, weekly dietary SAL intake, and acamprosate administration on GTM concentrations of SAL enantiomers and DA. As SAL enantiomers are hypothesized to be influenced by chronic alcohol exposure, it was expected that influence of the TLFB would contribute to the circulating plasma concentrations. The TLFB was subsequently incorporated into the ANCOVA model as a covariate, with the primary factors of smoking status and gender, and was found not to be significant for all analytes. Moreover, the ANCOVA model for assessment of the primary factors was not improved upon implementation of the TLFB covariate, for all GTM analytes.

Use of the acamprosate or SAL food Intake as a covariate was not significant and was not implemented into the full statistical model. The results of this covariate analysis suggest that the number of drinks consumed in the past ninety days, weekly dietary intake of total SAL, and administration of acamprosate do not influence the GTM levels of R/S-SAL or DA.

The GTM concentrations of the SAL enantiomers and DA represent the overall average behavior of concentrations throughout the in-patient detoxification period. The analysis was utilized to reflect the influence of smoking and gender, rather than time, during of circulating SAL levels throughout the abstinence period. For the SAL enantiomers, a significant effect of smoking status or gender was not observed on the GTM concentrations. This was unexpected as patients were able to smoke cigarettes throughout the detoxification period. A trend between the HS and NS was observed with HS possessing a higher GTM R/S-SAL concentration than NS, but statistical significance was not observed due to the within group variability. The within-group variability associated for the LS group was large for both SAL enantiomers, confounding the lack of a significant effect of smoking status. In addition, smoking variables such as the FTND and number of cigarettes smoked per day did not have a significant influence.

On the other hand, a significant effect of smoking status was observed with GTM DA concentrations, with NS possessing higher concentrations than that of both the smoking groups. Assuming that circulating plasma DA is reflective of CNS concentrations, this result supports the hypothesis of the reinforcing properties of

cigarette exposure. In other words, the LS and HS groups possess low concentrations of circulating DA compared to NS, and smokers require smoking to increase DA levels for homeostasis of central DA activity. Of note, biological sampling was performed prior to smoking the first cigarette of the day.

It is important to note that smoking behavior throughout the detoxification period was not recorded nor was dietary intake of SAL during the inpatient stay, both of which may obscure the primary factor analysis on the GTM concentrations. Interestingly, speculated influences of SAL exposure such as prior alcohol exposure or average dietary intake did not have an effect on GTM concentrations. Correlation analyses found no significant association between these variables and GTM concentrations. Throughout the inpatient abstinence period, the use of additional pharmacotherapies was employed for concomitant disease states other than alcoholism. The use of acamprosate did not have an effect on GTM concentrations. However, evaluation of additional therapies was not assessed for their influence on SAL or DA concentrations, which may confound the current results.

Of primary importance is the time-course and sampling schedule employed for the observations. Rommelspacher and co-workers found that, during an in patient detoxification period, the results of the R/S-SAL concentrations on each day observed were quite variable across subjects. For the first week R-SAL and S-SAL concentrations were statistically similar. Time-dependence of the SAL concentrations was not observed until 3 and 6-months after detoxification center admission. At these time points, the effect of the underlying alcoholism on SAL levels is presumably

negligible with similar SAL concentrations to non-alcoholics (Rommelspacher et al., 1995). The effect of smoking on SAL levels in alcoholics my not be observed within the time period assessed in our investigation, but rather at a later time of alcohol abstinence.

6.4.2c-3 Analysis of R/S-SAL and DA on Admission Day

Tables 6-14, 6-15 and 6-16 show the descriptive statistics for the admission day concentrations S-SAL, R-SAL and DA observed for each smoking status and gender, respectively.

Table 6-14: Descriptive statistics for admission S-SAL concentrations (pg/ml) divided into smoking status and gender.

S-SAL		Non-smoker	Light-smoker	Heavy-smoker	GRAND
Female	Mean	85	130	52	86
	Stdev	46	245	28	130
	%COV	54	189	53	151
	Median	72	15	49	58
	MIN	39	1	21	1
	MAX	147	567	95	567
Male	Mean	35	43	32	37
	Stdev	24	37	22	27
	%COV	69	85	68	74
	Median	30	36	33	33
	MIN	10	5	5	5
	MAX	66	106	61	106
GRAND	Mean	60	82	42	61
	Stdev	44	156	26	95
	%COV	73	191	62	156
	Median	51	36	35	41
	MIN	10	1	5	1
	MAX	147	567	95	567

Table 6-15: Descriptive statistics for admission R-SAL concentrations (pg/ml) divided into smoking status and gender.

R-SAL		Non-smoker	Light-smoker	Heavy-smoker	GRAND
Female	Mean	86	190	52	103
	Stdev	43	375	28	200
	%COV	50	197	53	194
	Median	91	15	49	46
	MIN	36	1	21	1
	MAX	157	860	95	860
Male	Mean	33	47	32	41
	Stdev	40	44	22	38
	%COV	122	94	68	93
	Median	17	30	33	28
	MIN	3	4	5	3
	MAX	108	119	61	119
GRAND	Mean	60	112	45	71
	Stdev	49	251	40	144
	%COV	82	223	88	201
	Median	45	19	31	36
	MIN	3	1	4	1
	MAX	157	860	133	860

Table 6-16: Descriptive statistics for admission DA concentrations (pg/ml) divided into smoking status and gender.

DA		Non-smoker	Light-smoker	Heavy-smoker	GRAND
Female	Mean	7.5	3.9	6.9	6.2
	Stdev	3.5	2.1	1.7	2.9
	%COV	47	54	25	47
	Median	7.0	3.6	6.9	5.8
	MIN	4.4	1.4	4.6	1.4
	MAX	13.6	6.7	9.7	13.6
Male	Mean	5.0	5.1	4.6	4.9
	Stdev	3.5	3.1	2.6	2.9
	%COV	69	60	56	59
	Median	4.3	4.0	3.2	4.0
	MIN	1.5	2.8	2.8	1.5
	MAX	11.2	11.2	8.8	11.2
GRAND	Mean	6.3	4.5	5.7	5.5
	Stdev	3.6	2.6	2.4	2.9
	%COV	57	58	42	53
	Median	5.1	3.7	6.2	4.6
	MIN	1.5	1.4	2.8	1.4
	MAX	13.6	11.2	9.7	13.6

The overall averages (mean and %COV), across all patients for S-SAL, R-SAL and DA were 61 pg/ml (156%), 71 pg/ml (201%) and 5.5 ng/ml (53%), suggesting that the admission plasma SAL and DA concentrations were variable between all patients. More variability was observed with the admission concentrations than that of the GTM concentrations. Upon comparison of the median concentrations, females were observed to have a slightly higher median S-SAL, R-SAL and DA concentrations compared to males.

With respect to smoking status, the ranking of median S-SAL concentration was NS > LS > HS with varying degrees of within-group variability observed. The NS and HS groups possessed similar %COV while the LS group had a much larger variability in admission S-SAL concentration. The ranking of NS > HS > LS was observed with median R-SAL concentrations with a highest amount of within-group variability for the LS group. Within each smoking status group, a gender difference in the ranking was observed with S-SAL. Females possessed the same overall smoking status group ranking while males had the ranking of LS > HS > NS. In addition, the difference in males between smoking status groups was observed to be small for median S-SAL admission concentrations. A discrepancy was also observed with R-SAL admission concentrations in which females had the similar overall smoking status group ranking, while in males the following ranking was observed of the median concentrations: HS > LS > NS. For DA, the HS > NS > LS ranking was observed. The inconsistency of ranking between genders was also observed with males possessing a rank for median concentration of NS > LS > HS, while the female counterparts NS ~ HS > LS.

The distribution of the admission data within each smoking group for R-SAL, S-SAL and DA followed a non-normality and unequal variance. Therefore, log-transformed data were used for the primary analysis. Box-plots are presented below for the admission log S-SAL (figure 6-25), log R-SAL (figure 6-26) and log DA (figure 6-27).

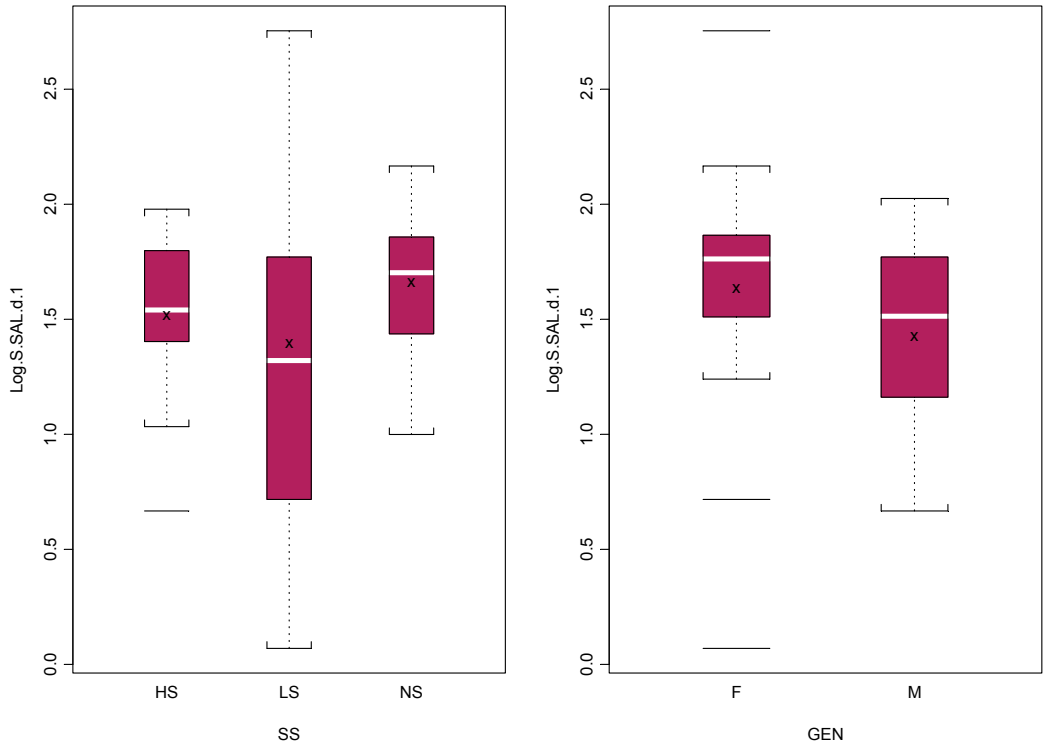


Figure 6-25: Boxplots of admission log S-SAL for smoking status (SS, left plot) and gender (GEN, right plot).

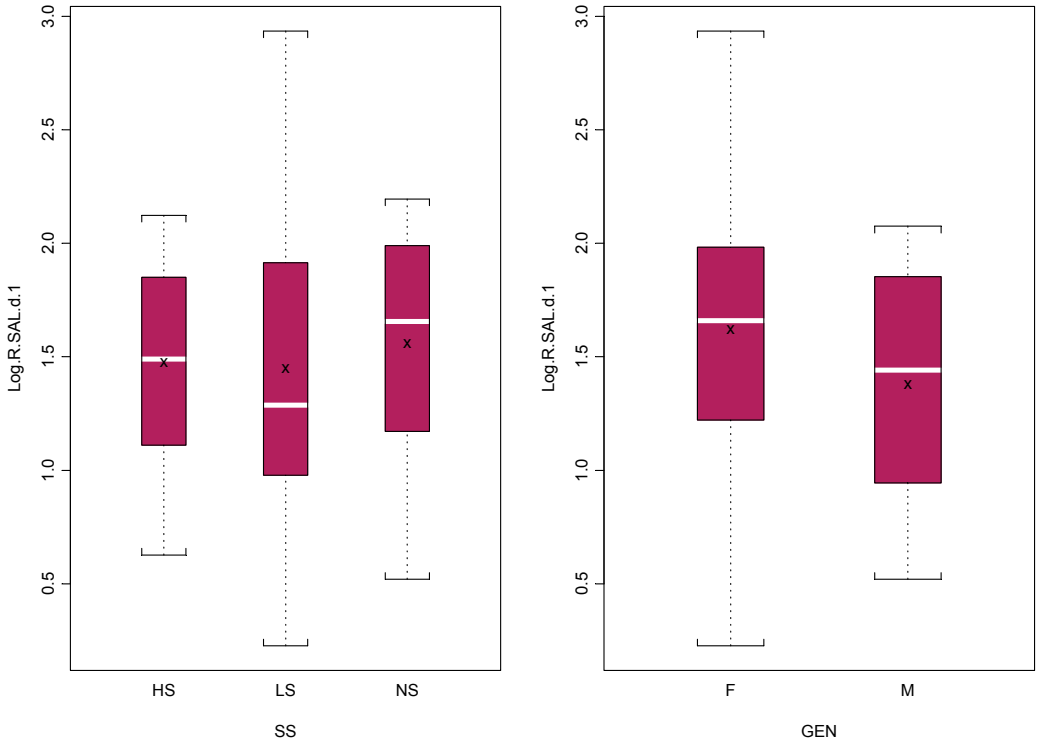


Figure 6-26: Boxplots of admission log R-SAL for smoking status (SS, left plot) and gender (GEN, right plot).

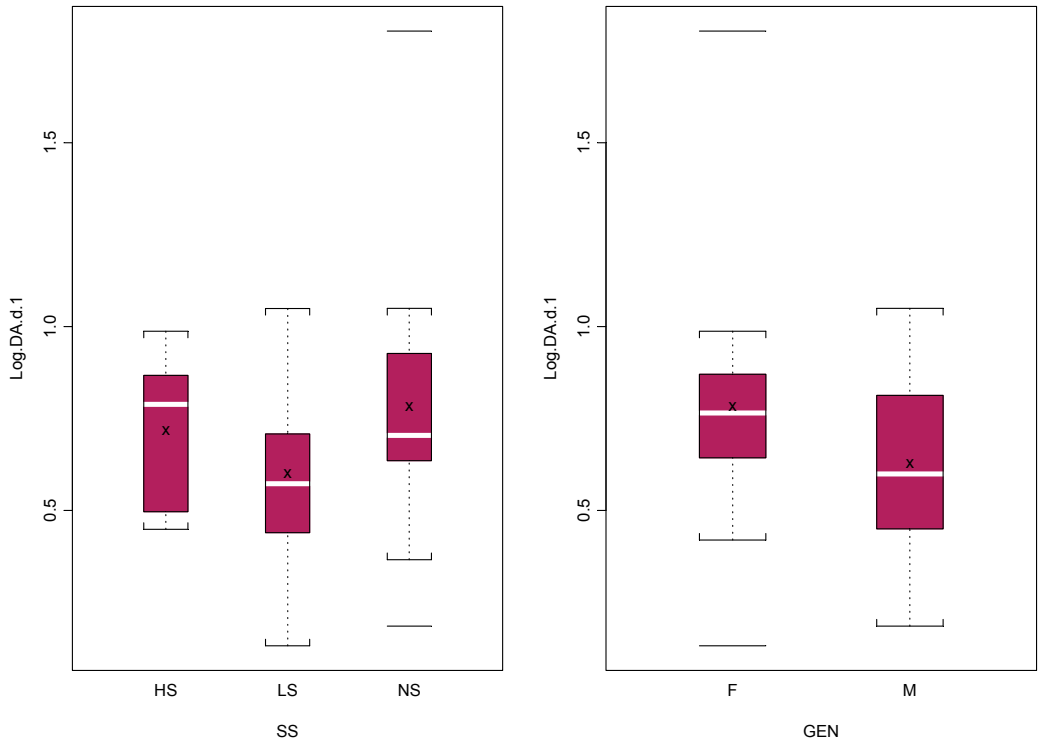


Figure 6-27: Boxplots of admission log DA for smoking status (SS, left plot) and gender (GEN, right plot).

Observation of the S-SAL, R-SAL and DA box-plots suggest that there was no significant difference between gender groups and a lack of a significant trend was observed between smoking groups. Two-way ANOVA was performed to evaluate the effects of gender and smoking on admission log S-SAL, log R-SAL and log DA concentrations. A significant effect of smoking status (SS) and gender (GEN) was not observed with respect to admission concentrations. No interaction between the SS and GEN factors was observed. Abbreviated ANOVA tables may be viewed in figure 6-28 below. Significance was not found with the main effects, thus further multiple comparisons were not performed.

```
*** log S_SAL Admission Analysis of Variance Model ***

Short Output:
Call:
   aov(formula = Log.S.SAL.d.1 ~ SS + GEN + SS:GEN, data =
       LOG.SAL.Data.NIAAA.Detox.d1.d15.AVG.041108, na.action = na.exclude)

          Df Sum of Sq   Mean Sq  F Value     Pr(F)
       SS  2  0.404638 0.2023190 0.790651 0.4630836
      GEN  1  0.357304 0.3573041 1.396324 0.2469397
   SS:GEN  2  0.531942 0.2659710 1.039400 0.3664710
Residuals 29  7.420785 0.2558891

Multiple R-Squared: 0.1485
F-statistic: 1.011 on 5 and 29 degrees of freedom, the p-value is 0.4289



*** log R_SAL Admission Analysis of Variance Model ***

Short Output:
Call:
   aov(formula = Log.R.SAL.d.1 ~ SS + GEN + SS:GEN, data =
       LOG.SAL.Data.NIAAA.Detox.d1.d15.AVG.041108, na.action = na.exclude)

          Df Sum of Sq   Mean Sq  F Value     Pr(F)
       SS  2  0.078214 0.0391069 0.118296 0.8888598
      GEN  1  0.500541 0.5005412 1.514103 0.2284020
   SS:GEN  2  0.807796 0.4038981 1.221764 0.3094337
Residuals 29  9.586996 0.3305861

Multiple R-Squared: 0.1264
F-statistic: 0.8388 on 5 and 29 degrees of freedom, the p-value is 0.5332



*** log DA Admission Analysis of Variance Model ***

Short Output:
Call:
   aov(formula = Log.DA.d.1 ~ SS + GEN + SS:GEN, data =
       LOG.SAL.Data.NIAAA.Detox.d1.d15.AVG.041108, na.action = na.exclude)

          Df Sum of Sq   Mean Sq  F Value     Pr(F)
       SS  2  0.196977 0.0984884 1.273453 0.2950514
      GEN  1  0.195106 0.1951058 2.522713 0.1230619
   SS:GEN  2  0.333961 0.1669806 2.159055 0.1336306
Residuals 29  2.242850 0.0773397


Multiple R-Squared: 0.2446
F-statistic: 1.878 on 5 and 29 degrees of freedom, the p-value is 0.1291
```

Figure 6-28: Two–way ANOVA output for the effects of SS and GEN on admission log S-SAL (top) log R-SAL (middle) and log DA (bottom) concentrations.

6.4.2c-4 Exploratory and Covariate analysis for admission day

A statistically significant effect of smoking status was not observed on log R-SAL and log S-SAL admission day plasma concentrations. Further evaluation of the variability associated with admission day R/S-SAL and DA exposure was performed by using the FTND score and number of cigarettes smoked per day as continuous dependent variables to explain the individual subjects' exposures.

For admission day log S-SAL, neither the FTND nor the number of cigarettes smoked per day had a significant influence on the ANOVA fit with resultant p-values being 0.704 and 0.799, respectively. The same observation was seen for admission log R-SAL with the FTND fit resulting in a p-value of 0.801 and the number of cigarettes having a p-value of 0.851. Results of the primary analysis (using smoking status as a factor) and the exploratory analysis (FTND or the number of cigarettes per day), suggest that smoking does not have a significant influence on admission day log S-SAL and log R-SAL concentrations.

With respect to admission day concentrations, it is important to note that biological sampling was conducted upon admission to the clinic. The time of day of sampling was variable across subjects, which may confound the analysis. More importantly, 13 of the 35 subjects evaluated (37.1%) tested positive for a drug of abuse other than alcohol and nicotine (see table 6-3). It is unknown to what extent drugs of abuse such as amphetamines, cocaine, or benzodiazepine influence circulating R/S-SAL plasma concentrations. Moreover, 13 of the 35 subjects entered the detoxification clinic

with a positive BrAC. This is the result of recent exposure to alcohol and therefore may influence the admission day concentration of log R/S-SAL.

Similar to the GTM analysis, information including alcohol intake and weekly exposure to dietary total SAL was evaluated for their effects on the admission day log R-SAL, S-SAL and DA. If recent alcohol intake (i.e., positive or negative BrAC), chronic alcohol intake (i.e., TLFB), dietary exposure, or drugs of abuse (i.e., positive or negative of admission) were found to significantly correlate with log R/S-SAL of DA exposure, they were considered a significant covariate and implemented into the statistical model.

Linear regressions were performed on log R-SAL, log S-SAL as a function of TLFB and average weekly dietary total SAL (Food Inventory) intake. An association of positive or negative BrAC, positive or negative admission drug of abuse as used to assess an influence of recent alcohol exposure or drug of abuse, respectively. Similar criteria aforementioned for covariate analysis were used for the SAL and DA analysis. Evaluation of the significance of the covariate was additionally assessed upon implementation into a final ANCOVA model. The p-value was evaluated for significance of the covariate. If considered significant, the fit of the entire model was evaluated for goodness of fit, with use of the covariate. For the case of BrAC and drug of abuse influence, a dummy-regression variable was used for implementation into ANCOVA model. Boxplots of these variables are included in figure 6-29. A table summarizing the covariate regression analysis for the TLFB, dietary SAL intake, BrAC and drugs of abuse (table 6-17).

Table 6-17: Covariate analysis results for admission log R-SAL, log S-SAL and log DA

Variable		TLFB (# of drinks in past 90 days)	Weekly Dietary Intake (ng SAL)	BrAC (positive or negative)	Drug of Abuse (positive or negative)
Log R-SAL	R^2	0.059	0.045	0.063	0.016
	p-value	0.180	0.438	0.146	0.464
	significance	NS	NS	NS	NS
Log S-SAL	R^2	0.065	0.155	0.077	0.009
	p-value	0.140	0.372	0.105	0.587
	significance	NS	NS	NS	NS
Log DA	R^2	0.017	N/A	0.001	0.003
	p-value	0.449	N/A	0.829	0.757
	significance	NS	N/A	NS	NS

NS: not significant
N/A: dietary intake of DA was not available

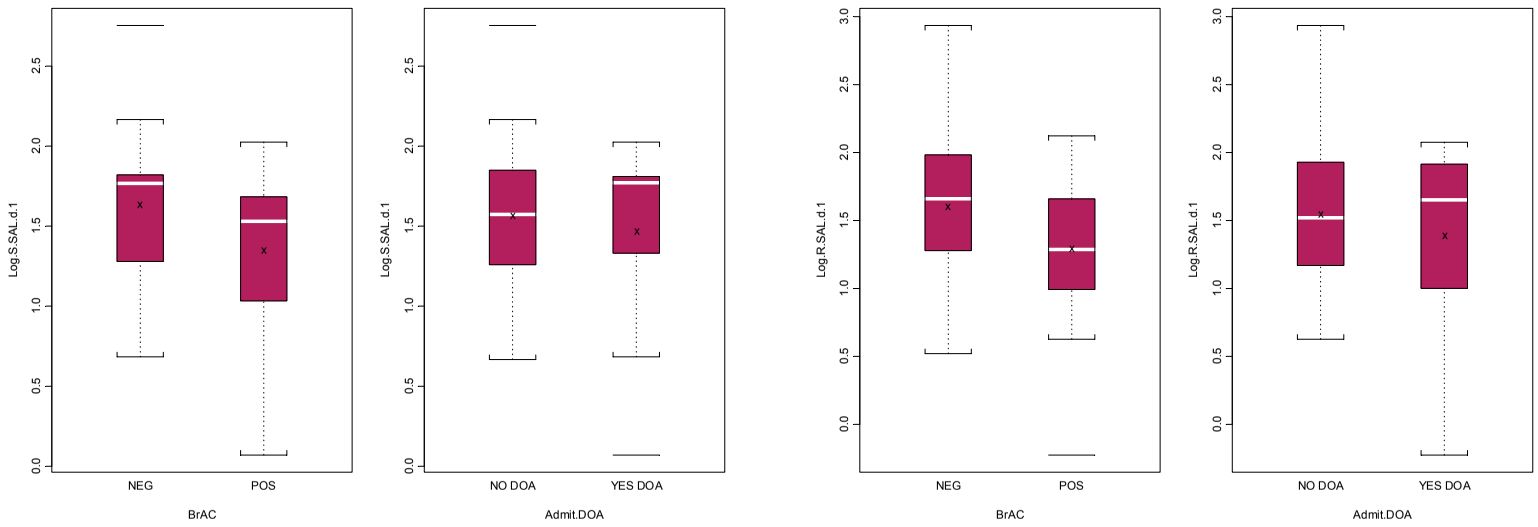


Figure 6-29: Admission log S-SAL and R-SAL boxplots of for breath alcohol (BrAC, left plot) and drugs of abuse (Admit DOA, right plot). Number of subjects for each group: n = 22 for NEG BrAC and n = 13 for POS BrAC, and n = 22 for NO DOA and n = 13 for YES DOA.

No correlation was observed between the TLFB, weekly dietary SAL intake, positive BrAC and positive drug of abuse for the admission concentrations of SAL enantiomers and DA. As SAL enantiomers are hypothesized to be influenced by acute alcohol exposure, it was expected that influence of the BrAC would contribute to the circulating plasma concentrations. The boxplot in figure 6-29 exemplifies a lack of a difference between positive admission BrAC and negative for both enantiomers, suggesting that the sole factor of BrAC is not descriptive of SAL concentrations. Interestingly, persons with a positive BrAC had a slightly lower average log concentration of both S-SAL and R-SAL. Employment of BrAC as a categorical regression variable in the ANCOVA models, accounting for the factors of smoking status and gender, found a lack of statistical significance for the potential covariate. Moreover, the fit for the primary factors did not show statistical significance with use of BrAC as a covariate. The same case was observed for persons showing positive results for the urine drug of abuse (DOA) screen.

Use of the TLFB or SAL food intake as a covariate was not significant and was not implemented into the full statistical model. The results of this covariate analysis suggest that the number of drinks consumed in the past ninety days, weekly dietary intake of total SAL, positive or negative BrAC, and positive or negative drug of abuse testing do not influence the admission levels of R/S-SAL or DA.

As a separate analysis, liver function as assessed by AST (aspartate aminotransferase) had a significant effect on admission SAL concentrations. While AST is considered a marker of acute ethanol exposure and hepatocellular damage,

correlation with the SAL enantiomers on day 1 was performed. Results showed that, for both enantiomers, a significant effect of AST was observed. AST as a predictor of day 1 log S-SAL and R-SAL was significant with the test statistic resulting in F (1, 32) = 5.98, p-value = 0.02 for log S-SAL and F (1, 32) = 7.42, p-value = 0.01 for log R-SAL. The regression plots of admission day log S-SAL and log R-SAL are shown in the figures 6-30 and 6-31 below.

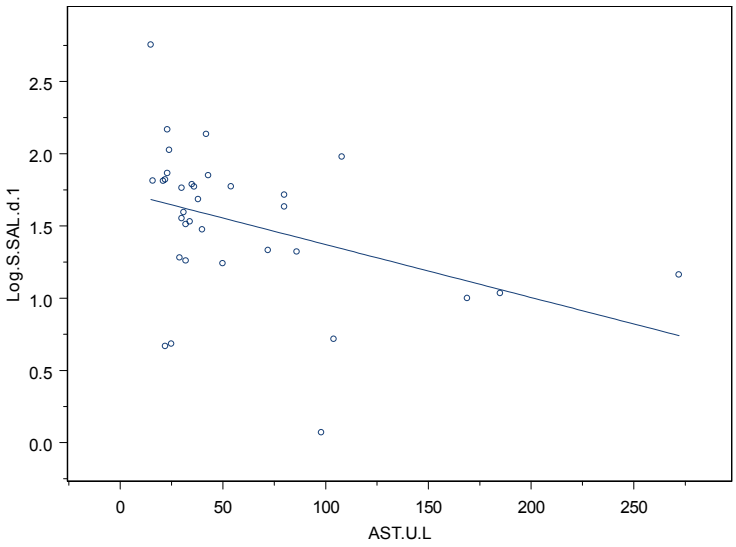


Figure 6-30: Admission log S-SAL as a function of AST (p-value = 0.02, R^2 = 0.158, log S-SAL = -0.0037 AST + 1.74)

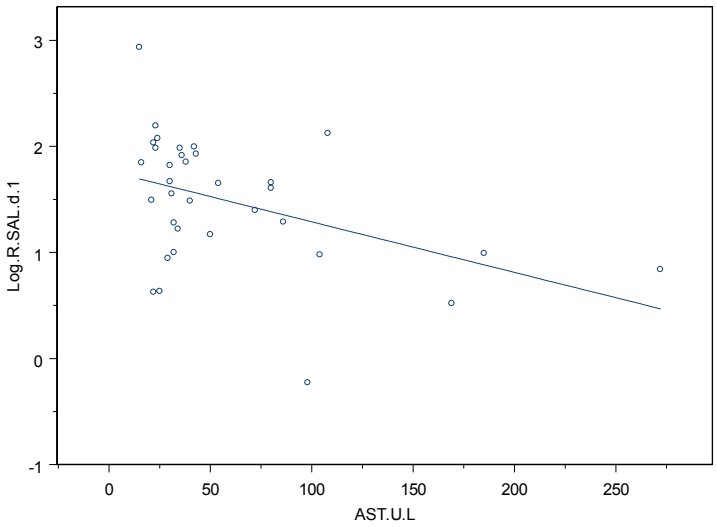


Figure 6-31: Admission log R-SAL as a function of AST (p-value = 0.01, R^2 = 0.188, log R-SAL = -0.0048 AST + 1.76)

According to the regression analysis, liver function as deemed by AST, may be an important variable when evaluating admission SAL enantiomer concentrations. As liver function declines, an observed decrease in the both enantiomers is present. For every U/L increase in AST a corresponding 0.0037 decrease in log S-SAL and 0.0048 decrease in log R-SAL concentration is observed. Of note, analysis of other liver function measures (e.g., alanine aminotransferase and albumin) yielded insignificant correlations. Therefore, as the relationship observed between SAL concentrations and AST was not strong, and other measures of liver function did not have a significant effect, the interpretability of the relationship should be taken with discretion.

The admission concentrations of the SAL enantiomers and DA represent the initial concentrations at the beginning of the in-patient detoxification period. The analysis was utilized to assess the influence prior to initial detoxification on circulating SAL levels. It is important to note that the SAL concentrations at the admission time-point possessed the greatest variability compared to that of the GTM and day 15. A lack of investigational control of the subjects prior to admission may account for this variability. Biological sampling was performed without information regarding the time relative to exposure of SAL-containing or aldehyde-containing foods and beverages.

A significant effect of smoking status or gender was not observed on the admission log SAL enantiomer concentrations. Knowledge of prior acute tobacco smoking exposure, before entrance into the detoxification center and before biological sampling, was unavailable and may have influenced the admission concentrations. In addition, smoking variables such as the FTND and number of cigarettes smoked per day did not have a significant influence. With respect to gender, a slight trend was observed with females possessing higher concentrations than males, but the difference was statistically insignificant.

Interestingly, hypothesized influences of SAL exposure, such as prior alcohol exposure, average dietary intake or positive drugs of abuse screen, did not have a significant influence on admission concentrations. Correlation analyses found no significant association between these variables and the admission concentrations.

Liver function, as assessed by AST, had an influence on admission R/S-SAL concentrations. For this reason, the applicability of BrAC or TLFB measures of

alcohol exposure may not have been reliable covariates. As the endogenous formation of the SAL enantiomer within the body may require consequent metabolism of ethanol, liver function may be an important determinant of SAL exposure upon acute ingestion of alcohol. Moreover, some researchers speculate that SAL enantiomers are formed via enzymatic routes (Naoi et al., 1996), therefore, hepatocellular function my play a role in the synthesis of SAL enantiomers.

6.4.2c-5 Analysis of R/S-SAL and DA on Day 15

Tables 6-18, 6-19 and 6-20 show the descriptive statistics for day 15 concentrations S-SAL, R-SAL and DA observed for each smoking status and gender, respectively.

Table 6-18: Descriptive statistics for day 15 S-SAL concentrations (pg/ml) divided into smoking status and gender.

S-SAL		Non-smoker	Light-smoker	Heavy-smoker	GRAND
Female	Mean	104	147	200	151
	Stdev	66	99	164	118
	%COV	64	68	82	78
	Median	99	122	139	122
	MIN	18	50	77	18
	MAX	217	314	504	504
Male	Mean	132	78	172	127
	Stdev	118	88	203	141
	%COV	89	113	118	111
	Median	76	41	128	73
	MIN	32	26	9	9
	MAX	337	253	560	560
GRAND	Mean	118	110	186	139
	Stdev	92	96	176	129
	%COV	78	87	95	93
	Median	94	85	136	102
	MIN	18	26	9	9
	MAX	337	314	560	560

Table 6-19: Descriptive statistics for day 15 R-SAL concentrations (pg/ml) divided into smoking status and gender.

R-SAL		Non-smoker	Light-smoker	Heavy-smoker	GRAND
Female	Mean	132	228	274	210
	Stdev	93	172	235	177
	%COV	70	75	86	84
	Median	113	185	236	166
	MIN	25	73	42	25
	MAX	300	524	704	704
Male	Mean	162	107	272	180
	Stdev	165	137	322	221
	%COV	102	127	118	123
	Median	92	43	217	92
	MIN	32	18	4	4
	MAX	456	367	883	883
GRAND	Mean	147	162	273	195
	Stdev	128	159	269	198
	%COV	88	98	99	102
	Median	107	150	217	149
	MIN	25	18	4	4
	MAX	456	524	883	883

Table 6-20: Descriptive statistics for day 15 DA concentrations (pg/ml) divided into smoking status and gender.

DA		Non-smoker	Light-smoker	Heavy-smoker	GRAND
Female	Mean	6.4	4.2	7.4	6.1
	Stdev	2.0	0.2	3.5	2.6
	%COV	31	5	47	43
	Median	6.1	4.2	6.2	5.0
	MIN	4.0	3.9	3.7	3.7
	MAX	9.6	4.5	12.7	12.7
Male	Mean	6.7	5.9	4.3	5.8
	Stdev	4.9	1.8	1.3	3.2
	%COV	73	31	30	55
	Median	5.1	5.8	4.1	5.2
	MIN	3.7	3.5	2.6	2.6
	MAX	16.6	9.1	6.3	16.6
GRAND	Mean	6.6	5.1	5.9	5.9
	Stdev	3.6	1.6	3.0	2.9
	%COV	55	31	51	49
	Median	5.5	4.5	5.1	5.2
	MIN	3.7	3.5	2.6	2.6
	MAX	16.6	9.1	12.7	16.6

The overall averages (mean and %COV), across all patients for S-SAL, R-SAL and DA were 139 pg/ml (93%), 195 pg/ml (102%) and 5.9 ng/ml (49%). Upon comparison of the median concentrations, females were observed to have a slightly higher day 15 median S-SAL and R-SAL concentrations compared to males, while DA concentrations were similar between genders.

With respect to smoking status, the ranking of median S-SAL concentration was HS > NS > LS with similar degrees of within-group variability observed. The ranking of HS > LS > NS was observed with median R-SAL concentrations on day 15. Within each smoking status group a gender difference in the ranking was observed with S-SAL. Males possessed the same overall smoking status group ranking while females had the ranking of HS > LS > NS. A discrepancy was also observed with R-SAL admission concentrations in which females had the similar overall smoking status group ranking, while in males the following ranking was observed of the median concentrations: HS > NS > LS. For DA, the NS > HS > LS ranking was observed, although the differences were negligible between NS and HS groups. The inconsistency of ranking between genders was also observed with males possessing a rank for median concentration of LS > NS > HS, while the female counterparts HS ~ NS > LS. Box-plots are presented below for the day 15 log S-SAL (figure 6-32), log R-SAL (figure 6-33) and log DA (figure 6-34).

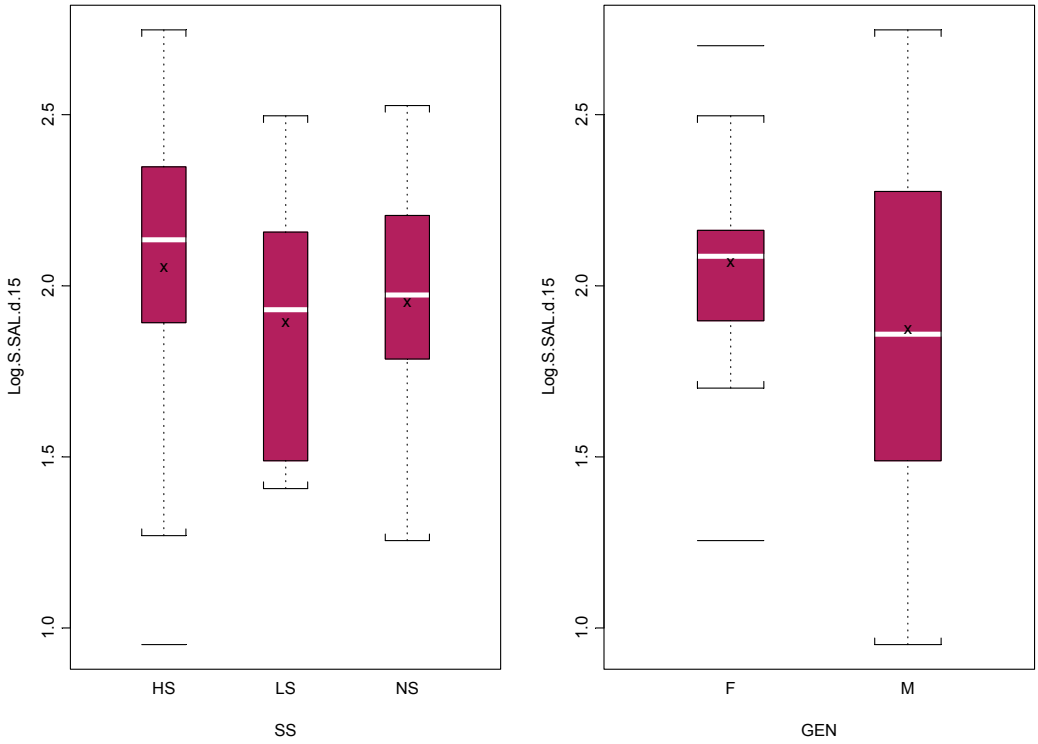


Figure 6-32: Boxplots of day 15 log S-SAL for smoking status (SS, left plot) and gender (GEN, right plot).

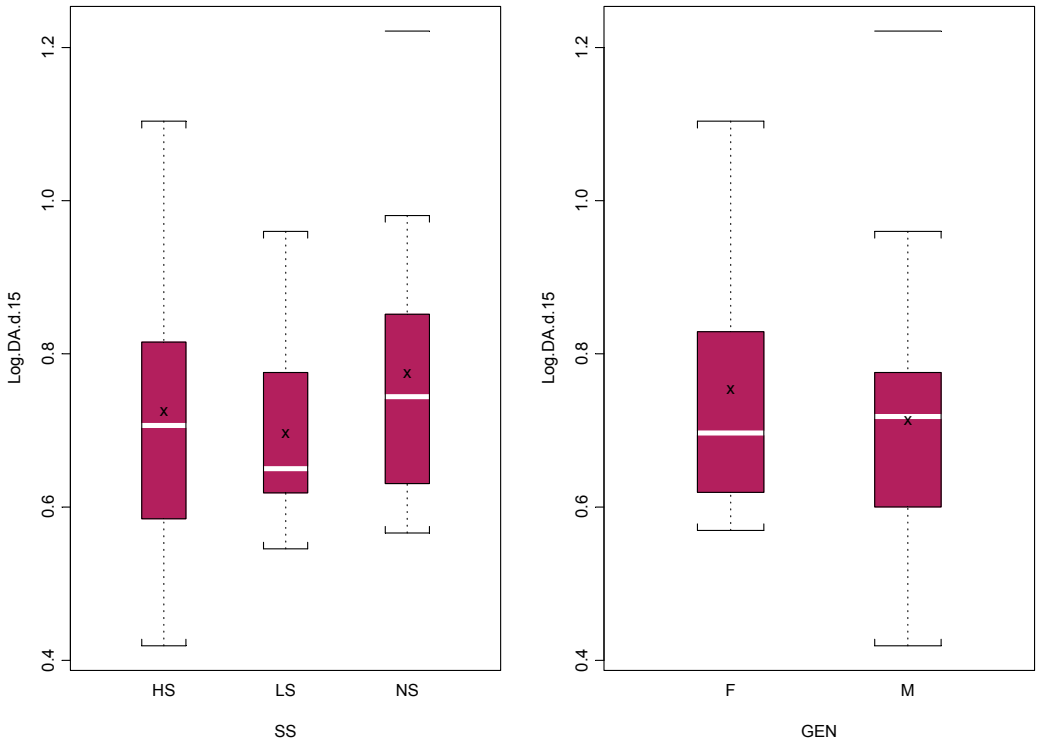


Figure 6-33: Boxplots of day 15 log R-SAL for smoking status (SS, left plot) and gender (GEN, right plot).

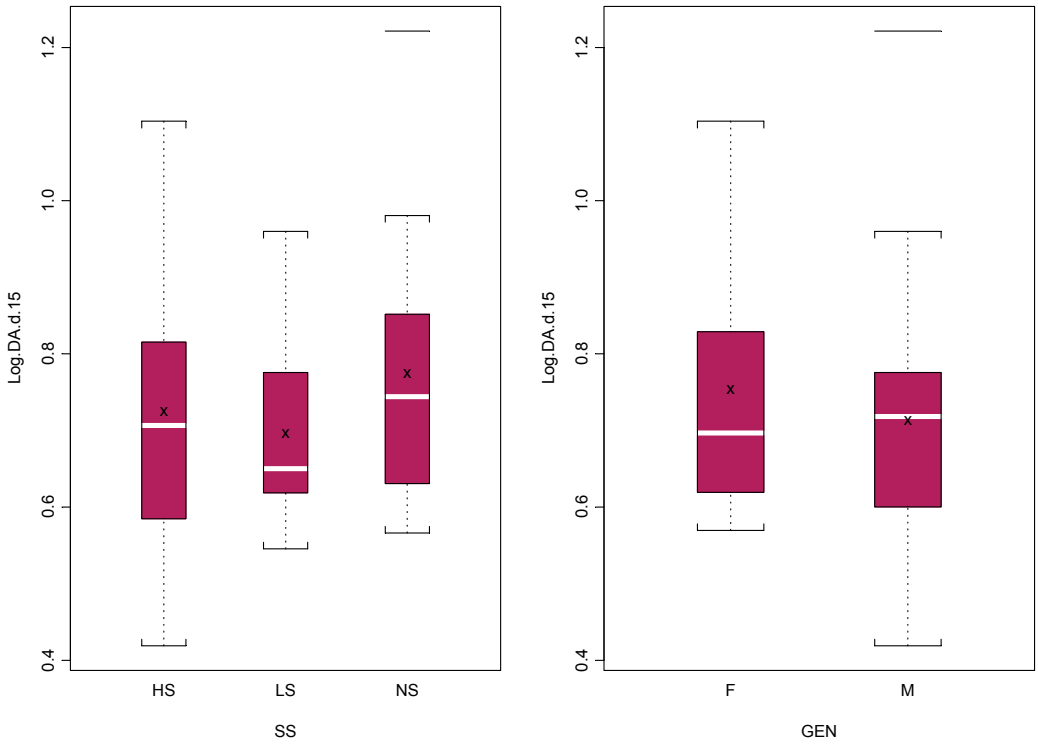


Figure 6-34: Boxplots of admission log DA for smoking status (SS, left plot) and gender (GEN, right plot).

Observation of the S-SAL, R-SAL and DA box-plots suggest that there was no significant difference between gender groups and a lack of a significant trend was observed between smoking groups on day 15. Two-way ANOVA was performed to evaluate the effects of gender and smoking on admission log S-SAL, log R-SAL and log DA concentrations. A significant effect of smoking status (SS) and gender (GEN) was not observed with respect to admission concentrations. No significant interaction between the SS and GEN factors was observed. Abbreviated ANOVA tables may be viewed in figure 6-35 below. Significance was not found with the main effects, therefore further multiple comparisons were not performed.

```
*** log S_SAL day 15 Analysis of Variance Model ***

Call:
   aov(formula = Log.S.SAL.d.15 ~ SS + GEN + SS:GEN, data =
       LOG.SAL.Data.NIAAA.Detox.d1.d15.AVG.041108, na.action = na.exclude)

          Df Sum of Sq   Mean Sq  F Value     Pr(F)
       SS  2  0.155235 0.0776174 0.432217 0.6531812
      GEN  1  0.327546 0.3275460 1.823960 0.1872881
   SS:GEN  2  0.309619 0.1548093 0.862065 0.4328294
Residuals 29  5.207807 0.1795795

Multiple R-Squared: 0.1321
F-statistic: 0.8825 on 5 and 29 degrees of freedom, the p-value is 0.5052


*** log R_SAL day 15 Analysis of Variance Model ***

Call:
   aov(formula = Log.R.SAL.d.15 ~ SS + GEN + SS:GEN, data =
       LOG.SAL.Data.NIAAA.Detox.d1.d15.AVG.041108, na.action = na.exclude)

          Df Sum of Sq   Mean Sq  F Value     Pr(F)
       SS  2  0.129790 0.0648950 0.229385 0.7964508
      GEN  1  0.616341 0.6163408 2.178589 0.1507193
   SS:GEN  2  0.392646 0.1963232 0.693947 0.5077060
Residuals 29  8.204339 0.2829082

Multiple R-Squared: 0.1219
F-statistic: 0.8051 on 5 and 29 degrees of freedom, the p-value is 0.5554


*** log DA day 15 Analysis of Variance Model ***

Call:
   aov(formula = Log.DA.d.15 ~ SS + GEN + SS:GEN, data =
       LOG.SAL.Data.NIAAA.Detox.d1.d15.AVG.041108, na.action = na.exclude)

          Df Sum of Sq    Mean Sq  F Value     Pr(F)
       SS  2 0.0369333 0.01846667 0.705681 0.5020544
      GEN  1 0.0133690 0.01336899 0.510880 0.4804731
   SS:GEN  2 0.1738083 0.08690416 3.320936 0.0502740
Residuals 29 0.7588887 0.02616858

Multiple R-Squared: 0.228
F-statistic: 1.713 on 5 and 29 degrees of freedom, the p-value is 0.1632
```

Figure 6-35: Two–way ANOVA output for the effects of SS and GEN on day 15 log S-SAL (top) log R-SAL (middle) and log DA (bottom) concentrations.

6.4.2c-6 Exploratory and Covariate analysis for day 15

A statistically significant effect of smoking status was not observed on log R-SAL and log S-SAL day-15 plasma concentrations. For admission day log S-SAL and log R-SAL, neither the FTND nor the number of cigarettes smoked per day had a significant influence on the ANOVA fit. Although a statistically significant difference was not observed, HS possessed and apparent higher average log S-SAL concentrations compared to that of the LS and NS groups on day 15. Results of the primary analysis (using smoking status as a factor) and the exploratory analysis (FTND or the number of cigarettes per day), suggest that smoking does not have a significant influence on log S-SAL and log R-SAL concentrations observed on day 15.

With respect to day 15 concentrations, it is important to note that biological sampling was conducted pre-prandially and before smoking a cigarette. The time of day of sampling was similar across subjects. Information including chronic alcohol intake and weekly exposure to dietary total SAL was evaluated for their effects on the admission day log R-SAL, S-SAL and DA. If chronic alcohol intake (i.e., TLFB) or dietary SAL was found to significantly correlate with log R/S-SAL of DA exposure, they were considered a significant and subsequently implemented into the statistical model. A table summarizing the covariate regression analysis for the TLFB and dietary SAL intake is shown below (table 6-21).

Table 6-21: Covariate analysis results for log R-SAL, log S-SAL and log DA concentrations in day 15.

variable		TLFB (# of drinks in past 90 days)	Weekly Dietary Intake (ng SAL)
Log R-SAL	R^2	0.0005	0.034
	p-value	0.901	0.221
	significance	NS	NS
Log S-SAL	R^2	0.001	0.093
	p-value	0.804	0.349
	significance	NS	NS
Log DA	R^2	0.032	N/A
	p-value	0.301	N/A
	significance	NS	N/A

NS: not significant
N/A: dietary intake of DA was not available

The covariate analysis yielded a lack of association between the TLFB and weekly dietary SAL intake for the day 15 concentrations of SAL enantiomers and DA. Therefore, use of the TLFB or SAL food intake as a covariate was not significant and was not implemented into the full statistical model.

A known biomarker of chronic alcohol consumption and liver damage, γ-glutamyl transferase (GGT), was used to assess its correlation with the SAL enantiomers. Liver function, as assessed by GGT did not have a significant effect on day 15 SAL concentrations. Results showed that, for both enantiomers, a lack of a significant effect of GGT was observed. GGT as a predictor of day 15 log S-SAL and R-SAL was insignificant with the test statistic resulting in $F(1, 32) = 1.09$, p-value = 0.302 for log S-SAL and $F(1, 32) = 0.774$, p-value = 0.385 for log R-SAL. According to the regression analysis, liver function as deemed by GGT, is not a good predictor of log SAL enantiomer concentrations on day 15. The results of this covariate analysis suggest that the number of drinks consumed in the past ninety days, weekly dietary intake of total SAL, and GGT do not influence the day 15 levels of R/S-SAL.

The day 15 concentrations of the SAL enantiomers and DA characterize the point during detoxification where, presumably, the subject is not recently exposed to any alcohol, and the acute, physiological effects of alcohol withdrawal are complete. This analysis assumes that the SAL concentrations reflected on day 15 are representative of either the physiological effects of chronic alcohol intake and/or continuous smoking throughout the detoxification period.

The analysis was done to assess the influence of smoking and gender on circulating SAL levels, two weeks after in-patient detoxification. It is important to note that the SAL concentrations at the day 15 time-point showed less variability than that admission day across all subject. A significant effect of smoking status or gender was not observed on the log R/S-SAL enantiomer concentrations on day 15. In addition, smoking variables such as the FTND and number of cigarettes smoked per day did not have a significant influence. Although a statistically significant difference was not observed with respect to smoking status, a divergence of median R- and S-SAL concentrations was observed with the HS compared to the LS and NS. For instance, R-SAL concentrations possessed a median concentration two-fold higher than that of the other smoking status groups. As reported in figure 6-3, the HS group smoked at least a two fold more cigarettes, on average than that of the LS group. This may account for the difference seen on median R-SAL concentrations on day 15, albeit statistically insignificant. With respect to gender, no observable or statistically significant difference was seen between males and females at day 15.

Chronic exposure measurements such as the TLFB and GGT did not show any significant influence on SAL exposure on day 15. This information suggests that neither variable is a good predictor of SAL exposure. It is interesting to note that, upon comparison of the known biomarker of chronic alcohol consumption GGT with TLFB, a significant relationship was not established (p-value 0.134, $R^2 = 0.066$).

6.4.2d CIWA-AR correlation with R/S-SAL and DA

A clinical endpoint, the CIW-AR was evaluated to assess if a relation between R-SAL and S-SAL plasma concentrations and withdrawal symptoms. Information regarding the time course of the CIWA-AR was gathered for the first three days during the detoxification period, in which coinciding SAL plasma samples were measured. Of note, biological specimen sampling and CIWA-AR scores occurred within 2 hours of each other. Exploratory evaluation of the distribution of day 1 and average CIWA-AR scores were assessed between smoking status and gender (see table 6-22 below).

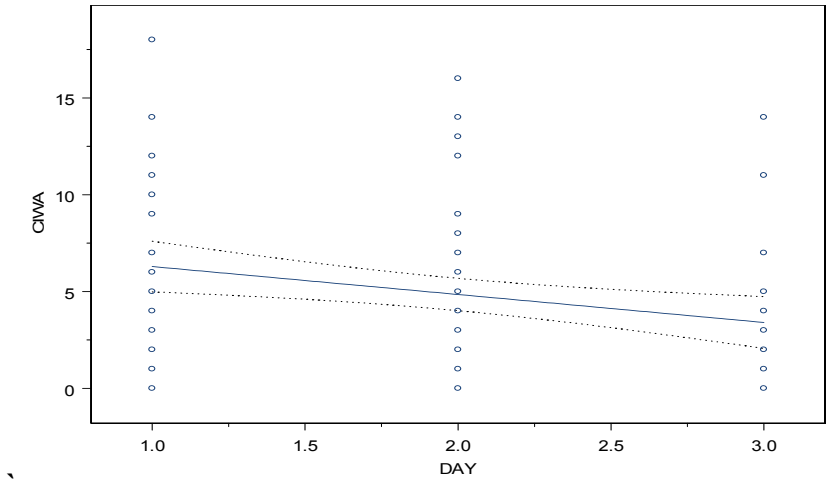
Table 6-22: Descriptive statistics for day 1 and average CIWA-AR score divided into smoking status and gender.

			Non-smoker	Light-smoker	Heavy-smoker	GRAND
CIWA-AR DAY 1	Male	Mean	3.8	4.8	7.8	5.5
		Median	2.0	3.5	9.0	4.0
		Range	0 - 12	2 - 12	1 - 14	0 - 14
	Female	Mean	5.0	5.0	7.8	6.0
		Median	5.5	5.0	6.0	5.0
		Range	0 - 12	2 - 10	3 - 18	0 - 18
	Overall	Mean	4.4	4.9	7.8	5.7
		Median	4.0	4.0	8.0	5.0
		Range	0 - 12	2 - 12	0 - 18	0 - 18

			Non-smoker	Light-smoker	Heavy-smoker	GRAND
CIWA-AR avg	Male	Mean	2.6	2.9	4.3	3.3
		Median	1.7	2.3	4.2	2.2
		Range	0 - 7.6	0.6 - 7.6	1 - 8	0 - 7.6
	Female	Mean	7.3	4.0	5.9	5.8
		Median	7.2	3.9	4.2	5.0
		Range	0 - 12.7	1.5 - 6.6	1.8 - 15.3	0 - 15.3
	Overall	Mean	5.0	3.4	5.1	4.5
		Median	4.3	2.6	4.2	3.6
		Range	0 - 12.7	0.6 - 7.6	1 - 15.3	0 - 15.3

Overall, the range of CIWA-AR scores was between 0 and 18. Of note the maximum score for the CIWA-AR is 67, therefore subjects who participated in the analysis possessed relatively low scores. Every individual had a positive score, with the exception of two NS who scored a "0" over the three days of initial detoxification. For day 1 CIWA-AR, a difference between genders in median scores was not different with males and females possessing median scores similar to the overall population. Conversely, a two-fold difference in median CIWA-AR scores was seen between HS and LS or NS groups. This is suggestive that HS underwent a more severe withdrawal that that of the LS and NS groups upon initiation of alcohol abstinence. With respect to average CIWA-AR scores, the effect of smoking status was not observed while the apparent effect of gender was seen. Females had a two-fold higher average CIWA-AR score as compared to males. This observation implies that females underwent more severe withdrawal symptoms than males throughout the detoxification period.

Over the initial time period of alcohol abstinence, the withdrawal symptoms in chronic alcoholics were suspected to decline. Of note, no subject possessed a positive CIWA-AR score after day three of detoxification. The effect of time was evaluated for the individual CIWA-AR scores over the first three days of detoxification for all subjects. Via linear regression, a significant difference in CIWA-AR was seen with the test statistic yielding $F(1, 98) = 7.78$, p-value = 0.006 ($R^2 = 0.074$). Time was able to only account for 7.4% of the variability associated with CIWA-AR scores. The regression may be viewed in the figure 6-36 below.




```
       *** Linear Model ***

Call: lm(formula = CIWA ~ DAY, data = CIWA.AR.ANAL.041208, na.action =
na.exclude)
Residuals:
    Min      1Q  Median      3Q  Max
 -6.295 -3.323 -0.8511 2.149 11.7

Coefficients:
              Value Std. Error t value Pr(>|t|)
(Intercept)  7.7389  1.1081     6.9839  0.0000
        DAY -1.4439  0.5178    -2.7886  0.0064

Residual standard error: 4.205 on 98 degrees of freedom
Multiple R-Squared: 0.07352
F-statistic: 7.776 on 1 and 98 degrees of freedom, the p-value is 0.00636
```

Figure 6-36: Regression of CIWA-AR vs. time (days) for all subjects until day 3 with 95% confidence bounds, along with full ANOVA output.

The effect of time on the CIWA-AR score was significant for each subject upon individual analyses. Of note, the effect of smoking status was not significant with respect to the overall CIWA-AR profile resulting in a test statistic F (2, 94) = 1.12, p-value = 0.329. On the other hand, gender showed a significant effect with F (1, 94) =

4.62, p-value = 0.034. Females possessed a greater difference in CIWA-AR score over time than their male counterpart. Interaction of time and smoking status or gender was not observed. The entire model, accounting for time, SS and GEN was able to account for 13.6% of the variability associated with CIAW-AR scores during the first three-days of detoxification.

```
*** Linear Model ***

Call: lm(formula = CIWA ~ DAY + SS + GEN, data = CIWA.AR.ANAL.041208,
na.action =
       na.exclude)
Residuals:
    Min     1Q Median    3Q   Max
 -6.638 -2.721 -1.005 2.123 10.42

Coefficients:
             Value Std. Error t value Pr(>|t|)
(Intercept)  9.4308  1.2817     7.3581  0.0000
        DAY -1.3893  0.5083    -2.7330  0.0075
       SSLS -1.2979  1.0339    -1.2553  0.2124
       SSNS -1.4035  0.9872    -1.4217  0.1584
        GEN -1.7750  0.8257    -2.1497  0.0341


Multiple R-Squared: 0.1359
F-statistic: 3.737 on 4 and 95 degrees of freedom, the p-value is 0.007216
```

Figure 6-37: Linear model output for the effects of time, smoking status and gender on CIWA-AR scores.

As an effect of time was present in the CIWA-AR scores, exploratory evaluation of an association between the SAL enantiomers was performed. Individual correlations were performed for each subject using log SAL concentration as the independent variable and CIWA-AR as the dependent variable. The regressions may be seen in Appendix O. For 28 of the 36 subjects, a significant relation was not observed

yielding p -values > 0.05 and R^2 ranging from 0.002 – 0.146. Of the remaining subjects, a significant relationship was observed with R^2 ranging from 0.532 – 0.924. Statistical significance and R^2 was not governed by the two factors of smoking status or gender. In addition, the direction of the relationship (negative or positive) varied across subjects and was not systematic with respect to smoking status or gender.

Day 1 concentrations of log S-SAL, log R-SAL and log DA were compared with initial CIWA-AR score to evaluate the admission concentration with severity of withdrawal. The regressions, including all subjects, of Day 1 log S-SAL, log R-SAL and log DA may be seen in figures 6-38, 6-39, and 6-40 below. An insignificant relation between admission concentrations was observed for either of the SAL enantiomers (p-values: S-SAL = 0.753, R-SAL = 0.694). In essence, both log R-SAL and log S-SAL were not able to explain any of the variability associated with the CIWA-AR scores. Moreover, a statistically significant association was not observed with log DA and CIWA-AR with p-value being 0.227.

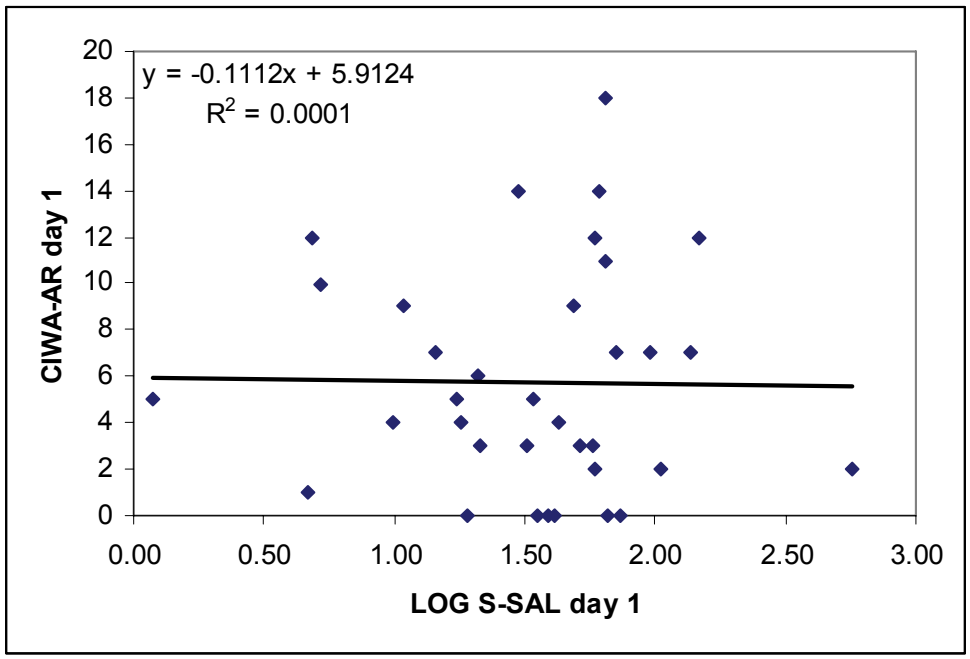


Figure 6-38: Day 1 log S-SAL vs. day 1 CIWA-AR score for all subjects.

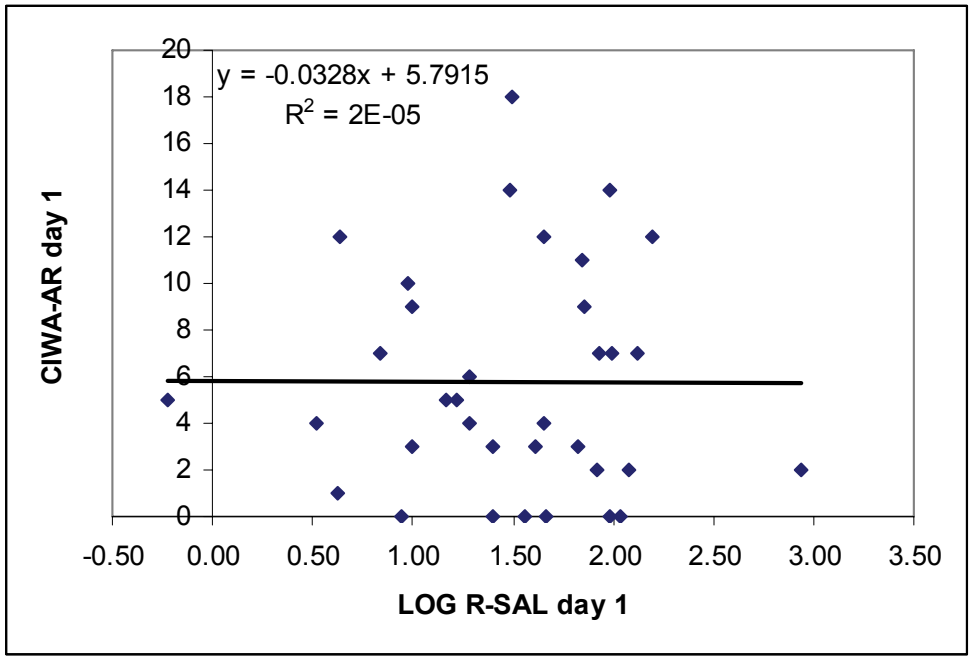


Figure 6-39: Day 1 log R-SAL vs. day 1 CIWA-AR score for all subjects.

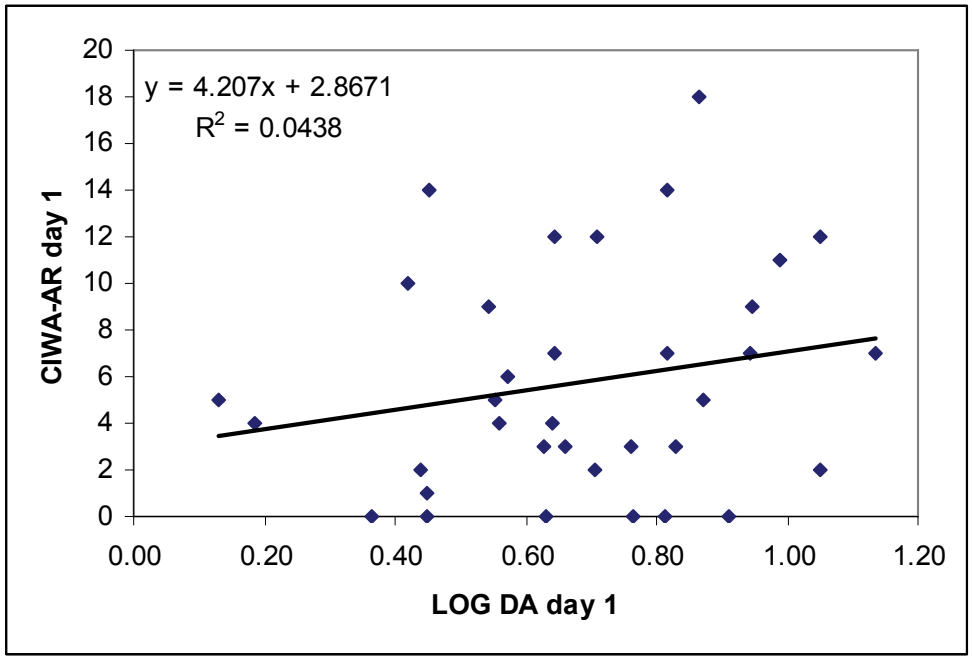


Figure 6-40: Day 1 log DA vs. day 1 CIWA-AR score for all subjects.

An analysis was performed on the change of CIWA-AR scores as a function of admission day concentrations. This assessment was executed to evaluate if initial SAL concentrations had an influence on the decline of CIWA-AR scores during the first three days. If a significant relationship were to be found, it could be deduced that recovery of withdrawal symptoms was influenced by circulating SAL concentrations. The regressions for each analyte are present in figures 6-41, 6-42 and 6-43 below.

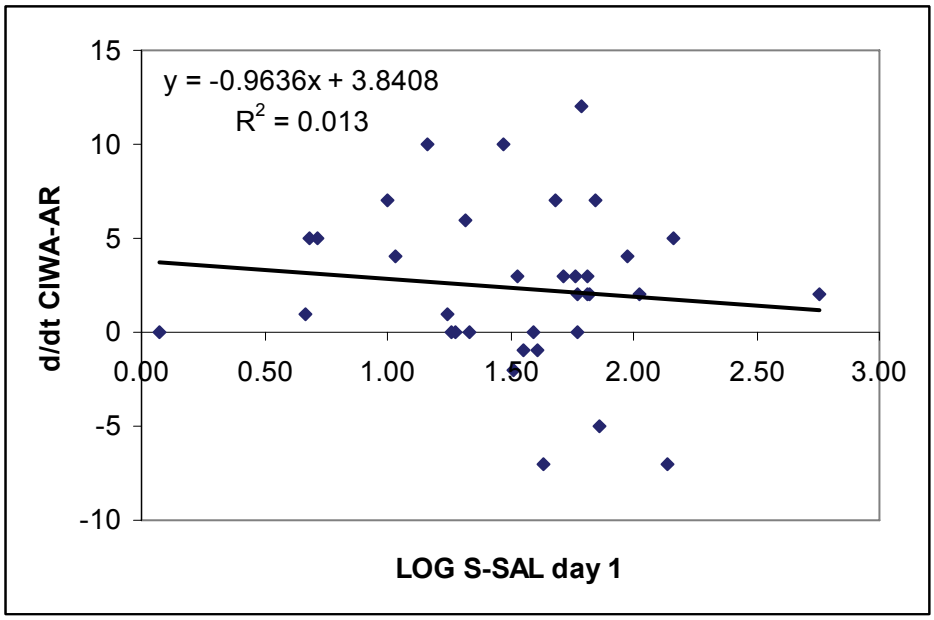


Figure 6-41: Day 1 log S-SAL vs. change in CIWA-AR scores over three days for all subjects.

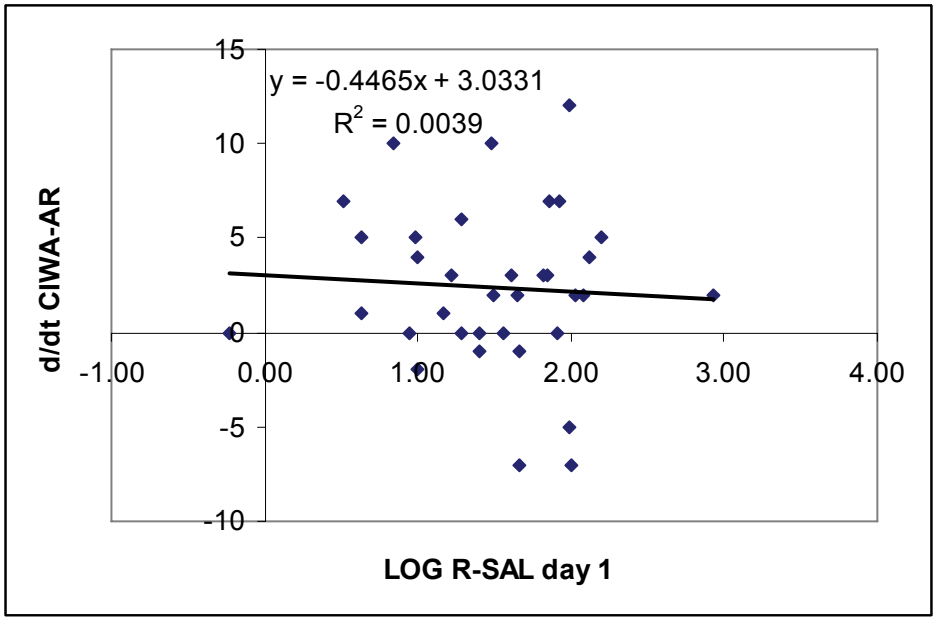


Figure 6-42: Day 1 log R-SAL vs. change in CIWA-AR scores over three days for all subjects.

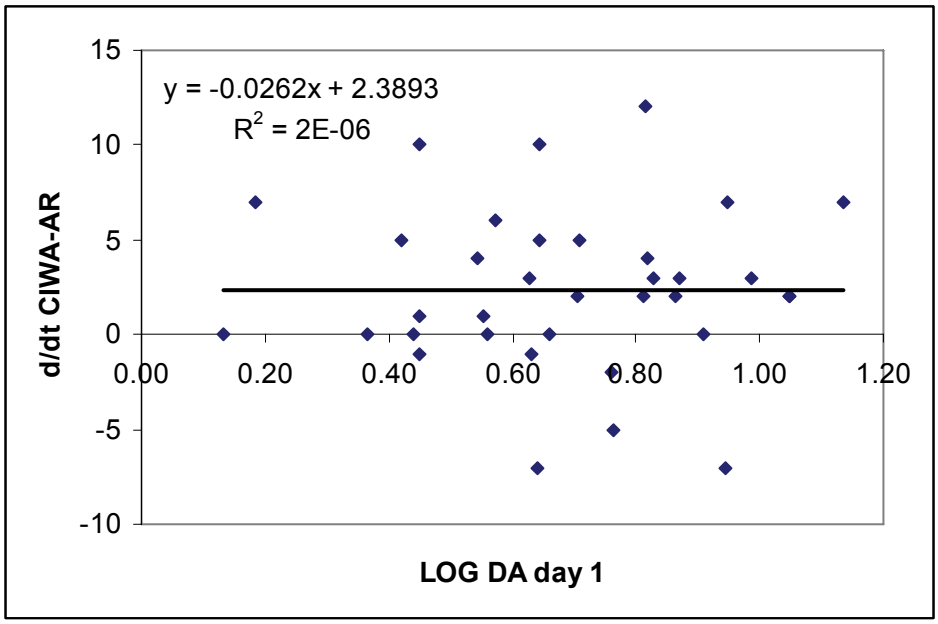


Figure 6-43: Day 1 log DA vs. change in CIWA-AR scores over three days for all subjects.

It can be concluded that no significant relationship between admission SAL or DA concentrations and a change in CIWA-AR scores exists. The regression for each SAL and DA analyte yielded statistically insignificant p-values for the association (p-values all greater than 0.467). In essence, admission SAL concentrations were not predictive of the recovery symptoms associated with alcohol withdrawal.

An exploratory analysis was performed on the change of CIWA-AR scores as a function of a change in analyte concentrations over three days. This evaluation was performed to assess if the change in SAL concentrations had an influence on the change of CIWA-AR scores during the first three days. The regressions for each analyte are present in figures 6-44, 6-45 and 6-46 below.

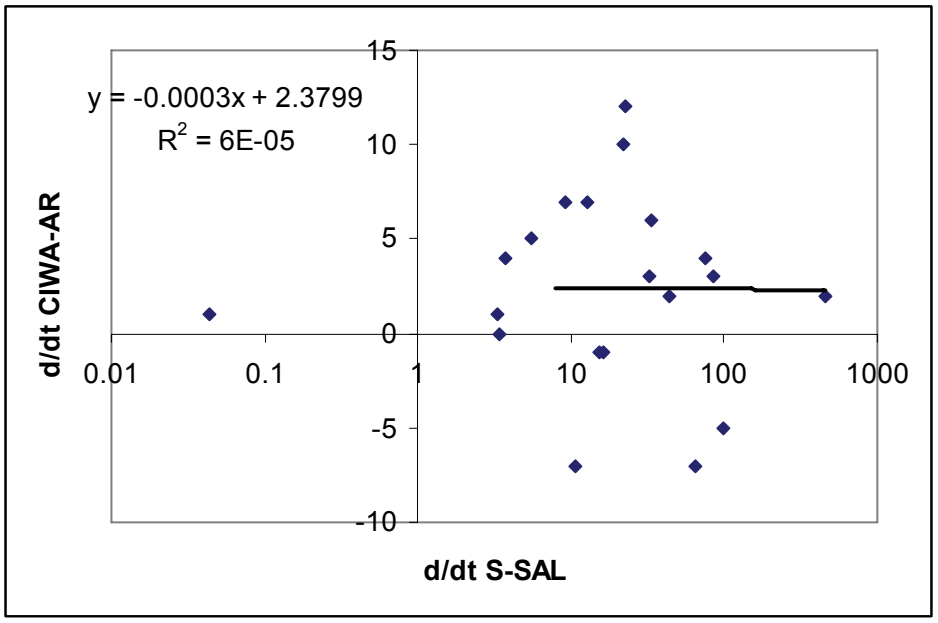


Figure 6-44: Change in CIWA-AR scores over three days as a function of change of S-SAL for all subjects.

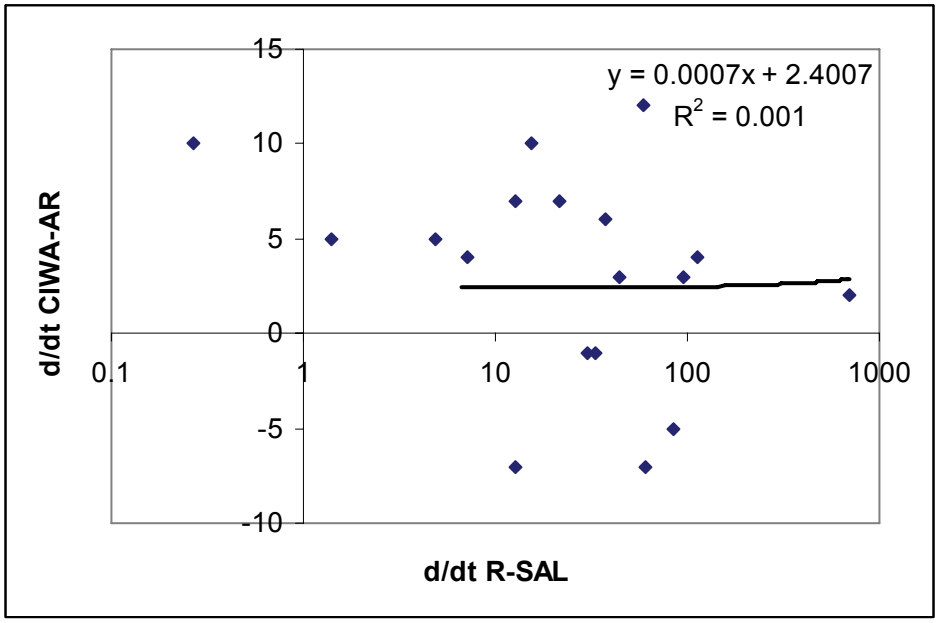


Figure 6-45: Change in CIWA-AR scores over three days as a function of change of R-SAL for all subjects.

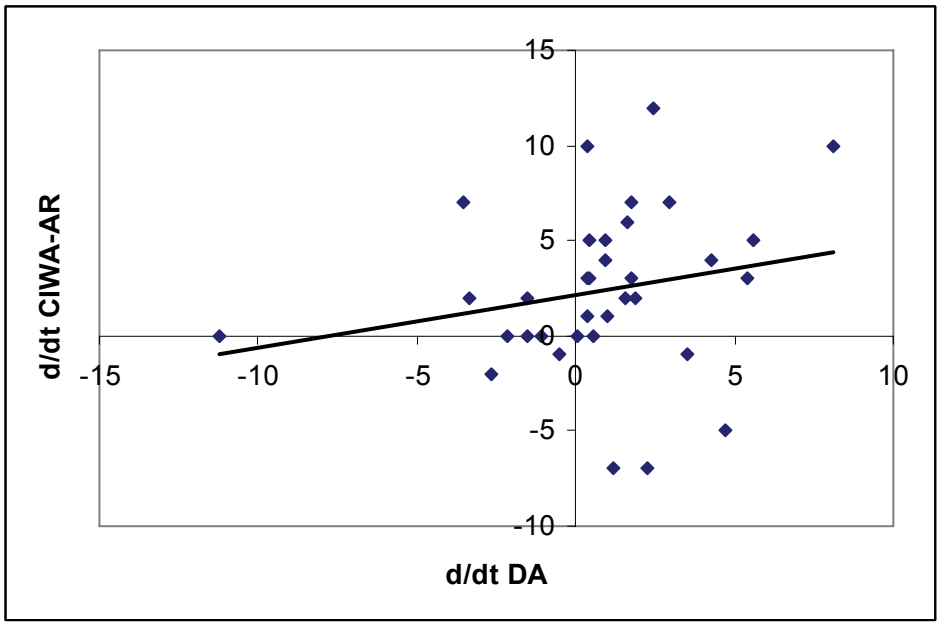


Figure 6-46: Change in CIWA-AR scores over three days as a function of change of DA for all subjects.

A significant relationship was not found for any of the analytes. The regression for each SAL and DA analyte yielded statistically insignificant p-values for the association (p-values all greater than 0.664). According to theses results, it could be inferred that recovery of withdrawal symptoms was not influenced by a change in SAL concentrations over the first three days of alcohol detoxification.

6.4.2e Summary of Primary Analysis

Large within-subject variability was observed with the SAL enantiomers over the first 15 days of detoxification. The levels \fluctuated throughout the detoxification period with individual subjects possessing both decreases and increases in concentrations of both R and S-SAL enantiomers from admission day to day 15. A

small effect of time was observed on the enantiomers with R-SAL and S-SAL concentrations, on average, increasing minimally from admission day to day 15 while DA concentrations remained constant. For individuals, covariates, such as smoking status or gender, did not explain the direction of increase or decrease in SAL concentrations during detoxification period. Therefore, three different measurements of time were evaluated to assess the influence of smoking and gender on SAL concentrations, admission day, day 15 and GTM concentrations throughout the in-patient period.

Admission day concentration assessment was to reflect the initial behavior of SAL concentrations at the start of alcohol detoxification. At this point in time, patients were expected to have recently consumed alcohol, compared to the rest of the time course. Of note, this time point showed the largest amount of between-subject variability as compared to the other time point assessments. An effect of smoking status or gender was not observed at this time point. Interestingly, recent exposure of alcohol or a drug of abuse did not have any bearing on SAL concentrations, which is contrary to the hypotheses involved with acute ethanol exposure effects on circulating SAL. Possibly confounding the results on admission day was presumably alcohol induced liver dysfunction, as assessed by AST. A relationship was observed on admission S-SAL and R-SAL and AST, suggesting that the degree of acute liver impairment has an influence. Exploratory covariate analysis evaluating the effects of the TLFB and food inventory yielded insignificant results. Grand total mean (GTM) concentrations were assessed for the influence of smoking and gender factors. This

assessment was to reflect the overall behavior of SAL and DA throughout the detoxification period. An effect of the primary factors was not seen upon evaluation of the GTM concentrations. Other potential covariates such as TLFB, dietary SAL intake and acamprosate administration was not able to explain the variability associated with the GTM measurements. Day 15 concentration evaluation resulted in an insignificant effect of the factors of smoking and gender. This time point was assumed to be a true reflection of SAL concentrations after prolonged abstinence of alcohol, and the significant withdrawal effects have subsided. At this time point, accounting for covariates such as the TLFB, dietary SAL assessment, a marker of chronic alcohol consumption, the GGT, did not explain any of the variability associated with the SAL concentrations.

The clinical endpoint, CIWA-AR, did not correlate with S-SAL or R-SAL concentrations suggesting that neither enantiomer is predictive of the withdrawal symptoms associated with alcohol detoxification. It is important to note that biological specimens were not obtained at the exact time point of CIWA-AR assessment. The acute effects of SAL concentrations on the time-course withdrawal symptoms have not been formally assessed. This discrepancy between sampling and CIWA-AR administration was a major limitation and may provide some explanation to the lack of association between the clinical endpoint and SAL concentrations.

Several shortcomings exist in this investigation on the influence of detoxification, smoking status and gender on SAL enantiomer concentrations. Of important note, the subject demographics, including age and disease status throughout

the detoxification period, are quite variable between and within smoking status groups. Moreover, different therapies were administered to each of the subjects including antihypertensives and antidepressants, both of which have not been assessed for their influence on SAL concentrations. A large majority of the subjects received thiamine as supportive therapy and vitamin supplementation. The precursor of SAL, acetaldehyde, is known to bind to thiamine (Takabe and Itokawa, 1983), which may disturb the endogenous synthesis of the analyte. The administration of daily thiamine at various times of the detoxification period may have confounded the assessment of SAL levels in these patients.

No statistically significant differences were observed at any time point with respect to gender or smoking status. Several short comings in this analysis precluded thorough interpretation of the results. As this was an observational study, a lack of investigational control was a major limitation. Admission concentrations were quite variable with patients possessing different exposures to ethanol and other drugs of abuse in the system, relative to the initial sampling time. Patients throughout the detoxification period were able to smoke cigarettes ad-libitum. An assessment of the number of cigarettes smoked during the detoxification period, via a self-report smoking log, would have been prudent to formally assess the effects of smoking on SAL levels during the detoxification period. Other functions of SAL exposure such as dietary intake was not controlled or accounted for throughout the detoxification period, which may have influenced the SAL measurements. Utilization of the Food Inventory attempted to capture the dietary intake of SAL but was unable to account for the

variability SAL concentrations. It is important to note that this in not a validated measure of SAL dietary intake. In essence, diet may have affected the measurements of SAL throughout the alcohol abstinence period, but may be unlikely as biological specimen sampling was primarily performed pre-prandial.

Two major validated measures of alcohol consumption, the TLFB and BrAC were assessed for possible effects on circulating SAL concentrations. The TLFB was an insufficient measure to characterize SAL concentrations at any of the time points assessed. This suggests that SAL was not influenced by chronic consumption of alcohol, which is contrary to other references. Moreover, the measure of acute alcohol consumption, the BrAC measurement, was also unable to provide explanation of the variability associated with plasma SAL. As endogenous SAL biosynthesis requires the enzymatic conversion of ethanol to acetaldehyde via alcohol dehydrogenase, liver function is presumed to be an important factor in the exposure of plasma SAL. It was found that a measure of acute liver damage, AST, had a significant effect on SAL exposure, with a decline in liver function resulting in decreased SAL concentration upon entry to the clinical unit. The correlation of AST with SAL enantiomers at different time-points throughout the detoxification period was not assessed, but the factor of liver function may have confounded the results associated with the primary analysis. In essence, the varying degrees of AST levels may have been responsible for some of the variability associated with SAL at each measurement in time. The acute effects of alcohol intake may have impaired the livers ability to synthesize acetaldehyde to yield the SAL product.

Upon further evaluation of the biosynthesis of SAL, both polymorphisms in alcohol dehydrogenase (ADH) and aldehydes dehydrogenase (ALDH) may contribute to varying exposures of SAL. The specific polymorphisms at the loci ADH1B, ADH1C, and ALDH2 can increase the levels of acetaldehyde more so than other alleles (Day et al., 1991). This can lead to increased circulating acetaldehyde and, in turn, the exposure of SAL. An abnormally high circulating level of SAL caused by a polymorphism in an alcohol metabolizing system is equally plausible. The intriguing possibility exists that the ALDH and ADH enzyme, in one or more of its isoforms, could partially be responsible for the formation of SAL in humans. As these polymorphisms are present in differing ethnicities, it would be prudent to genotype the populations being studied to determine if there is any effect of ALDH and ADH polymorphisms on circulating SAL levels.

The metabolic fate of the SAL enantiomers has not been addressed in published reports. This investigation principally evaluates exposure of SAL through different measures of intake of potential SAL sources such as cigarette smoking and chronic or acute alcohol consumption. However, this study does not evaluate the influence of varying degrees of SAL metabolism. The disposition for the SAL enantiomers have not been formally assessed, but according to SAL structural analysis, enzymes such as catechol-o-methyl transferase (COMT) and *N*-methyl transferase may play an important role. These enzymes are known to have major functional polymorphisms in an alcohol dependent population (Kauhanen et al., 2000; Oroszi et al., 2005). Results of both studies indicate that COMT and *N*-methyl transferase polymorphisms may contribute

significantly to alcohol intake not only in alcoholics but also in a general male population. As both enzymes are speculated to have a role in the metabolic fate of the SAL enantiomers, polymorphisms with these enzymes may explain the variability in SAL exposure. Moreover, as both of these enzymes are present in the liver, impairment of liver function may effect the metabolic disposition of SAL.

6.5 R/S-SAL and DA – Alcohol-dependent vs. control subjects

All information presented thus far summarizes R/S-SAL and DA exposure information obtained from non-alcohol dependent and alcohol-dependent NS, LS, and HS. In essence, the presented information examines the influence of smoking and gender within each population of healthy and alcoholic subjects undergoing detoxification. The significant relationship of smoking status and SAL exposure was observed from study #1, which may be resultant of acute inhalation of tobacco smoke and/or the inherent physiological difference of smokers to that of nonsmokers. No effect of smoking status was observed in the alcohol-dependent subjects at any point in time during the detoxification period. It is speculated that this discrepancy between study populations may have been confounded by the influence of alcoholism. To further clarify this relationship, a comparison was made from the subjects who were healthy nonsmokers and smokers to those patients who were alcohol dependent nonsmokers and smokers.

In brief, this analysis compared two different populations of healthy subjects to alcoholic subjects on admission day. The populations included 1) 41 healthy volunteers

including 19 NS and 11 LS and HS who smoked within 30 minutes of biological specimen sampling (Study #1) and 2) admission day concentrations of 35 alcohol dependent subjects undergoing detoxification including 12 NS, 11 LS and 12 HS. In the non smoking groups between populations, an assessment of the influence on alcoholism on circulating SAL levels can be performed. With respect to the smoking groups within each population, the comparison would evaluate the effects of recent smoking in the healthy population to that of alcoholic smokers who were assumed to have recently smoked a cigarette on admission to the detoxification clinic. For reference the subject demographics of both populations are shown in the tables 6-23 and 6-24 below.

Table 6-23: Demographic Results of Study #1 (healthy population acute cigarette smoking, mean ± SD)

Smoking Status	Demographic variable	Males	Females	Overall
NS	N	9	10	19
	Age	24.9 (2.5)	26.0 (3.1)	25.5 (2.8)
	FTND	0 (0)	0 (0)	0 (0)
	# Cig/day	0 (0)	0 (0)	0 (0)
LS	N	5	6	11
	Age	25.4 (4.1)	23.3 (3.8)	24.3 (3.9)
	FTND	2.4 (2.3)	1.5 (1.4)	1.9 (1.8)
	# Cig/day	9.0 (4.8)	8.3 (4.6)	8.6 (4.5)
HS	N	5	6	11
	Age	25.6 (3.1)	24.7 (4.0)	25.1 (3.5)
	FTND	6.8 (0.8)	5.5 (1.4)	6.1 (1.3)
	# Cig/day	17.4 (2.5)	25.8 (4.9)	22.0 (5.8)

Table 6-24: Demographic Results Study #2 (alcohol dependent population, mean ± SD)

Smoking Status	Demographic variable	Males	Females	Overall
NS	N	6	6	12
	Age	40.0 (6.4)	43.2 (10.3)	41.6 (8.3)
	FTND	0 (0)	0 (0)	0 (0)
	# Cig/day	0 (0)	0 (0)	0 (0)
LS	N	6	5	11
	Age	41.0 (10.7)	37.6 (9.4)	41.0 (10.7)
	FTND	4.7 (1.2)	3.6 (2.1)	4.2 (1.7)
	# Cig/day	10.2 (2.4)	9.5 (2.7)	9.9 (2.5)
HS	N	6	6	12
	Age	43.5 (11.7)	40.8 (8.5)	42.2 (9.9)
	FTND	7.3 (1.8)	8.8 (1.0)	8.1 (1.6)
	# Cig/day	32.1 (7.8)	26.8 (12.2)	29.5 (10.1)

With the exception of the number of subjects within each smoking status and gender group, the demographics between studies are similar with respect to FTND. There existed significant ethnicity differences between the two populations with the clinical study #1 having predominately Asian and Caucasian subjects, while subjects in clinical study #2 were predominately African American and Caucasian subjects (see tables 5-2 and 6-2). On average, the alcohol-dependent population smoked more cigarettes per day compared to the healthy population of smokers. It should be duly noted that the alcoholic subjects, on average, were at least twenty-years older than the healthy population, precluding a definitive interpretation of the comparative results between the populations.

A statistical analysis via two-way ANOVA was conducted on the individual SAL and DA concentrations between the populations. Log transformed values were compared evaluating the two primary factors of gender and smoking status.

Median smoking status concentrations, along with ranges, of the concentrations of Study #1 are compared to the median smoking status concentrations obtained from the admission concentrations from the alcoholic population in study #2 in table 6-25 below. Between studies, it was observed that the healthy population that recently smoked had a higher median concentration of all analytes as compared to the alcohol dependent population with approximately 2-fold difference seen with S-SAL, R-SAL, and DA concentrations. The discrepancy in concentrations between the studies is due, in part, to the smokers of the healthy population, who were exposed to cigarette smoking 30 minutes prior to biological specimen sampling. With respect to the NS groups between populations, it was observed that the concentrations were similar between the healthy and alcohol-dependent populations. Comparison via ANOVA found the comparison to be statistically insignificant across all analytes for the NS population. In other words, regardless of the dependency on alcohol, concentrations of S-SAL, R-SAL and DA are similar. Conversely, the populations showed a divergence when comparing both smoking groups of LS and HS. Healthy smokers who were recently exposed to tobacco had significantly higher median concentrations of R/S-SAL and DA than that of the alcoholic population on admission day. For instance, comparison of the S-SAL concentrations between LS populations showed that the healthy smokers have a three-fold higher median concentration than that of the LS

alcoholic population. This effect was more pronounced with the HS groups with healthy smokers, possessing a 9-fold higher median concentration of S-SAL than that of the alcoholic HS. Similar statistically significant trends were observed for the R-SAL and DA analytes.

Upon comparison with healthy patients that abstained from smoking for 15 hours, the NS group had statistically similar concentrations of all analytes across all populations. This suggests that there are no significant effects with regard to the sole factor of alcoholism on SAL concentrations. Moreover, even after smoking groups were abstinent from smoking for 15 hours, they still had higher median SAL concentrations as compared to their smoking status group counterparts from the alcohol-dependent patients on admission day. This infers that the effects of alcoholism itself may decrease the concentrations of SAL exposure. Of note, the ANOVA performed across all three populations resulted in the significant difference of SAL analytes being between the healthy recent smoking population and the alcohol dependent population on admission day. All of the information obtained from this comparison implies that alcoholism has no effect on SAL exposure while the acute exposure of tobacco smoke has the most significant effect.

Table 6-25: Comparison of median (range) between Study #1a (healthy, after smoking), Study #1b (healthy, 15 hours abstaining from smoking) and Study #2 admission S-SAL, R-SAL and DA.

Analyte	*Smoking status*	Study #1 ***median*** (range)		Study #2 ***median*** (range)	significance ANOVA* (p-value)
		a) Healthy, recent smoking NS, LS, HS = 19, 11, 11	b) Healthy, 15 hrs smoking abstinence NS, LS, HS = 6, 6, 6	NS, LS, HS = 12, 11, 12	
S-SAL	NS	***50*** (13 – 860)	*77* (51 – 437)	***51*** (10 – 147)	NS
	LS	***143*** (18– 1632)	***175*** (40 – 250)	***36*** (1 – 567)	< 0.05
	HS	***317*** (25 – 6084)	***228*** (81 – 693)	***35*** (5 – 95)	< 0.01
	total	***78*** (13 – 6084)	***44*** (51 – 693)	***41*** (1 – 567)	< 0.05
R-SAL	NS	***48*** (4 – 1210)	***45*** (2 – 437)	***45*** (3 – 157)	NS
	LS	***226*** (12 – 1478)	***277*** (148 – 705)	***19*** (1 – 860)	< 0.05
	HS	***324*** (25 – 7714)	***547*** (75 – 1002)	***31*** (4 – 133)	< 0.01
	Total	***87*** (4 – 7714)	***76*** (2 – 1002)	***36*** (1 – 860)	< 0.05
DA	NS	***5.9*** (1.5 – 16.6)	***2.3*** (1.0 – 9.5)	***5.1*** (1.5 – 13.6)	NS
	LS	***9.9*** (0.3 – 32.1)	***5.5*** (3.6 – 7.5)	***3.7*** (1.4 – 11.2)	< 0.05
	HS	***14.6*** (4.7 – 37.7)	***5.1*** (3.4 – 8.2)	***6.2*** (2.8 – 9.7)	< 0.05
	total	***8.7*** (0.3 – 37.7)	***4.8*** (51 – 437)	***4.6*** (1.4 – 13.6)	< 0.05

* ANOVA performed on log-analyte concentration between three studies.

It is interesting to note that the NS group amongst the healthy population had a higher median concentration than the HS group of the alcoholic population. This observation is conflicting with all reports of SAL measurement between alcoholic and control populations (see chapter 1). The rank observed for all analyte concentrations in the comparison between recently-smoking subjects to that of alcohol-dependent subjects on admission day yielded the following results: for S-SAL the HS Alcoholics < LS Alcoholics < NS Alcoholics ~ NS Healthy < LS Healthy < HS Healthy; for R-SAL the LS Alcoholics < HS Alcoholics < NS Alcoholics < NS Healthy < LS Healthy < HS Healthy; for DA the LS Alcoholics < NS Alcoholics < NS Healthy < HS Alcoholics < LS Healthy < HS Healthy.

Recall that the ANOVA analysis for the effects of smoking status resulted in significance for the healthy population, but did not yield significance for the alcohol dependent patients on admission day for all analytes. More investigational control was implemented in the healthy population from Study #1 as compared to Study #2. Study #1 implemented a requirement for healthy smokers to smoke one complete cigarette prior to sampling. The treatment effect of smoking was seen to significantly affect the circulating SAL levels (see table 5-25). For study #2, several confounding factors were present, hindering the interpretability of the results. For the most part, the alcoholic population presented to the clinic with additional factors that may influence circulating SAL levels such as recent alcohol exposure and other drugs of abuse. Moreover, opposite to the case for smokers in Study #1, the sampling time point of the admission sample, with respect to the subjects' last cigarette was not recorded. The variability

associated with the admission SAL concentrations within the alcohol dependent smoking groups may be due in part to the unknown pharmacokinetic characteristics of SAL in this population after smoking a cigarette.

The majority of the alcohol-dependent population either had a recent exposure to ethanol and/or a drug of abuse. Therefore, it was expected that SAL concentrations in this population would be elevated compared to that of the healthy population. Surprisingly, the healthy population, especially the subjects who were recently exposed to smoking, had much greater SAL concentrations compared to that of the alcohol-dependent population. From these results, it is concluded that the source of tobacco smoking is what influences the concentrations in a healthy population. In an alcoholic population, the SAL levels are comparable to the NS healthy population.

It should be noted that a difference in ethnicity distribution was observed between the alcohol-dependent and healthy population with majority of the population being Asian in the latter group. It is known that Asians possess polymorphisms in ADH and ALDH metabolizing systems (Yamamoto et al, 1993). The specific polymorphisms at the loci ADH1B, ADH1C, and ALDH2 can increase the levels of acetaldehyde more so than other alleles. This can lead to increased circulating acetaldehyde in the Asian population from Clinical Study #1 and, in turn, the exposure of SAL. It is also duly noted that alcohol-dependent patients may have induced alcohol and acetaldehyde metabolizing systems yielding a decrease in acetaldehyde. It is equally plausible that the decreased SAL concentrations in alcohol dependent patients compared to healthy subjects is due to a decrease in acetaldehyde levels due to metabolic induction.

Factors such as polymorphisms in metabolizing enzyme systems responsible for ethanol and SAL disposition along with liver pathophysiology in the alcoholic population may account for the difference is SAL exposures seen within this study.

6.6 Summary of Clinical Study #2

This investigation was designed to test the effects of smoking status and gender on plasma concentrations of R/S-SAL and DA in a population of alcohol dependent patients undergoing detoxification. Demographic information and plasma samples, along with clinical variables, were obtained from thirty-five subjects undergoing a four-week inpatient alcohol abstention program. Biological samples for R/S-SAL and DA measurements were obtained on days 1, 2, 3, 8, and 15 of the inpatient period. With respect to subject selection, patients were chosen based on health status, a complete sampling schedule over the sampling schedule, and stratification into smoking status groups, ensuring adequate distribution of smoking status and gender within each group. The observational study utilized a nicotine dependence scale and the number of cigarettes smoked per day in order to stratify the subjects into smoking status groups. Along with the alleged nicotine dependence difference between groups, all subjects were heterogeneous with respect to demographics. A large portion of subjects that participated in the study possessed a positive BrAC and drugs of abuse screen upon admission, which may confound results obtained on admission day. Throughout the inpatient detoxification, investigational control with respect to diet and smoking was not performed with patients allowing to smoke ad-libitum and consume foods that may

contain SAL. This information was not recorded and may have had influence on the SAL levels reported throughout detoxification. As biological specimen sampling was conducted early morning, pre-prandial and before the first cigarette of the day, it is unlikely that these variables would have an effect. Another confounding variable that may hinder interpretation of the results obtained from this study are the different pharmacotherapies used on the individual subjects. It is not known whether any of the therapies influence the distribution or disposition of the SAL enantiomers.

Overall, the variability observed within- and between-subjects was pronounced across all groups for all analytes tested. A general effect of time was observed with the both SAL enantiomers while DA concentrations were relatively consistent during alcohol abstinence. A slight increase was observed in average concentration across all subjects from admission day to day 15 of sampling. The variability in SAL concentrations associated with these time-points was large (> than 93% COV for S-SAL and R-SAL for admission day and day 15 across all subjects). Therefore, separate analyses for the factors of gender and smoking status were performed on the grand total mean, admission day, and day 15 concentrations for each analyte.

The effect of smoking status was not significant at any time-point evaluated. Although a trend was observed, with HS possessing higher SAL concentrations on day 15 compared to that of NS and LS groups, a statistically significant difference was not noticed. The effect of smoking status was further characterized into the design variables used for smoking status stratification, the FTND and cigarette smoking frequency. Characterization of subjects utilizing these variables did not yield a

significant result in explaining the variability associated with the SAL enantiomer concentrations. Further covariate analysis using the TLFB measure of chronic alcohol exposure, the BrAC measure of acute alcohol exposure, average dietary SAL intake, acamprosate administration throughout the detoxification period and admission drug of abuse screen, did not yield significant relation with R/S-SAL exposure. A measure of acute hepatocellular dysfunction, aspartate amino transferase (AST), showed an inverse relation to circulating SAL concentrations. An increase in AST (more hepatic impairment) yielded a decrease in plasma R/S-SAL enantiomers, suggesting that liver function was an important determinant of SAL exposure.

An attempt was made to assess a clinical endpoint, the CIWA-AR to SAL exposure, which proved unsuccessful. The lack of association could be due to the discrepancies involved with the biological sampling with the CIWA-AR measurement. Moreover a significant effect of time was observed with the CIWA-AR over three days with a decrease being observed in all patients. This time effect was not observed with the SAL enantiomers in the first three days, further precluding the correlation of these analytes with the alcohol withdrawal assessment.

Upon comparison with a healthy, non alcohol-dependent population, a surprising finding was observed. On average, the SAL concentrations observed in the healthy population were higher that that of the alcohol dependent population on admission day of detoxification, despite the fact that 37% of the alcohol dependent population has been recently exposed to alcohol (as deemed by entrance BrAC). Within each smoking status group, NS healthy and alcohol dependent patients showed

statistically equivalent concentrations. On the contrary, the LS and HS groups within the healthy population both possessed higher SAL concentrations than their alcohol dependent counterparts. A major limitation of this analysis is that the smoking population in the healthy subjects were required to smoke within thirty minutes of SAL sampling, while the alcohol dependent sampling was not controlled for this factor of recent smoking. The assessment of the time frame between last cigarette upon entrance into the clinic and the SAL sampling was not assessed in the alcohol dependent patients, hindering definitive interpretation of the results. Nevertheless, the results of clinical study #1 conclude that recent smoking of a cigarette influences SAL plasma concentrations. Throughout time, this observation was not observed in the alcohol dependent population as sampling, with the exception of admission day, occurred before the first cigarette of the day. The observational results from clinical study #2 may be confounded by several factors such as concurrent pharmacotherapies, other disease states, impaired liver function, and lack of rigid investigational control of smoking and dietary intake throughout the inpatient period.

A major critique of this analysis is that the two separate populations were compared to assess the circulating SAL differences, one study of a healthy population recently smoking and one study involving an alcohol dependent population on admission to a detoxification clinic that may or may not have recently smoked a cigarette. Ideally, a study consisting of observations in the same population, sampling before and after smoking, in a healthy and alcohol dependent population, with an

adequate sampling schedule would be needed to further support the notion of “true” smoking status differences.

CHAPTER 7

OVERALL CONCLUSIONS

TIQ and β-carboline exposure has been reported to be influenced by acute ethanol intake and chronic alcoholism. Significant variability within published studies has been reported, hindering the ability for these compounds to be an adequate marker for alcohol abuse. As the association of smoking and alcohol abuse is strong, it is suspected that the variability in TIQ and β-carboline exposure observed may be explained by tobacco smoking.

As the variability associated with measurements may be in part to the analytical methodology involved in quantification, two separate assays were developed and validated to assess biological concentrations of the β-carbolines, harman and norharman, and the TIQ's, R- and S-Salsolinol. Several of the reported bioanalytical methods used for the quantification of theses analytes in a biological matrix possessed many shortcomings including lack of internal standard use and unresolved chromatographic resolution between analytes of interest, decreasing the reliability of

accurate and precise quantification. Of utmost importance, none of the reported methodologies utilized a calibration matrix that was intended for use in actual sample analysis, presumably due to the lack of a true blank matrix. The bioanalytical methods developed in this investigation addressed the inadequacies associated with the reported methods. Significant attention of the analytical procedure development involved assessment of an appropriate surrogate matrix for calibration purposes. Using robust method development procedures and validation techniques, along with a thorough assessment of "surrogacy" of a modified matrix, two analytical methods used for the quantification of the β-carbolines and TIQ's in human plasma was established.

A robust, sensitive, selective and reproducible assay was developed for the quantification of the endogenous β-carbolines, harman and norharman in human plasma. This technique utilized protein precipitation via cold acetonitrile and phenyl SPE cartridges to isolate both β-carbolines from 2-ml plasma. Extraction efficiency was evaluated using yohimbine as an internal standard. Analyte separation was achieved via a commercial C_8 column with a ternary isocratic mobile phase consisting of methanol, acetonitrile and KH_2PO_4/H_3PO_4 buffer, resulting in acceptable resolution under optimal fluorescence detection conditions. A modified matrix, using a minimal dilution factor, was proven to show similarity in analytical response as that of unmodified, plasma sample matrix. The surrogate matrix was used to decrease the response of the observed constitutive harman and norharman concentrations, thus providing a "blank" matrix. Therefore, this modified matrix was used for calibration purposes and subsequently used for validation.

At physiologically relevant concentrations, method precision and accuracy were found to be acceptable for both inter- and intra-day measurements. A linear response was observed in 2 ml plasma for both analytes from the LLOQ of 6.3 pg/2 ml to the ULOQ of 1.0 ng/2 ml for each analyte, yielding acceptable linear model fits. The evaluation of endogenous plasma concentrations in healthy humans yielded vales that were consistent with literature values.

The current developed method for the β-carbolines, harman and norharman, has maintained resolution between analytes, utilized a novel internal standard to assess sample loss from extraction and was fully validated using and appropriate surrogate matrix. Moreover, this new method has maintained adequate sensitivity for physiological studies. The chromatographic separation conditions along with the optimized extraction technique and surrogate matrix calibration was used to support clinical investigation for the quantification if the β-carbolines in human plasma.

In the case of the TIQ's, a robust, sensitive, selective and reproducible assay was developed for the quantification of the endogenous, S- and R-SAL, along with their precursor DA, in human plasma. A direct single-step pentafluorobenzyl derivatization scheme in an aqueous media, without extractive alkylation using phase transfer catalysts, was devised for the enantioseparation of SAL with simultaneous detection of DA. In brief, this technique utilized phenylboronic acid cartridges to isolate the analytes from 1-ml plasma. An elution aliquot of acidified methanol was pH adjusted for subsequent, optimized, analyte derivatization with pentafluorobenzyl bromide. The final product was hexane extracted, evaporated to dryness, and the residue was

dissolved in methanol for the analysis by HPLC-ESI-MS/MS. Chiral separation was obtained via a commercial amylose-derivate based stationary phase HPLC column with a binary mixture of isopropanol and methanol as mobile phase. Deuterium-labeled individual SAL enantiomers along with deuterium labeled dopamine were used as internal standards. Detection was carried out via tandem mass-spectrometry and ESI mass spectra were acquired in positive ion mode with selected reaction monitoring. The resultant derivatives were stable and base-line resolved both SAL enantiomers as well as the DA precursor. A modified matrix involving destruction of constitutive SAL and DA was proven to be surrogate and subsequently used for calibration purposes.

At physiologically relevant concentrations, method precision and accuracy were found to be acceptable for both inter- and intra-day measurements. A linear response was observed in 1-ml plasma for both SAL enantiomers from the LLOQ of 20 pg to the ULOQ of 4 ng for each racemate, yielding acceptable correlation coefficients. For the DA precursor the range observed was between the LLOQ of 100 pg to the ULOQ of 10 ng, resulting in adequate linear calibration fits. For both SAL enantiomers, the average extraction recovery was 56 ± 5% within the concentration range. Evaluation of endogenous concentrations in healthy, human plasma yielded results that were in the reported physiological range for R/S-SAL and DA.

In comparison to reported methodologies for the quantification of R/S-SAL and DA in human plasma, this procedure has surmounted the limitations aforementioned. The optimized chromatography has preserved the baseline resolution of the both enantiomers throughout the concentration range, improving the reliability of

quantification. Quantification with the isotopically labeled internal standards of R- and S-SAL with DA yielded suitable assay performance results for the quantitative bioanalytical HPLC-MS/MS assay.

Employment of both of the sensitive and reproducible assays was suspected to circumvent any variability associated with the quantification of the β-carbolines and TIQ's in human plasma. Therefore, these assays were subsequently used to support the two separate clinical investigations.

Clinical Study #1 was a pilot study in forty-one male and female volunteers to study the effects of gender smoking on TIQ and β-carboline exposure. The outpatient study was non-interventional, designed to evaluate measurements of plasma TIQ's and β-carbolines in nonsmokers and in a smoking population who had just recently smoked a cigarette. Subjects were stratified into groups of non-smokers (NS), light-smokers (LS) and heavy smokers (HS), based on the number of cigarettes smoked per day and the Fagerström Test for Nicotine Dependence (FTND). Subject participation involved two morning outpatient visits in which a single blood sample was taken on each visit for the quantification of plasma TIQ's and β-carbolines.

Overall, the variability observed between subjects was pronounced across all groups for both β-carbolines and the SAL enantiomers. The effect of smoking status was significant within this study with the primary difference being between nonsmokers and smokers. Although a trend was observed, a statistically significant difference was not noticed between the two LS and HS smoking groups. The difference in exposures of the β-carbolines between smokers and nonsmokers are presumed to be resultant of

the acute inhalation of β-carbolines from tobacco smoke. Along with the inhaled β-carbolines, the endogenous formation of the β-carbolines via condensation of acetaldehyde biogenic amines may also contribute to the overall exposure. On the other hand, the SAL enantiomers and dopamine are not known to be constituents of tobacco smoke. It is presumed that the acute exposure of acetaldehyde from the tobacco smoke, along with the subsequent condensation with dopamine, is responsible for the divergence of SAL concentrations between nonsmokers and smokers. It was also observed that there were inherent dopamine differences between smoking status groups. This effect was presumed to be caused by induction of a stress response upon smoking, thereby releasing dopamine.

An attempt was made to characterize true baseline differences between smoking status groups with evaluation of a population of smokers who abstained from smoking for 15 hours. A significant baseline difference was observed for both salsolinol enantiomers and norharman within this study with the primary difference being between the heavy-smokers and nonsmokers. This is presumed to be a function of a true constitutive difference between smokers and nonsmokers or an additive accumulation of SAL enantiomer and norharman concentrations within the body. When compared to the study involving smoker's recent exposure to tobacco smoke, the difference between nonsmokers and smokers was more pronounced. This suggests that, in addition to a supposed baseline difference, inhalation of tobacco smoke provides additional exposure to circulating TIQ and β-carbolines contributing to the incongruity of concentrations between smoking status groups. As the difference between the smoking status groups

was much greater after smoking a cigarette, it was concluded that the effect on the analyte concentrations are due to recent tobacco smoke inhalation.

The design of the investigation of baseline difference between smoking status groups possessed few shortcomings. The primary study was not balanced with respect to gender and smoking status. Moreover a strong correlation was observed between the smoking status groups and measures of annual ethanol intake, hindering interpretability of the resultant smoking status effect. For all analytes, a large inter-occasion variability was observed between observational periods. The sampling schedule, with respect to inhalation of tobacco smoke, required more rigid control to minimize variability associated with the separate sampling occasion. A formal conclusion with respect to this study cannot be deduced without full understanding of the pharmacokinetics of the analytes in question. These insufficiencies confound the results and variability associated between smoking status groups. Of note, a gender difference was not observed but the true difference may have been masked due to the unbalanced design for this factor.

The interrelationship between smoking and alcoholism is strong and has been exemplified by several researchers. As smoking status had a significant effect on TIQ and β-carboline exposure in a healthy population, it is expected that this effect would be observed in an alcoholic population. Therefore, a comparison was made to a second study involving an alcohol-dependent cohort undergoing detoxification treatment at the National Institutes of Health - National Institute on Alcohol Abuse and Alcoholism. This investigation evaluated detoxification-induced changes in plasma TIQ

concentrations in n = 35 alcoholics undergoing a four-week, inpatient alcohol abstinence program. Using the same criteria used from study #1, subjects were stratified with respect to smoking status of NS, LS and HS. Converse to the study involving healthy patients this analysis was balanced for gender.

Plasma samples were collected during the first two weeks of detoxification: on admission, day 2, 3, 8, and 15 days after enrollment. A clinical endpoint, the Clinical Institute Withdrawal Assessment-Alcohol Revised (CIWA-AR), was used to assess a possible correlation of these levels to withdrawal symptoms. Plasma TIQ levels were assessed along with CIWA-AR, smoking history and exposure, and alcohol dependence measurements in order to assess their feasibility as a clinical biomarker for smoking and alcohol dependence. Importantly, the time-course of these compounds during early abstinence in an alcohol-dependent cohort was assessed. Evaluation of the contribution of smoking to levels of these compounds and, ultimately, the time-course was the primary objective of this study.

Overall, the variability observed within- and between-subjects was pronounced across all groups for both SAL enantiomers. A slight increase was observed in average R- and S-Salsolinol concentrations across all subjects from admission day to day 15 of sampling, while DA concentrations were relatively consistent during alcohol abstinence. Separate analyses for the factors of gender and smoking status were performed on the grand total mean, admission day, and day 15 concentrations for each analyte.

At every time point assessed, the effect of smoking status or gender was not significant. Further covariate analysis using the a measure of chronic alcohol exposure the TLFB, the BrAC measure of acute alcohol exposure, average dietary SAL intake, acamprosate administration throughout the detoxification period and admission drug of abuse screen, did not yield significant relation with R/S-SAL exposure. It is interesting to note that measures of alcohol intake such as the TLFB and acute ethanol intake, the BrAC did not yield a significant correlation. These results refutes majority of the published literature with regard to SAL concentrations and ethanol intake. A measure of acute hepatocellular dysfunction, aspartate amino transferase (AST), showed an inverse relation to circulating SAL concentrations at admission day suggesting that SAL exposure may be dependent on liver pathophysiology. It was concluded in the investigation that the discrepancies observed may have been due, in part, to liver dysfunction.

Upon comparison with a healthy, non alcohol-dependent population, the SAL concentrations observed in the healthy population were higher that that of the alcohol dependent population on admission day of detoxification. These results may have been due to the fact that the healthy subjects were required to smoke within thirty minutes of SAL sampling, while the alcohol dependent sampling was not controlled for this factor of recent smoking. The assessment of the time frame between last cigarette upon entrance into the clinic and the SAL sampling was not assessed in the alcohol dependent patients, hindering definitive interpretation of the results. The observational results from clinical study #2 may be confounded by several factors such as concurrent

pharmacotherapies, other disease states, impaired liver function, and lack of rigid investigational control of smoking and dietary intake throughout the inpatient period, other drugs of abuse and the heterogeneity of the subject demographics.

It was found that, in a healthy population, a noteworthy trend was observed between smoking status and TIQ and β-carboline exposure. This trend is hypothesized to be a product of a combination of constitutive endogenous differences between smoking status groups and exposure via the inhalation of the analytes themselves and/or inhalation of precursors required for endogenous synthesis, acetaldehyde. Moreover, TIQ exposure has been reported to be influenced by acute ethanol intake and chronic alcoholism. Significant variability within these studies has been reported, hampering the ability for these compounds to be an adequate marker for alcohol abuse. As the association of smoking and alcohol abuse is strong, it is suspected that the variability in TIQ exposure observed may be explained by tobacco smoking. In the investigation with alcohol dependent subjects smoking status or gender was not significant with respect to TIQ levels, at any point during detoxification. Moreover, our study concluded that alcohol-dependent patients did not have higher concentration of circulating TIQ's compared to the healthy patients, which contests all published reports.

From the information provided from this investigation, SAL enantiomers do not seem to be viable biomarker candidates for alcoholism. The specificity of these compounds as state markers of alcoholism is compromised by factors such as liver dysfunction. Moreover, other factors such as other drugs of abuse or additional pharmacotherapies may further complicate the use for SAL as a biomarker. Of most

importance, the use of the SAL measurement was unable to discriminate between a healthy population and an alcohol dependent population, further negating the use of SAL and an alcoholism marker. It was observed that smoking does influence SAL and β-carboline concentrations in a healthy population. Further research is warranted in the evaluation of the pharmacokinetics of these compounds to support additional smoking biomarker studies.

The plasma concentrations of TIQ and β-carboline exposure are assumed to be reflective of central dopaminergic activity. The concentration difference between smokers and nonsmokers suggest that nicotine dependent subjects may require maintenance of these higher concentrations in order to experience feelings of pleasure, simultaneously circumventing negative symptoms of nicotine withdrawal. In the case of alcohol dependent subjects, concentrations of TIQ's were actually lower than that of healthy subjects. This difference between the populations infers that the alcohol-dependent person may drink in order to attain higher concentrations of TIQ's for feelings of pleasure and reward. Further studies evaluating the acute cravings of alcoholics and smokers need to be conducted in order to substantiate these hypotheses.

Behavioral studies do indicate that nicotine and alcohol are addictive and that these drugs reinforce self-administration. This phenomenon is reported to be governed by the mesolimbic dopamine system. A major assumption in this investigation was that central TIQ and β-carboline concentrations reflect to those observed in plasma. Further investigations, including pharmacokinetic and central nervous system concentration assessment, would help corroborate the plasma TIQ and β-carboline

concentrations within the presumed *in-vivo* effects within the mesolimbic dopaminergic system. TIQ and β-carboline exposure within this "reward pathway" may play a synergistic role, along with the pharmacological actions of nicotine and alcohol, in the reinforcing aspects of tobacco smoking and chronic alcoholism.

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APPENDICES

Appendix A

Fagerström Test for Nicotine Dependence
Heatherton R, Kozlowski L, Frecker R and Fagerström K.
Br. J. Addiction. 1991; 86, 1119-1127.

1. How soon after you wake up do you smoke your first cigarette?

_____ After 60 minutes

_____ 31 – 60 minutes

_____ 6 – 30 minutes

_____ Within 5 minutes

2. Do you find it difficult to refrain from smoking in places where it is forbidden (e.g. library, cinema, church?)

_____ No

_____ Yes

3. Which cigarette would you be the most unwilling to give up?

_____ First one in the morning

_____ Any other

4. How many cigarettes a day do you smoke?

_____ 1 –10

_____ 11-20

_____ 21-30

_____ 30 +

5. Do you smoke more during the first hours in the morning than during the rest of the day?

_____ No

_____ Yes

6. Do you smoke if you are so ill that you are in bed all day?

_____ No

_____ Yes

Appendix B

Subject Stratification Criteria

Subject Group	**Inclusion/Exclusion Criteria**
Nonsmoker **(NS)**	1. No current tobacco product use 2. Not a smoker for the past 5 years 3. If previously a smoker, did not smoke more than once a year continuously and < 10 cigarettes/year 4. FTND score = 0
Light-smoker **(LS)**	1. Current smoker of cigarettes (No other tobacco products) 2. Smokes at least 10 cigarettes/day 3. Smoked for at least 1 year continuously 4. FTND score 1-7
Heavy-smoker **(HS)**	1. Current smoker of cigarettes (No other tobacco products) 2. At least more than 20 cigarettes/day 3. Smoked for at least 1 year continuously 4. FTND score > 7

Appendix C

Annual Alcohol Intake (AAI) Inventory

Khavari KA and Farber PD 1978. J. Stud. Alcohol. 39: 1525-1539.

Please answer the following questions as accurately as you can:

1. How often do you drink beer?
   _____ Never had beer
   _____ Tried, but not currently drinking
   _____ Once a year
   _____ Twice a year
   _____ Three or four times a year
   _____ Once a month
   _____ Twice a month
   _____ Three or four times a month
   _____ Once a week
   _____ Twice a week
   _____ Three or four times a week
   _____ Daily

2. What is the amount of beer that you usually drink per occasion?

3. What is the maximum amount of beer you drink on any one occasion?

4. How often do you drink this maximum amount of beer?
   _____ Never had beer
   _____ Tried, but not currently drinking
   _____ Once a year
   _____ Twice a year
   _____ Three or four times a year
   _____ Once a month
   _____ Twice a month
   _____ Three or four times a month
   _____ Once a week
   _____ Twice a week
   _____ Three or four times a week
   _____ Daily

5. How often do you drink wine?
   _____ Never had wine
   _____ Tried, but not currently drinking
   _____ Once a year
   _____ Twice a year
   _____ Three or four times a year
   _____ Once a month
   _____ Twice a month

_____ Three or four times a month
_____ Once a week
_____ Twice a week
_____ Three or four times a week
_____ Daily

6. What is the amount of wine that you usually drink per occasion?

7. What is the maximum amount of wine you drink on any one occasion?

8. How often do you drink this maximum amount of wine?
_____ Never had wine
_____ Tried, but not currently drinking
_____ Once a year
_____ Twice a year
_____ Three or four times a year
_____ Once a month
_____ Twice a month
_____ Three or four times a month
_____ Once a week
_____ Twice a week
_____ Three or four times a week
_____ Daily

9. How often do you drink distilled spirits?
_____ Never had distilled spirits
_____ Tried, but not currently drinking
_____ Once a year
_____ Twice a year
_____ Three or four times a year
_____ Once a month
_____ Twice a month
_____ Three or four times a month
_____ Once a week
_____ Twice a week
_____ Three or four times a week
_____ Daily

10. What is the amount of distilled spirits that you usually drink per occasion?

11. What is the maximum amount of distilled spirits you drink on any one occasion?

12. How often do you drink this maximum amount of distilled spirits?

_____ Never had distilled spirits
_____ Tried, but not currently drinking
_____ Once a year
_____ Twice a year
_____ Three or four times a year
_____ Once a month
_____ Twice a month
_____ Three or four times a month
_____ Once a week
_____ Twice a week
_____ Three or four times a week
_____ Daily

Appendix D

TIQ/BC Food Inventory

INSTRUCTIONS: Place a check mark in the appropriate column to indicate how frequently you consume each the following foods.
Then place a check mark to indicate your typical serving size when consuming these foods.

TYPE OF FOOD	HOW OFTEN									HOW MUCH			
	Never or less than 1 per month	1-3 per month	1 per week	2-4 per week	5-6 per week	1 per day	2-3 per day	4 per day	5+ per day	Medium Serving Size	Your Serving Size		
											Small	Medium	Large
Tomatoes										1/2 cup			
Tomato Sauce										1/2 cup			
Tomato Paste										1/2 cup			
Bananas										1 Medium Size			
Chocolate Covered Doughnuts										1 Doughnut			
Chocolate Filled Doughnuts										1 Doughnut			
Chocolate Cookie Sandwich										1 Cookie			
Chocolate Covered Raisins										10 pieces			
Chocolate Syrup										1 T			
Chocolate Pie										1/8 pie			
Chocolate Cake										1/6 cake			
Chocolate Fudge										.5 oz piece			
Chocolate Cereal										3/4 cup			
Chocolate Candy Bar										1 bar (regular size)			
Chocolate Chip Cookie										1 medium cookie			
Chocolate Candy (M&M, Rolos)										1 pkg			
Chocolate Covered Ice Cream Bar										1 bar			
Chocolate Ice Cream/ Yogurt										1/2 cup			
Chocolate Pudding/Mousse										1/2 cup			
Chocolate Milk										8 fluid oz			
Cocoa										8 fluid oz			
Hot Chocolate										8 fluid oz			
Charred Beef										3 oz			
Charred Chicken										3 oz			
Charred Fish (specify type)										3 oz			
Charred Pork										3 oz			
Fish, other than charred (specify type)													

TYPE OF FOOD	HOW OFTEN									HOW MUCH			
	Never or less than 1 per month	1-3 per month	1 per week	2-4 per week	5-6 per week	1 per day	2-3 per day	4 per day	5+ per day	Medium Serving Size	Your Serving Size		
											Small	Medium	Large
Soy Sauce										3oz			
Ketchup										1 T			
Fish Supplements/Oils													
Instant Caffeinated Coffee										6 fluid oz			
Brewed Caffeinated Coffee										6 fluid oz			
Cappuccino										6 fluid oz			
Mocha										6 fluid oz			
Caffeinated Soda										12 fluid oz			
Instant Caffeinated Tea										8 fluid oz			
Brewed Caffeinated Tea										6 fluid oz			
Supplements (Chocolate Flavored)										8 fluid oz			
Red Wine										3.5 fluid oz			
White Wine										3.5 fluid oz			
Beer										12 fluid oz			
Distilled spirits (liquor)										1.5 fluid oz			
Liqueurs										1.5 fluid oz			
Alcohol Coolers										12 fluid oz			
Champagne										3.5 fluid oz			
Other (please Specify)													

Appendix E

Protocol and Informed Consent Form – Clinical Study #1

Project Title:	**A Pilot Study to Determine Tetrahydroisoquinoline (TIQ) and β-Carboline Levels in Nonsmokers, Light-Smokers and Heavy Smokers**
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Table of Contents

A Pilot Study to Determine Tetrahydroisoquinoline (TIQ) and β-Carboline Levels in Nonsmokers, Light-Smokers and Heavy Smokers

Abstract

Nicotine dependence is a complex biological and behavioral problem that can be extremely difficult to overcome. An improved overall understanding of drug dependence, coupled with the identification of nicotine as a drug with dependence potential, has been instrumental in the development of medications and behavioral treatments for nicotine dependence. Researchers are beginning to find out that there may be chemical ingredients other than nicotine in cigarette smoke that contribute to tobacco's addicting potential.

Two classes of endogenously formed compounds, tetrahydroisoquinolines (TIQ) and β-carbolines, have been involved in adaptive brain mechanisms that may advance to drug dependence. These substances have been found to react with specific CNS neuro-receptor system activity (notably dopaminergic and serotonergic receptors, see below). Of primary importance is that TIQ's and β-carbolines may activate the brain circuitry that regulates feelings of pleasure, the so-called "reward pathways". In turn, both of these substances might be responsible in nicotine seeking behavior and dependence. The anticipated results of the proposed pilot study are essential to assess a possible relationship between blood TIQ's and β-carbolines with smoking history and dependence, if it is likely to exist. The study is also intended to evaluate statistically the inter- and intra-individual variability in the TIQ and β-carbolines levels as well as the smoking history/nicotine dependence scores of nonsmokers, light-smokers, and heavy-smokers.

In the proposed study, sixty (60) healthy young volunteers (twenty nonsmokers, twenty light-smokers, and twenty heavy-smokers) will supply blood samples for determination of baseline levels of TIQ's and β-carbolines. During outpatient visits, nicotine and cotinine plasma concentrations will also be measured from the urine collected to evaluate systemic self-exposure. An evaluation of their smoking history and nicotine dependence score will be used along with the baseline levels of TIQ's and β-carbolines to determine any correlation between the compounds of interest and the degree of nicotine exposure and dependence.

If such a relationship can be revealed, future, more detailed, interventional studies will be designed to assess the suitability of these endogenous compounds as possible biomarkers of nicotine dependence.

Background

In the search for an explanation for the mechanism in which drug dependence develops, researchers have explored possible theories for why individuals become addicted to specific drugs including alcohol, nicotine, opiates and other drugs of abuse. Of note, two classes of endogenously formed compounds, tetrahydroisoquinolines (TIQ) and β-carbolines, have been indicted as chemicals that may mediate mechanisms thought to be involved in dependence.

TIQ's are a class of partially aromatic alkaloids that include salsolinol, 1-carboxysalsolinol, tetrahydropapaveroline, and salsoline. TIQ's are compounds that are formed as a result of the condensation reaction between dopamine and acetaldehyde or pyruvate (1) and are natural metabolites of dopamine produced in the brain as well as other organs (2). TIQ's also occur naturally such as in wine and bananas (3). Tetrahydropapaveroline (THP) is the dopamine - 3,4, -dihydroxyphenyl acetaldehyde condensation product whereas salsolinol (SAL) is the dopamine-acetaldehyde condensation product (4). It is known that SAL exists in two chemical conformations, the S-enantiomer and the R-enantiomer (2). Previous studies have found that the R-enantiomer or racemic mixture of SAL predominates in the urine of normal healthy subjects whereas the S-enantiomer predominates in the urine of alcoholics (3).

β-carbolines are another class of compounds such as noreleagnine, harman and norharman. β-carbolines are aromatic alkaloids that are formed via the condensation of tryptophan or indolealkylamines with aldehydes (5). These compounds exist endogenously in humans under normal conditions, however some are also formed after the ingestion of alcohol and further more have been shown to increase ethanol consumption in rats (6). These compounds also exist in plants that have psychotropic properties as well in food such as in charred meat and fish as well as in cigarette smoke (5). Studies have shown that norharman may interact with several receptor systems including benzodiazepines as well as serotonin and dopamine in higher concentrations (7).

Interestingly, previous studies have found that upon chronic injection of TIQ's and β-carbolines causes an increase in alcohol intake (4, 6, 8). Specifically, rats infused intraventricularly with 4.0 μg of salsolinol increased alcohol intake from 0.74 to 4.9 gm/kg/day (8). Additionally, 4 rats that were infused with 4.0 μg of noreleagnine also had an increase in alcohol consumption from 0.75 to 6.0 gm/kg/day (8). In another study, single infusions of THP ranging from a dose of 0.1 - 1.0 μg increased alcohol consumption from 0.62 to 4.38 g/kg/day in the non-alcohol-preferring strain of Sprague-Dawley rats (6). Furthermore, when unanesthetized rats were infused with THP and tryptoline (TLN), a β-carboline, the release of C-dopamine in the caudate nucleus and nucleus accumbens was significantly increased (9). A later study (7) involving injections of a range of doses on norharman showed that administration of doses of 2.44 μmol/kg and 43.97 μmol/kg induced an increase of dopamine efflux by 70% and 160%, respectively; however, with the administration of 7.33 μmol/kg there was a 72% decrease in dopamine efflux from baseline (7). This was thought to indicate that norharman must influence the mesolimbic dopaminergic neurons in a U-shaped dose-response curve, and the authors suggest that norharman is affecting the dopaminergic system via different receptors, namely MAO-A, MAO-B and non-MAO binding site.

As it has been noted in the literature, the modulation of drinking behavior and the consequent interaction of TIQ's and β-carbolines with the dopaminergic system demonstrate that these compounds may have a role in alcohol and drug dependence. The dopaminergic system has been well established as the "reward system" in the brain. Therefore, compounds that interact with the nucleus accumbens and the dopaminergic neurons could have a significant role in drug dependence. Furthermore, as the dopaminergic system has a significant role in the drug-seeking behavior, it is possible that other drugs of dependence such as nicotine or other ingredients of cigarette smoke may also interact with the TIQ's and β-carbolines. Additionally, these compounds could possibly mediate smoking behavior.

In this study, we seek to collect preliminary data on the endogenous baseline levels of TIQ's and β-carbolines in nonsmokers, light-smokers and heavy-smokers to assess if there are any gross differences between the three groups. This information may aid the development of further interventional studies where the administration of these compounds could be performed to investigate if there is an effect on cigarette smoking behavior.

Objectives

The objective of this study is to obtain preliminary information on the baseline levels of TIQ's and β-carboline concentrations in the blood of non-, light-, and heavy-smokers. Additionally, venous nicotine and cotinine plasma levels will be determined as a measure of systemic exposure due to smoking. Specific aims of this study include:

1. Assessment of a possible association between the levels of blood TIQ's and β-carbolines with the smoking history and dependence of the volunteers to determine if the line of research in smokers should be continued;
2. Statistical measurement of inter- and intra-individual variability in the levels and smoking history/nicotine dependence scores, which would permit formal sample size calculations in future studies.
3. Classification of volunteers according to smoking history and nicotine dependence with the Fagerström Scale (Appendix I) in nonsmokers, light smokers, and heavy smokers; further

classification by personality type using the TCI personality survey (Temperament and Character Inventory).

The anticipated follow-up study would involve a larger sample size along with possible interventions to further characterize plasma levels of TIQs and β-carbolines in order to:

1. establish TIQs and β-carbolines as biomarkers of nicotine dependence.
2. be able to predict treatment success of nicotine-replacement and cessation therapies for individuals of varying degrees of nicotine dependence.

Methods

Study Design

Sixty young healthy male and female volunteers, aged 21-35, will be recruited, including 20 nonsmokers, 20 light-smokers, and 20 heavy-smokers as follows:

Nonsmoker	Male	10
	Female	10
Light-Smoker	Male	10
	Female	10
Heavy-Smoker	Male	10
	Female	10

The study will involve two visits in which the volunteer will present to the Clinical Research Center. Prior to participation, the subject will be required to take a telephone interview for qualification purposes (Appendix VI). Upon qualification, the subject will be notified of any abstentions required before each visit. The abstentions include: no prescription/OTC/herbal medications or caffeinated products for 72 hours prior to each visit (with exception of oral contraceptives prescription for females) and no alcoholic beverages 12 hours prior to each visit. The subject will be given a chance to decline participation in the study or, if necessary, to seek his/her personal physician's advice as to whether to discontinue any medications he/she may be on. During the first visit, the volunteer will complete forms including: Medical History (Appendix. V), Subject Entry Probe (Appendix. IV), Smoking History (Appendix. II), Annual Alcohol Intake (AAI, Appendix. IX), and Zuckerman's Sensation Seeking Scale (Appendix. VIII). Additionally, during the first visit, the subject will take a Breathalyzer and carbon monoxide test as well as give a 60-ml blood sample for estimation of TIQ and β-carbolines levels. The volunteer will also provide a urine sample for drugs of abuse screen and nicotine/cotinine levels. During the second visit, only the breath tests, blood and urine sample will be repeated. Additionally, the Temperament and Character Inventory (TCI) (App. X) will be administered as a computer test. A pregnancy test will be given to female subjects and repeated if the time elapsed between the two visits exceeds one week.

Inclusion/Exclusion Criteria

Potential subjects will be screened over telephone for inclusion/exclusion criteria. A general health questionnaire will be administered over the telephone regarding their medical history, substance use, medications, and patient characteristics such as height and weight (Appendix VII). In addition to the general health questionnaire, the Fagerström Test of Nicotine Dependence, (FTND) (10,11, Appendix I) will be administered to determine their dependence on nicotine in order to classify potential subjects into three groups: Nonsmokers, Light-smokers, and Heavy-smokers. The following criteria must be met for subjects in each group:

Subject Group	Inclusion/Exclusion Criteria
Nonsmoker	1. No current tobacco product use 2. Not a smoker for the past 5 years 3. If previously a smoker, did not smoke more than once a year continuously and no more than 10 cigarettes/year 4. FTND score = 0
Light-smoker	1. Current smoker of cigarettes (No other tobacco products) 2. Smokes at least 10 cigarettes/day 3. Smoked for at least 1 year continuously 4. FTND score 1-7
Heavy-smoker	1. Current smoker of cigarettes (No other tobacco products) 2. At least more than 20 cigarettes/day 3. Smoked for at least 1 year continuously 4. FTND score > 7

In the situation that the smoking history and FTND scores put them in two different categories (specifically for light- and heavy-smokers), preference will be given to the smoking history (number of cigarettes/day) over the FTND score.

Along with the Fagerström Test for Nicotine Dependence scores, personality surveys will be administered in order to characterize the study population in terms of their personality traits and alcohol use. Personality surveys including the Temperament and Character Inventory, TCI (17,18, App. X), Zuckerman's Sensation Seeking Scale, ZSSS (12, App. VIII). In addition, the Annual Alcohol Intake, AAI (13, App. IX), will be administered to all subjects to assess concomitant alcohol intake as a potential major covariate for TIQ's.

Cloninger et al. (17, 18, 19) developed a Temperament and Character Inventory (TCI) designed to assess personality traits that may be associated with activity of central neurotransmitter systems. The TCI (App. X) consists of 240 true/false questions that will
provide the basis for determining specific personality traits. The personality characteristics measured incorporate: 1) novelty seeking, 2) harm avoidance, 3) reward dependence, 4) persistence, 5) self-directedness, 6) cooperativeness and 7) self transcendence (17, 18). This inventory was originally based on the concept that each factor is a function of brain neurochemical transmitter systems, i.e., dopamine, serotonin, and/or norepinephrine. The original research suggested that three of the characteristics evaluated were a function of one of these neurotransmitters: novelty seeking to dopamine; harm avoidance to serotonin; and reward dependence to norepinephrine (17,18). Human research suggests that there is a correlation between personality and neurochemistry but the data are not overwhelming (19).

The ZSSS (12, App VIII) is designed to characterize subjects according to their interests and preferences. The survey features 40 questions of two choices of answers.

The AAI (13, App. IX) survey will be primarily used to assess a potentially significant covariate for TIQs: Several human studies have noted that norharman (a β-carboline) levels are significantly elevated in chronic alcoholics (15). Furthermore, chronic alcoholics who have undergone controlled abstinence have shown two-fold higher levels of norharman compared to control subjects at the beginning of the abstinence period, with a gradual decline in levels at the end of the 3-week abstinence period (16).

The results of these surveys will allow for better characterization of the study population and permit comparison with other clinical studies.

Female subjects must not be pregnant during the clinical study and must be using acceptable methods of contraception (abstinence, barrier methods, or oral contraceptives). However, females that are not using oral contraceptives must have regular menstrual cycles of 28-32 days on average and must not have dysmenorhea. Subjects who satisfactorily pass the screening exam will be enrolled in the study.

Volunteers who have passed the initial telephone screening will be invited to the General Clinical Research Center (GCRC) for an out patient visit including medical history (particularly personal or family history of psychiatric disorders and/or drug dependence other than smoking), blood pressure, and vital signs in order to ensure the health of the subject. Urine test for drugs of abuse, breath alcohol and breath carbon monoxide tests will be done to ensure that the subject does not abuse other drugs, is abstinent from alcohol, and to measure nicotine exposure, respectively. Personality surveys including the Temperament and Character Inventory (TCI) (17,18, App. X), Zuckerman's Sensation Seeking Scale (12, Appendix. VIII), and AAI (Annual Alcohol Intake (13, Appendix IX) will be administered to all subjects. The results of these surveys will allow for better characterization of the study population and permit comparison with other clinical studies.

Endpoints and Measurements

For each visit period, the subject will be admitted as an outpatient to the GCRC between 8 AM-10AM. During this time, subjects classified as smokers will smoke a cigarette, and blood and urine samples will be collected within 30 minutes of smoking. Upon entrance to the GCRC, a breath carbon monoxide test and Breathalyzer test will be administered. A subject entry probe will also be given to determine their adherence to the 12-hour abstention from alcohol and caffeinated products in addition to the 72-hours abstention from medications.

Before the blood samples are collected from the subject, Annual Alcohol Intake and Zuckerman's Sensation Seeking Scale are administered (Appendices VIII and IX). Subsequently, a urine specimen will be taken to evaluate drugs of abuse test and nicotine/cotinine levels. A 60-ml blood sample will be collected from the non-dominant forearm in a reclined, seated position during the study. Sitting blood pressure, heart rate, and body temperature will be measured as safety precautions. The blood samples will be centrifuged to obtain plasma and serum, and plasma samples will be stored at -70°C until analysis. Nicotine and cotinine plasma and urine concentrations will be determined by gas chromatography/mass spectroscopy method developed and validated by the Biopharmaceutical Analysis Laboratory at the Virginia Commonwealth University Department of Pharmaceutics (14). A blood sample will be sent to Dr. H. Rommelspacher at Freie Universität Berlin in Berlin, Germany to determine levels of tetrahydroisoquinolines and β-carbolines (1,2); this sample will not have any identifying information about the subject to ensure confidentiality.

The subject will discharged in approximately two hours pending an evaluation by the nursing staff for lack of adverse events.

The second visit will consist of all of the aforementioned with exception of the medical history, smoking history, and personality survey, Annual Alcohol Intake, and Zuckerman's Sensation Seeking Scale). The Temperament and Character Inventory (TCI, App. X) will also be administered during this period.

Data Analysis

Intra-individual variability (e.g., COV%) will be calculated for all measured endpoints, i.e., TIQ and β-carboline concentrations, nicotine and cotinine urine concentrations for each volunteer. Appropriate summary statistics such as mean, median, COV%, percentiles and range will be computed.

Inter-individual variability (i.e., COV% and range) will be calculated for the above endpoints as well as all the rating scale scores for each of the three groups and across all groups (see ANOVA, below). These variability measures will allow formal sample size calculations for future crossover or parallel-group studies.

Both TIQ and β-carboline mean concentrations will be correlated with the measures of cigarette exposure (nicotine and cotinine mean urine concentrations) and dependence (Fagerström rating scale scores) using Spearman's rank sum correlation coefficient.

All the above endpoints will be compared with smoking history and gender by means of two-way ANOVA. If the overall ANOVA is significant at the $p<0.05$ level, this will followed by a Scheffé test to isolate group differences.

For all tests and statistics, the raw data may be log- or rank-transformed to comply with the parametric assumptions of equal variance across groups and normal distribution of the residuals. If necessary, appropriate nonparametric tests, e.g., Wilcoxon U-test, will be performed.

Description of Human Subject Protection

Subject Selection

Subjects participating in the study will have passed outpatient screening including past medical history and drug screen. This will exclude participation of subjects with significant disease states including renal, hepatic, neurological, cardiovascular, gastrointestinal, pulmonary, neurological and psychiatric diseases. Female subjects must not be pregnant during the clinical study and must be using acceptable methods of contraception.

Prior to enrollment, all subjects will be explained the objectives, methods, benefits, risks, and inconveniences of the study: they will be required to sign the VCU IRB-approved Informed Consent Form. They will be paid an honorarium for the time commitment and inconveniences that the study may entail.

Inclusion of Children

Children (defined as individuals under the age of 21 years) are to be excluded from the study. For this particular study, it would be inappropriate to recruit smoking adolescents since it may reward their smoking habits, which should be discouraged.

Risks and Safety Monitoring

This investigation does not require the introduction of a study drug or device. Therefore, any risks or side effects associated with a therapeutic drug or device should not be observed.

A total of one (1) blood sample will be drawn during each study period. The total amount of blood from each session will be 60 ml (about 4 tablespoons) and about a total of 120ml (less than half a blood donation) over the entire duration of the study. Obtaining these blood samples may cause some discomfort, pain, or slight bruising around the site of the needle stick. Sometimes, fainting or infection may occur.

During the two outpatient periods, the subjects' vital signs will be recorded and will be monitored for the appearance of any adverse events by the GCRC nursing staff. In addition, a Medical Monitor will be available to monitor for signs of adverse events associated with blood drawing such as: mental confusion, dizziness, and weakness.

Risks associated with the aforementioned procedures involving the subject are generally minimal to none. During participation of the study, there is no direct benefit for the subject. If necessary, adverse events will be treated and followed up until resolution.

Confidentiality of Records

Medical records, consent forms, and collected data, which identify the subject, may be looked at and/or copied for research or regulatory purposes by:

- the FDA;
- Department of Health and Human Services (DHHS) agencies;
- Virginia Commonwealth University (VCU);
- Governmental and/or regulatory agencies to the extent required by law.

Blood samples will be sent to our collaborator, Dr. Rommelspacher without any identifying confidential patient information. Absolute confidentiality will not be guaranteed because of the need to give information to these parties. The results of this research study may be presented at meetings or in publications. The identity of the subject will not be disclosed in those presentations.

Dose Selection:

A therapeutic drug dose or medical device will not be introduced to the subject by the investigators or the GCRC staff at any time throughout the study.

Expected Results and Directions for Future Research

Building on a series of recent scientific findings suggesting that, independent of a drug's initial site of action, a number of drugs of abuse appear to increase the levels of the neurotransmitter dopamine in a particular brain pathway, TIQ's and β-carbolines may act as a link between nicotine dependence and this common reward producing pathway.

Several studies have reported that with injection of TIQ's and β-carbolines, there is an increase in alcohol intake (4, 6, 8). Several human studies have noted that norharman levels are significantly elevated in chronic alcoholics (15). Furthermore, chronic alcoholics who have undergone controlled abstinence have shown two-fold higher levels of norharman compared to control subjects at the beginning of the abstinence period, with a gradual decline in levels at the end of the 3-week abstinence period (16). As nicotine is also a drug of dependence that may interact with the dopaminergic reward pathways, we expect that smokers should have higher levels of TIQ's and β-carbolines compared to nonsmokers. Moreover, heavy smokers are expected to have higher levels of TIQ's and β-carbolines as compared to light-smokers.

With data from this pilot study, a larger follow-up study will be done to further characterize plasma levels of TIQs and β-carbolines in order to establish TIQs and β-carbolines as biomarkers of nicotine dependence and consequently be able to predict or identify individuals of varying nicotine dependencies.

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RESEARCH SUBJECT INFORMATION AND CONSENT FORM

Title:	Tetrahydroisoquinoline and β-carboline Levels in Healthy Nonsmokers, Light-Smokers, and Heavy Smokers
Protocol No.:	VCU IRB #1990
Principal Investigator:	Jürgen Venitz, M.D., Ph.D. (804) 828-6249 (804) 997-9261 (pager) jvenitz@vcu.edu
Co-Investigators:	Satjit Brar, B.S., Pharm.D./Ph.D. candidate
Site:	Virginia Commonwealth University Medical College of Virginia Campus Department of Pharmaceutics Smith Building 410 North 12th Street, Room 450-B Richmond, VA 23298-0533 Virginia Commonwealth University/VCU Health System General Clinical Research Center (GCRC) North Hospital, 8th Floor 1300 East Marshall Street Richmond, VA 23298

This consent form may contain words that you do not understand. Please ask the study doctor or the study staff to explain any words or information that you do not clearly understand. You may take home an unsigned copy of this consent form to think about or discuss with family or friends before making your decision.

Introduction

This study is designed to measure the blood and urine levels of chemicals that already exist in your body and to study possible differences between nonsmokers, light-smokers, and heavy-smokers. In this study, no drug(s) or medical device(s) will be given. If you agree to participate, you will have to complete paperwork including your medical history, some personality surveys, and give a blood and urine sample on two occasions. Your urine will be tested for drugs of abuse**. If you do not want to get involved with the study because of concerns about illicit drug use, please let us know immediately and you can opt out of the study**. If you are female, a pregnancy test will be done as well. Approximately 60 subjects are expected to participate in this study.

Before coming in for this study, you will not be allowed to take any prescription medications for three (3) days before the start of the study. However, females will be allowed to use oral contraceptives throughout the duration of the study. Prior to discontinuing any medication, you are strongly advised to contact your primary care physician. You will not be allowed to take over-the counter medications or drink any beverages containing caffeine for 72 hours, and not permitted to drink any beverages containing alcohol for the 12 hours before and during the in-house study periods. Finally, if you are a smoker, you will be allowed to smoke only one cigarette starting from when you enter the General Clinical Research Center (GCRC) (i.e., from approximately 8:00am until discharge at 10am).

You will come to the General Clinical Research Center (GCRC) at Virginia Commonwealth University / VCU Health System, two times for outpatient visits (spaced at least one week apart from each other). Each time, you will come to the GCRC in the morning (about 8:00 a.m.) and will be released around 2 hours later the same morning.

During the first visit when you come to the GCRC, you will complete several forms including your medical history and smoking habits, as well as several personality surveys. You will also take a breath test for carbon monoxide and alcohol. You will have to give a urine sample of which your urine will be tested for drugs of abuse as well as for nicotine. One (1) blood sample of 60 ml (approximately 4 tablespoonfuls) will be collected from a vein in your arm by sticking a needle directly into the vein. For your safety, your blood pressure will be taken before and after the blood sample.

The second visit when you come to the GCRC, everything is the same as the first visit, except you do not have to complete the forms on your medical history, smoking history, and personality surveys. One additional personality survey will be taken during the second visit in the form of a computer test.

This study is being conducted at the Virginia Commonwealth University / VCU Health System by Jürgen Venitz, M.D., Ph.D., Satjit Brar, Pharm.D./Ph.D. candidate, and John Clore, M.D.. Dr. Clore is the Medical Monitor for this study and is the first person to be contacted in the case of a medical emergency.

Risks, Inconveniences, Discomfort

Since this investigation does not require the introduction of any study drug or device, there should be no risks or side effects which might be associated with therapeutic drugs or devices.

A total of one (1) blood sample will be drawn during each study period. The total amount of blood from each session will be 60 ml (about 4 tablespoons) and about a total of 120 ml (less than half a blood donation) over the entire duration of the study. Obtaining these blood samples may cause some discomfort, pain, or slight bruising around the site of the needle stick. Sometimes, fainting or infection may occur.

You will report any adverse events after the end of the study up to 30 days after the final discharge from the GCRC. If any undesirable effects occur, you should report them directly to the study doctors. Dr. Clore is the Medical Monitor for this study, and is the person you should contact in the case of a medical emergency. If you cannot reach Dr. Clore, you may contact any of the study doctors.

Pregnancy

There are no effects on pregnancy in this study since there is no drug being given. However, for female subjects of childbearing potential who wish to participate in the study, a negative pregnancy test is required for entry into the study. A pregnancy test will be repeated upon entry to the GCRC, unless a test has been performed within seven (7) days prior to admission. These tests must be negative in order to qualify for participation in the study, as there may be an effect of pregnancy on the blood chemicals that are being measured.

Women who are pregnant or nursing a child may not participate in this study. The use of reliable birth control is required for sexually active women to enter this study. This may include barrier methods, intrauterine devices (IUDs) or being surgically sterile; however, no birth control method completely eliminates the risk of pregnancy. Females using birth control pills may be included in the study. Females not using birth control pills may also be included in the study, provided that their periods are within 28 to 32 days on average (with no history of abnormal periods). Before entering this study, you and your study doctor must agree on the method of birth control you will use during the entire study.

If you suspect that you have become pregnant during the study, you must notify the study doctor promptly.

Benefits

You are being asked to participate in this study as a volunteer. This is not a treatment study, and you are not expected to receive any direct medical benefits from your participation in the study. The information from this research study may lead to a better treatment in the future for people with smoking dependence.

Costs of Participation

There will be no charge to you for any laboratory tests, GCRC visits, or other tests related to the conduct of this study.

Payment for Participation

This is a time-consuming study that may interfere with your employment or other activities. You will be at the study unit two (2) times in the morning for the study.

You will be paid $40.00 for the completion of both outpatient visits. If you withdraw early or are discontinued for medical reasons, you will be paid $20.00 for each visit you complete.

If you decided to withdraw from the study, you may be paid based on the amount of usable information that has been collected. If it is determined that you did not give an accurate history or did not follow the guidelines of the study and the regulations of the General Clinical Research Center, you will be withdrawn from the study without compensation.

Alternative Treatment

This is not a treatment study. You may choose not to participate.

Compensation for Injury

Virginia Commonwealth University and the VCU Health System (formerly known as Medical College of Virginia Hospitals) have no plan for providing long-term care or compensation in the event that you suffer injury as a result of your participation in this research study.

If you are injured or if you become ill as a result of your participation in this study, contact your study doctor immediately. Your study doctor will arrange for short-term emergency care or referral if it is needed.

Fees for such treatment may be billed to you or to appropriate third party insurance. Your health insurance company may or may not pay for treatment of injuries as a result of your participation in this study.

Sources of Funding

Funding for this research study will be provided from ongoing research overhead monies.

Confidentiality of Records

Confidentiality of personal information about you – including your medical records and personal research data gathered in connection with this study – will be maintained in a manner consistent with federal and state laws and regulations.

You should know that research data or medical information about you may be reviewed or copied by the sponsor of the research or by Virginia Commonwealth University. Personal information about you might be shared with or copied by authorized official s of the Federal Food and Drug Administration, or the Department of Health and Human Services.

Although results of this research may be presented at meetings or in publications, identifiable personal information pertaining to participants will not be disclosed.

Voluntary Participation/Withdrawal

Your participation in this study is voluntary. You may decide to not participate in this study. If you do participate, you may freely withdraw from the study at any time. Your decision will involve no penalty or loss of benefits to which you are otherwise entitled.

Your participation in this study may be stopped at any time by the study doctor or the sponsor without your consent. The reasons might include:

- the study doctor thinks it necessary for your health or safety;
- you have not followed study instructions;
- the sponsor has stopped the study; or
- administrative reasons require your withdrawal.

If you leave the study before the final regularly scheduled visit, you may be asked by the study doctor to make a final visit for some of the end of study procedures.

Questions

In the future, you may have questions about your study participation. You may also have questions about a possible side effect or a possible research-related injury. If you have any questions at any time concerning the study procedures, contact the study doctors:

	Office	Pager
Jürgen Venitz, M.D., Ph.D.	(804) 828-6249	(804) 997-9261
Satjit Brar, Pharm.D./Ph.D. 2006	(804) 828-6136	
John Clore, M.D.	(804) 828-9349	

Dr. John Clore is the Medical Monitor for this study. He is the first person to be contacted in the case of an emergency.

If you have any questions regarding your rights as a volunteer in a clinical research study, you may contact:

VCU Office of Research Subjects Protection
Bio-Tech Research Park, Building 1
800 E. Leigh St., Suite 111
Richmond, VA 23298=0568
Telephone: 804-828-0868

Do not sign this consent form unless you have had a chance to ask questions and have received satisfactory answers to all of your questions.

Consent

I have been provided with an opportunity to read this consent form carefully. All of the questions that I wish to raise concerning this study have been answered.

By signing this consent form, I have not waived any of the legal rights or benefits, to which I otherwise would be entitled. My signature indicates that I freely consent to participate in this research study.

Printed _____
(subject name)

Signed _____ Date _____
(subject)

Printed _____
(witness name)

Signed _____ Date _____
(witness)

Signed _____ Date _____
(person conducting informed consent discussion)

Signed _____ Date _____
(investigator – if different from above)

Appendix F

Activity Flow Sheet - Clinical Study #1

Outpatient Period 1	Outpatient Period 2
ICF	SEP
SEP	Breathalyzer Test
Weight and Height Recording	CO
MHx, SH	TCI
ZSSS	**SMOKE***
AAI	VS
Breathalyzer Test	Blood Sample
CO	VS
SMOKE*	Urine Sample
VS	Drug Screen / PT**
Blood Sample	
VS	
Urine Sample	
Drug Screen / PT**	

* for smokers only
** for female subjects only

AAI: Annual Alcohol Intake
CO: Breath Carbon Monoxide Test
ICF: Informed Consent Form
MHx: Medical History
PT: Pregnancy Test
SEP: Subject Entry Probe
SH: Smoking History
TCI: Temperament and Character Inventory
VS: Vital Signs
ZSSS: Zuckerman's Sensation Seeking Scale

Appendix G

Patient Summary – Clinical Study #1

A Pilot Study To Determine TIQ and Beta-Carboline Levels in Nonsmoker, Light-Smokers And Heavy Smokers

Three groups of Nonsmokers, Light Smokers and Heavy Smokers (n=15 Females and 15 Males per Group)

Observational, Two-Period Study

Internal Funding, Personal, J. Rosecrans

VCU IRB #1990: , approved: 08/29/01, 10/16/01, 12/07/02 (advertisement), 02/08/02 (advertisement), 07/25/02, 05/16/03, 04/11/04, 04/28/05 (advertisement *2)

GCRC: #686; approved 6/28/01.

Protocol: 07/07/00, 08/21/01, 09/17/01, 06/25/02

ICF: 08/21/01, 09/10/02, 05/16/03, 06/23/03, 05/27/04, 04/28/05, 03/23/06, 02/22/07

SCREENING I

No	Init	ID	Screening Date	Abnormalities	Repeats Date	Abnormalities	Comments	Race	Sex	Age [yrs]	Weight [kg]	Height [cm]	Previous Study	Smoking FTD Score
* 101	ADS	1980	Nov-16-01	none			ELIGIBLE	White	F	21	68.0	162	none	0
* 102	JKB	2232	Dec-17-01	(recent stomach cramps) -ncs			ELIGIBLE	Black	F	22	52.7	159	none	0
* 103	HAG	6554	Dec-19-01	none			ELIGIBLE	White	F	31	86.2	173	none	0
* 104	RPR	6651	Dec-19-01	none			ELIGIBLE	White	F	21	51.2	153	none	4
* 105	BAC	6603	Dec-21-01	(h/o chronic shoulder injury, c/o shoulder pain) -ncs			ELIGIBLE	Hispanic	M	26	70.2	171	none	0
* 106	LGK	7838	Jan-25-02	ncs			ELIGIBLE	Asian	F	26	52.2	151	none	0
* 107	TSL	3465	Feb-22-02	(borderline HTN) -ncs			ELIGIBLE	White	M	22	88.1	175	none	0
* 108	KBR	5471	Mar-15-02	ncs			ELIGIBLE	Asian	M	24	67.7	171	none	6
* 109	LMD	7823	Jun-06-02	ncs			ELIGIBLE	White	F	23	49.5	158	none	4
* 110	JCD	8124	Jun-12-02	ncs			ELIGIBLE	White	F	23	59.0	164	none	5
* 111	TAT	3529	Jun-14-02	ncs			ELIGIBLE	White	F	22	60.9	169	intranasal r	0
* 112	CDP	5306	Jul-11-02	ncs			ELIGIBLE	White	F	21	48.7	156	none	6
* 113	PRB	0020	Jul-17-02	ncs			ELIGIBLE	White	M	25	71.3	181	none	0
114	SAT	5433	Jul-29-02	DS: Cocaine POSITIVE			INELIGIBLE	White	F	29	45.5	162	none	2
115	M-G	5263	Jul-30-02	h/o current depression/anxiety; DS: THC: POSITIVE			INELIGIBLE	Black	F	23	50.3	166	none	0
* 116	P-G	8471	Jul-25-02	ncs			ELIGIBLE	Asian	M	24	71.3	157	none	3
* 117	JOF	2678	Aug-21-02	ncs			ELIGIBLE	White	M	32	78.5	177	none	7
* 118	MRH	6018	Sep-19-02	ncs			ELIGIBLE	White	F	24	79.7	172	intranasal r	7
* 119	JMK	3716	Oct-08-02	h/o single marijuana use 3 weeks prior			ELIGIBLE	White	F	21	86.0	176	none	2
* 120	JIM	3916	Oct-07-02	ncs			ELIGIBLE	White	F	26	73.3	171	none	0
* 121	BMR	3461	Oct-14-02	ncs			ELIGIBLE	Asian	M	26	81.0	176	none	0
* 122	ZER	6322	Jul-24-03	ncs			ELIGIBLE	White	F	21	58.7	160	none	1
* 123	FLW	7447	Jul-25-03	ncs			ELIGIBLE	Asian	F	30	65.3	167	none	0
* 124	AJC	5839	Aug-13-03	ncs			ELIGIBLE	Asian	M	26	67.5	174	none	0
* 125	JBH	1461	Sep-17-03	ncs			ELIGIBLE	White	M	22	103.6	178	none	0
* 126	CLM	6562	Feb-24-04	ncs			ELIGIBLE	White	F	28	83.6	169	none	7
* 127	KAB	1800	Mar-10-04	ncs			ELIGIBLE	White	M	23	107.4	188	none	6
* 128	JAG	3606	Sep-25-07	ncs			ELIGIBLE	White	F	25	65.1	159	none	0
* 129	NFL	2060	Oct-01-07	ncs			ELIGIBLE	White	F	31	54.1	157	none	1
* 130	KAS	2911	Oct-05-07	ncs			ELIGIBLE	Asian	M	26	90.8	178	none	0
* 131	DSL	4231	Oct-05-07	ncs			ELIGIBLE	Asian	M	30	83.7	173	none	0
* 132	CNL	0092	Oct-22-07	ncs			ELIGIBLE	White	F	31	83.6	172	Migraine st	4
* 133	C-M	0000	Nov-05-07	ncs			ELIGIBLE	White	F	22	70.2	174	none	1
* 134	KDC	5842	Nov-21-07	ncs			ELIGIBLE	Asian	M	23	72.5	177	none	0
* 135	B-S	4592	Nov-27-07	ncs			ELIGIBLE	Asian	F	26	52.6	164	none	0
* 136	PSB	9265	Nov-29-07	ncs			ELIGIBLE	Asian	F	28	51.8	156	none	0
* 137	JSS	9263	Nov-29-07	ncs			ELIGIBLE	Asian	F	25	56.8	157	none	0
* 138	ABB	1255	Nov-30-07	ncs			ELIGIBLE	Asian	M	24	81.1	175	none	2
* 139	GKJ	4593	Nov-30-07	ncs			ELIGIBLE	White	M	24	78.8	190	none	8
* 140	DRD	6917	Nov-30-07	ncs			ELIGIBLE	White	M	25	79.8	170	none	6
* 141	AAA	2976	Dec-10-05	ncs			ELIGIBLE	Asian	M	25	79.7	167	none	7
* 142	TFP	9482	Dec-19-07	ncs			ELIGIBLE	White	M	31	82.6	175	none	6
* 143	M-R	6798	Jan-14-08	ncs			ELIGIBLE	Asian	M	21	66.5	171	none	1

SCREENING II

	No	Init	ID	Screening Date	Preexisting Illnesses	Concurrent Medications
*	101	ADS	1980	Nov-16-01	(h/o seasonal allergies) -ncs (LMP: XXX)	Orthocyclen QD, Fe supplement for 5 days, Flunase PRN
*	102	JKB	2232	Dec-17-01	(h/o seasonal allergies) -ncs (LMP: XXX)	Excedrin PRN, multivitamin QD
*	103	HAG	6554	Dec-19-01	(LMP:12/3-12/9/01)	Ibuprofen PRN, multivitamin QD
*	104	RPR	6651	Dec-19-01	(LMP:12/3-12/9/01)	none
*	105	BAC	6603	Dec-21-01	(h/o inguinal hernia OP in 1999) -ncs	Ibuprofen PRN, multivitamin QD
*	106	LGK	7838	Jan-25-02	ncs	Naproxen 220 mg, vit. C
*	107	TSL	3465	Feb-22-02	(h/o childhood asthma, cat allergy) -ncs	Ipratroprium and albuterol 110 mcg puffs BID
*	108	KBR	5471	Mar-15-02	ncs	Dayquil on 3/11/02
*	109	LMD	7823	Jun-06-02	(h/o UTI, 1998; asthmatic bronchitis, 1997; chem. ind. Bipolar disorder, 1998) -ncs (LMP:5/11/02)	none
*	110	JCD	8124	Jun-12-02	(h/o bronchitis, 2000; minor cyst surgery 2001) -ncs (LMP: 6/5/02)	Advil, Tylenol PRN; triphasil QD
*	111	TAT	3529	Jun-14-02	(h/o intermittent insomnia) -ncs (LMP: 5/27/02)	Ibuprofen PRN, Unisom on 6/9/02
*	112	CDP	5306	Jul-11-02	(h/o depression 1998, codeine allergy, intermittent migraines)-ncs (LMP: 7/2/02)	Necon 1 mg QD, metabolife PRN, tylenol PRN
*	113	PRB	0020	Jul-17-02	(h/o GERD) -ncs	Pepcid, Inbuprofen PRN; multivitamines and glucos/chondr. Q
	114	SAT	5433	Jul-29-02	ncs	none
	115	M-G	5263	Jul-30-02	(h/o current depression/anxiety) -ncs	none
*	116	P-G	8471	Jul-25-02	none	multivitamins QD
*	117	JOF	2678	Aug-21-02	(h/o allergies) -ncs	none
*	118	MRH	6018	Sep-19-02	(h/o miscarriage (DNC) 1996) -ncs	ASA PRN, Robitussin PRN, DepoVera since 2000
*	119	JMK	3716	Oct-08-02	(h/o borderline anemia, coconut allergy) -ncs (LMP: 9/21/02)	none
*	120	JIM	3916	Oct-07-02	(h/o mitral prolapse) -ncs (LMP: 9/23/02)	orthocept QD
*	121	BMR	3461	Oct-14-02	(h/o mild asthma, sulfa-allergy) -ncs	TUMS PRN (10/12/02), Advil PRN
*	122	ZER	6322	Jul-24-03	(h/o IBS, acid reflux, anemia, PCN allergy) -ncs (LMP: XXX)	Advil, Rolaids, Immodium PRN
*	123	FLW	7447	Jul-25-03	(h/o exercise-induced asthma, pediatric heart murmur) -ncs (LMP:XXX)	Albuterol inhalation, Tums PRN
*	124	AJC	5839	Aug-13-03	ncs	none
*	125	JBH	1461	Sep-17-03	ncs	Ibuprofen PRN
*	126	CLM	6562	Feb-24-04	(h/o asthma, seasonal allergies) -ncs	APAP PRN, cortisol cream, Depo-Provera since 2002
*	127	KAB	1800	Mar-10-04	(h/o asthma, allergy to erythromycin) -ncs	Ibuprofen PRN, Nyquil PRN, multivitamins QD
*	128	JAG	3606	Sep-25-07	(h/o GERD, depression long time ago) -ncs (LMP: 9/2/07)	Jonel QD, Nexium 40 mg, ranitidine 300 mg -ncs
*	129	NFL	2060	Oct-01-07	(h/o of kidney infection in 2000, recent hepatitis vaccination) -ncs (LMP: 9/7/07)	ibuprofen PRN, OC QD, multivitamins
*	130	KAS	2911	Oct-05-07	ncs	none
*	131	DSL	4231	Oct-05-07	(h/o hyperthyroidosis in 2000; tx with metimazole; recent hepatitis vaccination) -ncs	multivitamins QD
*	132	CNL	0092	Oct-22-07	(h/o childhood asthma; ovarian cysts - surgery) -ncs (LMP: 10/20/07)	occasional naproxen
*	133	C-M	0000	Nov-05-07	(h/o anxiety/public speaking - escitalopran 2/07) (LMP: 11//07)	Minerva, Nasonex
*	134	KDC	5842	Nov-21-07	ncs	none
*	135	B-S	4592	Nov-27-07	(h/o mild anemia in 2005; childhood asthma) -nca (LMP: 11/14/07)	occasional ibuprofen
*	136	PSB	9265	Nov-29-07	(h/o hypothyroidism since age 10) - (LMP: 11/4/07)	carbinizole QD
*	137	JSS	9263	Nov-29-07	(h/o low BP, childhood asthma) - (LMP: 11/27/07)	albuterol inhaler PRN; ibuprofen PRN
*	138	ABB	1255	Nov-30-07	ncs	none
*	139	GKJ	4593	Nov-30-07	(h/o perforated appendix-AE in 1990; occas. asthma) -ncs	occas. ibuprofen
*	140	DRD	6917	Nov-30-07	(h/o bronchitis, 2002; TE in 1988) -ncs	occas. ibuprofen
*	141	AAA	2976	Dec-10-05	(AE in 1997; h/o nephrolithiasis-lithotripsy in 2002) -ncs	ncs
*	142	TFP	9482	Dec-19-07	(h/o heart murmur, high cholesterol, GERD) -ncs	Liptor 20 mg QD; multivitamins; occ. Advil, TUMS
*	143	M-R	6798	Jan-14-08	ncs	Advil PRN

FINAL DEMOGRAPHICS

	No	Init	ID	Screening Date	Race	Sex	Age [yrs]	Weight [kg]	Height [cm]	Previous Study	FTD Score	Visit Date #1	Visit Date #2
*	1	ADS	1980	Nov-16-01	White	F	21	68.0	162	none	0	Nov-16-01	Nov-22-01
*	2	JKB	2232	Dec-17-01	Black	F	22	52.7	159	none	0	Dec-17-01	Jan-07-02
*	3	HAG	6554	Dec-19-01	White	F	31	86.2	173	none	0	Dec-19-01	Jan-11-02
*	4	LGK	7838	Jan-25-02	Asian	F	26	52.2	151	none	0	Jan-25-02	Feb-01-02
*	5	JIM	3916	Oct-07-02	White	F	26	73.3	171	none	0	Oct-07-02	Oct-14-02
*	6	FLW	7447	Jul-25-03	Asian	F	30	65.3	167	none	0	Jul-25-03	Aug-18-03
*	7	JAG	3606	Sep-25-07	White	F	25	65.1	159	none	0	Sep-25-07	Oct-02-07
*	8	B-S	4592	Nov-27-07	Asian	F	26	52.6	164	none	0	Nov-27-07	Nov-29-07
*	9	PSB	9265	Nov-29-07	Asian	F	28	51.8	156	none	0	Nov-29-07	Dec-04-07
*	10	JSS	9263	Nov-29-07	Asian	F	25	56.8	157	none	0	Nov-29-07	Dec-04-07
*	11	BAC	6603	Dec-21-01	Hispanic	M	26	70.2	171	none	0	Dec-19-01	Jan-11-02
*	12	TSL	3465	Feb-22-02	White	M	22	88.1	175	none	0	Feb-22-02	Mar-01-02
*	13	PRB	0020	Jul-17-02	White	M	25	71.3	181	none	0	Jul-17-02	Jul-24-02
*	14	P-G	8471	Jul-25-02	Asian	M	24	71.3	157	none	0	Jul-27-02	Aug-14-02
*	15	AJC	5839	Aug-13-03	Asian	M	26	67.5	174	none	0	Aug-13-03	Aug-20-03
*	16	JBH	1461	Sep-17-03	White	M	22	103.6	178	none	0	Sep-17-03	Sep-25-03
*	17	KAS	2911	Oct-05-07	Asian	M	26	90.8	178	none	0	Oct-05-07	Oct-22-07
*	18	DSL	4231	Oct-05-07	Asian	M	30	83.7	173	none	0	Oct-05-07	Oct-12-07
*	19	KDC	5842	Nov-21-07	Asian	M	23	72.5	177	none	0	Nov-21-07	Nov-27-07
	20												
*	21	LMD	7823	Jun-06-02	White	F	23	49.5	158	none	4	Jun-06-02	Jun-27-02
*	22	TAT	3529	Jun-14-02	White	F	22	60.9	169	intranasal nic	0	Jun-14-02	Jun-21-02
*	23	JMK	3716	Oct-08-02	White	F	21	86.0	176	none	2	Oct-08-02	Oct-15-02
*	24	ZER	6322	Jul-24-03	White	F	21	58.7	160	none	1	Jul-24-03	Aug-27-03
*	25	NFL	2060	Oct-01-07	White	F	31	54.1	157	none	1	Oct-01-07	Oct-08-07
*	26	C-M	0000	Nov-05-07	White	F	22	70.2	174	none	1	Nov-05-07	Nov-08-07
	27												
	28												
	29												
	30												
*	31	KBR	5471	Mar-15-02	Asian	M	24	67.7	171	none	6	Mar-15-02	Mar-22-02
*	32	JOF	2678	Aug-21-02	White	M	32	78.5	177	none	3	Aug-21-02	Aug-28-02
*	33	BMR	3461	Oct-14-02	Asian	M	26	81.0	176	none	0	Oct-14-04	Oct-22-02
*	34	ABB	1255	Nov-30-07	Asian	M	24	81.1	175	none	2	Nov-30-07	Dec-07-07
	35	M-R	6798	Jan-14-08	Asian	M	21	66.5	171	none	1	Jan-14-08	Jan-18-08
	36												
	37												
	38												
	39												
	40												
*	41	RPR	6651	Dec-19-01	White	F	21	51.2	153	none	4	Dec-19-01	Jan-16-02
*	42	JCD	8124	Jun-12-02	White	F	23	59.0	164	none	5	Jun-12-02	Jun-21-02
*	43	CDP	5306	Jul-11-02	White	F	21	48.7	156	none	6	Jul-11-02	Jul-22-02
*	44	MRH	6018	Sep-19-02	White	F	24	79.7	172	intranasal nic	7	Sep-19-02	Sep-26-02
*	45	CLM	6562	Feb-24-04	White	F	28	83.6	169	none	7	Feb-24-04	Mar-02-04
*	46	CNL	0092	Oct-22-07	White	F	31	83.6	172	Migraine stuf	4	Oct-22-07	Oct-30-07
	47												
	48												
	49												
	50												
*	51	KAB	1800	Mar-10-04	White	M	23	107.4	188	none	6	Mar-10-04	Mar-17-04
*	52	GKJ	4593	Nov-30-07	White	M	24	78.8	190	none	8	Nov-30-07	Dec-03-07
*	53	DRD	6917	Nov-30-07	White	M	25	79.8	170	none	6	Nov-30-07	Dec-07-072
*	54	AAA	2976	Dec-10-05	Asian	M	25	79.7	167	none	7	Dec-10-07	Dec-11-07
*	55	TFP	9482	Dec-19-07	White	M	31	82.6	175	none	6	Dec-19-07	Dec-21-07
	56												
	57												
	58												
	59												
	60												

		Age [yrs]	Weight [kg]	Height [cm]
Females	*mean*	24.9	64.1	163.5
	S.D.	3.6	12.9	7.5
	median	24.5	60.0	163.1
	min	21.0	48.7	151.0
	max	31.0	86.2	176.0
	n	22	22	22
Males	*mean*	25.2	80.1	174.9
	S.D.	3.0	11.3	7.1
	median	25.0	79.7	175.0
	min	21.0	66.5	157.3
	max	32.0	107.4	190.0
	n	19	19	19

PERIOD 1

No	Init	ID	Dosing Date	Labs (Predose)	Vital Signs	Adverse Events
1	ADS	1980	Nov-16-01	DS/HCG: ncs	ncs	none
2	JKB	2232	Dec-17-01	DS/HCG: ncs	ncs	none
3	HAG	6554	Dec-19-01	DS/HCG: ncs	ncs	none
4	LGK	7838	Jan-25-02	DS/HCG: ncs	ncs	none
5	JIM	3916	Oct-08-02	DS/HCG: ncs	ncs	none
6	FLW	7447	Jul-24-03	DS/HCG: ncs	ncs	none
7	JAG	3606	Sep-25-07	DS/HCG: ncs	ncs	none
8	B-S	4592	Nov-27-07	DS/HCG: ncs	(SBP: 97) -ncs	none
9	PSB	9265	Nov-29-07	DS/HCG: ncs	ncs	none
10	JSS	9263	Nov-29-07	DS/HCG: ncs	ncs	none
11	BAC	6603	Nov-16-01	DS: ncs	ncs	none
12	TSL	3465	Feb-22-02	DS: ncs	ncs	none
13	PRB	0020	Jul-17-02	DS: ncs	ncs	none
14	P-G	8471	Jul-27-02	DS: ncs	(asx hypoTN>91 mmHg) -ncs	none
15	AJC	5839	Aug-13-03	DS: ncs	ncs	none
16	JBH	1461	Sep-17-03	DS: ncs	ncs	none
17	KAS	2911	Oct-05-07	DS: ncs	ncs	none
18	DSL	4231	Oct-05-07	DS: ncs	ncs	none
19	KDC	5842	Nov-21-07	DS: ncs	ncs	none
20						
21	LMD	7823	Jun-06-02	DS/HCG: ncs	ncs	none
22	TAT	3529	Jun-06-02	DS/HCG: ncs	ncs	none
23	JMK	3716	Oct-08-02	DS/HCG: ncs	(asx hypoTN>88 mmHg) -ncs	none
24	ZER	6322	Jul-24-03	DS/HCG: ncs	ncs	none
25	NFL	2060	Oct-01-07	DS/HCG: ncs	ncs	none
26	C-M	0000	Nov-05-07	DS/HCG: ncs	ncs	none
27						
28						
20						
30						
31	KBR	5471	Mar-15-02	DS: ncs	ncs	none
32	JOF	2678	Aug-21-02	DS: ncs	ncs	none
33	BMR	3461	Oct-14-04	DS: ncs	ncs	none
34	ABB	1255	Nov-30-07	DS: ncs	ncs	none
35	M-R	6798	Jan-14-08	DS: ncs	ncs	none
36						
37						
38						
39						
40						
41	RPR	6651	Dec-19-01	DS/HCG: ncs	ncs	none
42	JCD	8124	Jun-12-02	DS/HCG: ncs	ncs	none
43	CDP	5306	Jun-12-02	DS/HCG: ncs	(asx hypotn: >91/58) -ncs	none
44	MRH	6018	Sep-19-02	DS/HCG: ncs	ncs	none
45	CLM	6562	Feb-24-04	DS/HCG: ncs	ncs	none
46	CNL	0092	Oct-22-07	DS/HCG: ncs	ncs	none
47						
48						
49						
50						
51	KAB	1800	Mar-10-04	DS: ncs	(HR: 90 bpm) - ncs	none
52	GKJ	4593	Nov-30-07	DS: ncs	ncs	none
53	DRD	6917	Nov-30-07	DS: ncs	ncs	none
54	AAA	2976	Dec-10-07	DS: ncs	ncs	none
55	TFP	9482	Dec-19-07	DS: ncs	ncs	none
56						
57						
58						
59						
60						

PERIOD 2

No	Init	ID	Dosing Date	Labs (Predose)	Vital Signs	Adverse Events
1	ADS	1980	Nov-27-01	DS: ncs	ncs	none
2	JKB	2232	Jan-07-02	DS/HCG: ncs	ncs	none (took APAP)
3	HAG	6554	Jan-11-02	DS/HCG: ncs	ncs	none (took biaxin, decongest for sinusitis until 1/7/02)
4	LGK	7838	Feb-01-02	DS/HCG: ncs	ncs	none
5	JIM	3916	Oct-14-02	DS: ncs	ncs	none
6	FLW	7447	Aug-18-03	DS: ncs	ncs	none
7	JAG	3606	Oct-02-07	DS: ncs	ncs	none (h/a, gastroenteritis)
8	B-S	4592	Nov-29-07	DS: ncs	ncs	none
9	PSB	9265	Dec-04-07	DS: ncs	(105/59, 78) - ncs	none
10	JSS	9263	Dec-04-07	DS: ncs	ncs	none
11	BAC	6603	Jan-16-02	DS: ncs	ncs	none
12	TSL	3465	Mar-01-02	DS: ncs	ncs	none
13	PRB	0020	Jul-24-02	(h/o claritin on 7/20) -ncs	ncs	none
14	P-G	8471	Aug-14-02	DS: ncs	ncs	none
15	AJC	5839	Aug-20-03	DS: ncs	during blood draw: 95/45 mmHg, 50bpm	feeling faint (no LOC) during blood draw
16	JBH	1461	Sep-25-03	DS: ncs	ncs	ncs
17	KAS	2911	Oct-22-07	DS: ncs	ncs	ncs
18	DSL	4231	Oct-12-07	DS: ncs	ncs	ncs
19	KDC	5842	Nov-27-07	DS: ncs	(HR > 96) -ncs	ncs
20						
21	LMD	7823	Jun-27-02	DS: ncs	ncs	none
22	TAT	3529	Jun-21-02	DS: ncs	ncs	none
23	JMK	3716	Oct-15-02	DS: ncs	ncs	none
24	ZER	6322	Aug-27-03	DS: ncs	(HR prior to blood draw: 112 bpm) -ncs	none
25	NFL	2060	Oct-08-07	DS: ncs	ncs	none
26	C-M	0000	Nov-08-07	DS: ncs	ncs	none
27						
28						
20						
30						
31	KBR	5471	Mar-22-02	DS: ncs	ncs	ncs
32	JOF	2678	Aug-28-02	DS: ncs	ncs	ncs
33	BMR	3461	Oct-22-02	DS: ncs	ncs	ncs
34	ABB	1255	Dec-07-07	DS: ncs	ncs	ncs
35	M-R	6798	Jan-18-08	DS: ncs	ncs	ncs
36						
37						
38						
39						
40						
41	RPR	6651	Jan-09-02	DS/HCG: ncs	(SPB>96; HR>96 bpm) -ncs	transient mild lightheadedness during blood draw
42	JCD	8124	Jun-21-02	DS/HCG: ncs	ncs	none
43	CDP	5306	Jul-22-02	DS/HCG: ncs	ncs	none
44	MRH	6018	Sep-26-02	DS/HCG: ncs	ncs	none
45	CLM	6562	Mar-02-04	DS/HCG: ncs	ncs	none
46	CNL	0092	Oct-30-07	DS/HCG: ncs	(HR>97) - ncs	none
47						
48						
49						
50						
51	KAB	1800	Mar-17-04	DS: ncs	ncs	none
52	GKJ	4593	Dec-03-07	DS: ncs	ncs	none
53	DRD	6917	Dec-07-072	DS: ncs	ncs	none
54	AAA	2976	Dec-11-07	DS: ncs	ncs	none
55	TFP	9482	Dec-21-07	DS: ncs	ncs	none
56						
57						
58						
59						
60						

Appendix H

Protocol and Informed Consent Form – Clinical Study #2

Date:	March 13, 2007	
To:	Barbara Karp, M.D.	Chair, CNS IRB
Recommended by:	_____	Clinical Director, NIAAA
	_____	Chief, LCTS, NIAAA
Protocol title:	Assessment and treatment of people with alcohol drinking problems	
Abbreviated title:	Alcoholism assessment and treatment	
Identifying words:	Dependence, CIWA, Addiction severity index, CPRS-S-A, combined behavioral intervention, medical management	
Principal Investigator:	David Herion, M.D.	
Associate Investigators:	Linda Doty, R.N., M.S.W. David T. George, M.D. Markus Heilig, M.D., Ph.D. Debby Hill, M.S.W. Dan Hommer, M.D. Judie Johnson, R.N. Tom Lionetti, R.N. Vijay Ramchandani, Ph. D. Dave Spero, R.N. John Umhau, M.D. Margaret Weiser, R.N. Marjorie Wright, RN	
Estimated duration of study:	Five years	

Participants in study

Number	Gender	Age range
1000	Male	18 years and older
1000	Female	18 years and older

Project uses ionizing radiation:	No
Project involves use of Durable Power of Attorney	No
Off-site project:	No
Multi-institutional project:	No

1. Précis

The purpose of this protocol is to create a mechanism whereby the intramural program of the NIAAA can evaluate and treat a broad range of people with drinking problems at the NIH Clinical Center (CC) in Bethesda, MD. Through this program, participants will receive comprehensive, state-of-the-art treatment for their alcohol, psychosocial and medical problems and the program will be able to evaluate and recruit participants for other, more focused clinical research efforts to advance its research goals. Additionally, this will allow investigators and staff to gain broad training experience in alcohol and addiction medicine through the clinical care of such patients. The protocol is open to any adult who is seeking help for a drinking problem and who is likely to qualify to participate in another NIAAA protocol. Participants will be recruited through local media and professional avenues in the Washington, DC Metro area. They will be evaluated by a nurse and physician, among others, who will determine the need for hospitalization, detoxification and to address other issues. For those needing medically supervised detoxification, a standard program of monitoring and treatment with benzodiazepines and other medications will be instituted. A standard battery of screening blood, urine and other clinically indicated tests, an electrocardiogram, chest x-ray and MRI of the brain will be done as part of the comprehensive medical and neurological assessment. Following at least five days of abstinence from alcohol, participants will undergo a series of verbal and observational-type assessments designed to evaluate psychiatric co-morbidity, psychopathology, psychosocial problems, neurocognitive function, personality and other factors relevant to alcoholism treatment. Participants will then be offered a 12-16 week course of outpatient treatment, consisting of either of two, manual-based therapies used in Project COMBINE, a large, NIAAA-sponsored national trial of counseling and medication therapies for alcohol dependence(The COMBINE Study Research Group, 2003a; The COMBINE Study Research Group, 2003b). The first is an intensive counseling approach (12 sessions) called Combined Behavioral Intervention (CBI) and the second, Medical Management (MM), is a series of brief counseling sessions every 2-4 weeks. At five points during the outpatient phase participants will come to the clinic for selected blood and urine tests, interviews and verbal/observational assessments to evaluate abstinence from alcohol and identify change in various psychological dimensions. During their participation in this protocol, participants will be approached to consider enrolling in other clinical research protocols such as imaging studies and drug-treatment trials. For participants willing to participate in these other protocols, other appropriate consent(s) will be obtained.

2. Introduction

People who have serious problems related to alcohol drinking have heterogeneous historical courses, the most serious of which may come to clinical attention (Institute of Medicine, 1990). Some people independently experience resolution of their drinking problems, while others go through variable cycles of adverse consequences; various non-clinical and clinical treatments and other efforts at abstinence or reduced drinking; lapse (first drinking episode); relapse (recurring sustained heavy drinking episode); and reemergence of old and new untoward consequences (Institute of Medicine, 1990). It is in recognition of this cycle that alcoholism, here equated with alcohol dependence, has been dubbed a chronic, relapsing disease, analogous in some respects to diabetes (Institute of Medicine, 1990; O'Brien, 1994; O'Connor and Schottenfeld, 1998). However, as medical, psychiatric and psychological research has advanced, the understanding of how alcohol affects the body's (brain and other systems) health and how coping difficulties maintain problem drinking and, indeed, the concept of disease itself are changing (Monti et al., 1989).

Against this changing knowledge, treatments for clinically manifest alcohol problems are emerging. The most commonly employed psychosocial treatment options, cognitive-behavioral coping skills therapy, motivational enhancement therapy, and twelve-step facilitation, are equally effective with abstinence rates of 19-35% and relapse rates of 40-46% at one year, under optimal conditions, delivered either in inpatient, day treatment, residential or outpatient settings (Project MATCH Research Group, 1998; Longabaugh and Wirtz, 2001). Neuro-pharmacological treatments are being increasingly studied; thus far two drugs, naltrexone and acamprosate, have demonstrated benefit (Kranzler and Van Kirk, 2001; Anton and Swift, 2003; Soyka and Chick, 2003). Both are to be used in conjunction with

counseling and supportive approaches, where they yield abstinence rates at one year of (17-47%), with most relapse occurring at three months (35-60% relapsed) under typical clinical trial conditions (O'Malley et al., 1992; Anton et al., 1999; Kiefer et al., 2003). Furthermore, response to treatment in "real-world" care delivery environments is very sparsely documented (O'Malley et al., 2003; The COMBINE Study Research Group, 2003a; The COMBINE Study Research Group, 2003b).

Given the challenges of understanding and treating clinical problems related to alcohol use, the primary thrust of the Laboratory of Clinical and Translational Studies (LCTS) at the intramural research program of the NIAAA is to investigate the neurochemistry of alcohol dependence and withdrawal (which itself may play a role in relapse), mechanisms of relapse and craving and their possible inter-relationship and the short-term efficacy of candidate drugs in promoting abstinence. The main approaches used by the LCTS are translational research from pre-clinical to clinical models using sensitive observational techniques and tools, collaborations with other intramural scientists and scientifically sound and relevant clinical research projects.

One important tool for the translational studies envisioned by the lab and created within this protocol is the evaluation of genes that may underlie processes involved in the more fine-grained behavioral and cognitive (so-called "intermediate") phenotypes seen in clinical populations and relevant animal model systems. Among them are neuropeptide and monoaminergic genes. We therefore propose to collect genetic information from alcoholics for the purpose of studies of the association of various genes with endophenotypes, primarily regional brain volumes and/or functional brain imaging results.

For example, due to the well-documented role of dopamine (DA) in drug reinforcement processes (Koob and Nestler, 1997), and the role of serotonin (5-HT) in affective psychopathology pertinent to substance abuse (Heinz et al., 2001), we propose to obtain allelic data on the following genes: 1) The 5-HT transporter, the expression of which is reduced in alcoholism (Heinz et al., 1998), and a genotype of which has been linked to vulnerability of the brain to excessive alcohol (Heinz et al., 2000), 2) the 5-HT 2a receptor, which has been linked to impaired impulse control in the context of history of psychopathology (Bjork et al., 2002), 3) the DA DR4 receptor, an allelic variant of which has been linked to attentional deficits and conduct-disordered behavior (Faraone et al., 2001), 4) the catechol-O-methyltransferase gene, which has been linked to working memory functioning (Heinz et al., 2001), and 5) the DA DR2 receptor gene, which has been linked to severity/age of onset of alcoholism (Geijer et al., 1994), and extraversion in an interaction with presence versus absence of an alcoholic father in the home (Ozkaragoz and Noble, 2000). Recently a number of research groups have reported that various genes are associated with differences in brain structure as well as function (Mustovic et al., 2005; Wrase et al., 2005) (Gordon et al., 2005).

In addition to the genes discussed above, we will also examine other genes that affect normal brain metabolism or development. In no case will we characterize a gene that has been established to be useful in the diagnosis of any medical or psychiatric disorder. In summary, we wish to explore how variants of these genes may relate to behavioral, cognitive, neurophysiological and neuroanatomical features of subjects, which are ascertained in this and other NIAAA protocols.

For this final approach, the NIAAA intramural program needs a steady base of people who are seeking treatment for problems related to alcohol drinking and willing to participate in clinical studies. Furthermore, a busy and broadly-based alcoholism unit supports another important program goal: to train clinicians and investigators in alcohol studies.

The evaluation and treatment plan offered in this protocol represents a state-of-the art clinical addiction medicine program. Evaluation, including assessments of alcohol use, negative consequences, alcohol dependence, co-morbid psychiatric conditions, family history, neuropsychological functioning and physical sequelae, has a strong evidence base and has been recommended for clinical use, particularly to enhance motivation, plan for treatment and to provide a basis for follow-up(Institute of Medicine, 1990; Project MATCH Research Group, 1997; O'Connor and Schottenfeld, 1998; Miller et al., 2003). Unfortunately, such recommendations have not yet been routinely implemented outside leading treatment institutions. However, in accordance with the NIAAA mission and responsibility to lead the community not only by performing state-of-the-art, cutting-edge research in alcoholism, but we also plan for the

intramural program, in this protocol, to lead by example in the delivery of treatment by offering an evaluation and treatment program that vastly exceeds the standard practice in the community.

Because of the evidence-based nature of all assessments used in this protocol, data obtained through them can serve at least three purposes: first, to provide a basis for individualizing patient treatment, enhancing motivation to change, and following-up such treatment for the sole purpose of providing optimal clinical care; second, to provide patient characteristics and outcome data for specific research protocols to which the patient may additionally and separately consent; and third, to provide data for monitoring the performance and functioning of the program as a whole.

To provide the framework for operational management of the LCTS research program and conducting clinical trials in coordination with the NIH CC operation as well as to achieve the goal of recruiting participants for research, we have written this protocol as a hybrid training and short-term natural history protocol. Through it, we offer patients a state-of-the-art clinical work-up that represents a research evaluation and a 12-16 week course of standard psychosocial treatment for alcoholism using well-documented, effective approaches. Within this framework, we will seek subject participation in other protocols focused on more specific questions, including those related to alcohol withdrawal and the neuropharmacology of relapse (prevention). The data collected in this protocol may be used in the future for research purposes.

Subjects enrolled in this protocol may participate in other protocols, including clinical trials or mechanism studies using experimental compounds. The data collected in this protocol will be shared and used for analysis in those protocols. The protocols whose enrollment is explicitly contingent on prior enrollment in this protocol include:

- 05-AA-0120: Acamprosate for Central Nervous System Hyperexcitability and Neuroadaptation in Alcohol Withdrawal
- 06-AA-0129: NK1 Receptor Antagonism for Treatment of Anxiety and Craving in Anxious Alcohol Dependent Subjects During Early Abstinence

Furthermore, as part of a longitudinal effort to characterize the cohort of subjects seen at the NIAAA, data from this protocol will be combined with data from 98-AA-0009, Screening Evaluation for NIAAA Protocols, particularly for rating scale information (such as personality factor assessment, mood and anxiety scales, etc.), correlation with brain MRI volumes in alcoholics, organ damage and genetic tests.

3. Objectives

This protocol has several purposes:

1) It is meant to serve as an entry mechanism to authorize a subject's admission to the NIH CC in Bethesda, Maryland under the care of the NIAAA LCTS and the CC nursing staff (NIAAA Inpatient and Outpatient Care Units).
2) It authorizes the provision of state-of-the-art care for individuals with alcohol and drug problems, including detoxification, assessments, and outpatient counseling approaches.
3) It provides a high-quality, research-driven venue for training physicians, fellows, residents, medical students and other health-care professionals in the diagnosis and management of alcohol use disorders.
4) It provides a set of standard measures, serving a dual purpose:
   a) To provide a basis for optimal treatment planning/matching and follow-up for the benefit of the individual patient
   b) To potentially provide patient characteristics and follow-up measures for participants in other NIAAA clinical research protocols.
5) It can serve as a reference document for community facilities that may be interested in referring clients to the NIAAA intramural program.
6) To collect blood samples from alcoholic subjects for the purpose of analyses of DNA and biomarkers of alcohol exposure

4. Study Design and Methods

This protocol does not involve experimental procedures or therapeutics. Rather, it follows the typical clinical course of events in people with alcohol dependence and abuse over a brief, intensive time period. It therefore consists of a series of phases including:

- pre-NIH visit gathering of subject information (which generally takes 1 week in our experience),
- physical evaluation at NIH CC (1-2 days),
- inpatient alcohol withdrawal and psychosocial management when necessary (5+ days, 2+ weeks, respectively),
- baseline observation (3-5 days)
- and outpatient treatment (12 weeks).

In summary, the planned procedures throughout this protocol involve routine verbal and observational procedures, such as "pen-and-paper"-style self-reports and interviews, and minimally invasive procedures, such as phlebotomy and urine collection, electrocardiogram (ECG), chest x-ray (CXR) and magnetic resonance imagining (MRI) of the brain to provide a comprehensive medical, psychiatric and addiction medicine evaluation. The treatment involves standard outpatient counseling-type therapy for alcohol dependence.

Since the NIAAA clinical program also serves to train physicians, nurses and other health professionals in the practice of addiction medicine, trainees will be involved in the evaluation of, and have direct contact with patients in this protocol. Involvement of trainees will always be under supervision of Senior Medical Staff of the Clinical Center, according to the general regulations of clinical privileges of the Clinical Center.

Following recruitment to the CC, patients will be examined by the medical and nursing staff to determine medical and psychiatric stability and to evaluate inclusion and exclusion criteria. Selected blood and body fluid tests, among others, will be done as clinically indicated.

Patients may need to be hospitalized for monitoring and treatment for problems and certain conditions, such as severe alcohol withdrawal, which, when uncomplicated, typically lasts about a week (Sullivan et al., 1989b; Kosten and O'Connor, 2003). Currently, the standard care for the treatment of the alcohol withdrawal syndrome is to provide close monitoring, supportive care and symptomatic treatment with benzodiazepines (diazepam, oxazepam or lorazepam), using the Clinical Institute Withdrawal Assessment-Alcohol Revised (CIWA-AR), a validated tool to categorize severity of alcohol withdrawal based on symptoms and physical signs (Sullivan et al., 1989b). Which of the three benzodiazepines to use and in which dosing regimen, are ultimately clinical decisions, and will be so taught to trainees.

Additionally, intravenous fluids and parenteral medications, such as thiamine, haloperidol, and antibiotics, may be required. Patients will receive daily folic acid supplements. The successful treatment and resolution of the alcohol withdrawal syndrome, for example as measured by consecutive CIWA-Ar scores below 7 and a clear sensorium, constitutes the detoxification phase, after which begins the baseline period.

During the baseline period, participants will undergo various verbal and observational assessments (detailed below). Based on these assessments, multidisciplinary treatment planning will be undertaken by the staff with the active participation of the patient. They will then be offered an opportunity to enroll in a 12-16 week treatment course of counseling that would consist of one of the following: CBI or MM, run through the NIAAA/CC outpatient clinic (see 4.C., "Therapy", below). Alternatively, patients may be referred to a suitable non-NIH program for further treatment, depending on their preferences and treatment availabilities.

During the outpatient follow-up phase they will also be scheduled to be seen for clinic visits at weeks 1, 2, 4, 8 and end-of-therapy for brief medical and psychiatric check-ups, and selected blood and body fluid tests will be performed, as well as selected verbal assessments (see below). The assessment instruments; blood, body fluid and body tests; and therapies, with schedules are:

A. Structured assessments of a subject's history and internal psychological experiences are performed using pen-and-paper and computerized tools after a subject has been abstinent form alcohol for at least 5 days.

a. The Addiction Severity Index (ASI) is an instrument used extensively in the Addiction Medicine field to comprehensively identify problems in multiple dimensions including medical, employment, drug and alcohol use, legal, family and social and psychiatric (McLellan et al., 1980). It is a 200-item interview that takes about 60 minutes. It will be done prior to and at the end of the outpatient treatment phase, i.e. baseline and end-of-therapy.
b. The Structured Clinical Interview for Diagnostics and Statistics Manual-IV (DSM-IV) (SCID-I) is another widely-used, standard clinical interview to establish criteria for psychiatric diagnoses (First et al., 2002). It is a structured interview consisting of 11 modules with between 35-292 items/module that takes about 120-180 minutes. It will be done at baseline. DSM-IV diagnoses will be established via a consensus, or "blind-rating" process involving trained psychiatrists.
c. The Alcohol Dependence Scale assesses severity of alcohol dependence in a variety of clinical settings (Skinner and Allen, 1982). It consists of 25 questions and takes about 5-10 minutes to complete. It will be done at the baseline.
d. The Obsessive-Compulsive Drinking Scale (OCDS) assesses craving and urges for alcohol (Anton et al., 1995). It is a 10-item self-report that takes about 5-10 minutes. It will be done at baseline and at each clinic visit during the outpatient treatment phase.
e. The affective symptoms (anhedonia, depression, anxiety, and dysphoria) following removal of alcohol and other drugs, the so-called motivational effects of alcohol, will be assessed with self-administered subscales derived from the Comprehensive Psychopathological Rating Scale Self-rating Scale for Affective Syndromes (CPRS-S-A) (Mattila-Evenden et al., 1996; Svanborg, 1999). It is a 19-item self-report that takes 5-10 minutes to complete. It will be done every three days during the admission and baseline phases and during the outpatient phase, at clinic visits.
f. The Timeline Follow-Back (TLFB) technique collects drinking information using personal historical events recounted over a fixed time period (Sobell and Sobell, 1992b; Sobell and Sobell, 1996). It is a commonly used technique to assess alcohol drinking patterns and quantification in treatment programs. The number of items corresponds to the number of days of interest, up to 360 which usually takes about 30 minutes. It will be done at baseline to cover the prior 360 days and at each clinic visit during the outpatient treatment phase.
g. The University of Rhode Island Change Assessment Scale (URICA) is used to indicate a subject's motivation and readiness for treatment. It is URICA is a 32-item self-report that takes about 5-10 minutes. It will be done at baseline.
h. The Neurotocism-Extroversion/Introversion-Openess to Experience (Five Factor) Personality Inventory- Revised (NEO PI-R) provides scores on various dimensions of personality (Costa and McCrea, 1997; Costa and McCRea, 2002; Costa et al., 2002). It is a 240-item self-report that takes up to 35-45 minutes to complete. It will be done at baseline.
i. The Wechsler Adult Intelligence Scale-Revised (WAIS-R) is the standard technique to determine Intelligence Quotient (IQ). We use the Block Design and Vocabulary components to measure IQ. In total the tasks comprise 50 items that take about 15-30 minutes to complete. It will be done at baseline.
j. Trails A and B have been extensively used to globally measure executive cognitive performance and frontal lobe function. They take about 5 minutes to complete.
k. The Family Tree Questionnaire (FTQ) is an interview about a subject's family history of alcohol and drug problems (Mann et al., 1985). The duration of the interview depends on the number of first- and second-degree relatives, but generally takes about 5-10 minutes. It will be done at baseline.
l. The Important People and Activities Instrument (IPA) is a structured interview about interpersonal and social networks, especially as they relate to alcohol drinking (Longabaugh, 1991; Zywiak et al., 2002). It covers 19 items and takes about 20-30 minutes. It will be done at baseline.
m. The Fagerström nicotine dependence scale is a 5-item self-reported questionnaire about smoking. It takes 1-2 minutes to complete.

n. The modified Overt Aggression Scale (m-OAS) is a scale to screen for a history of violent behavior. It is a single page of questions which takes about a minute to complete. It is an interview.
o. The Lifetime Drinking History estimates the total amount of alcohol consumed over a lifetime. It is an interview that takes about 20 minutes to complete.

B. Neurocognitive tests of frontal lobe function:

Several reports collectively suggest that alcoholism-prone individuals are characterized by frontal lobe hypofunction (Ciesielski et al., 1995; Giancola and Moss, 1998), specifically deficits in executive cognitive functioning (ECF). These include response inhibition, attention, working memory, strategy, and assessment of behavior consequences. Patients need these cognitive abilities, at least to some degree, for the successful treatment of their alcohol use disorders. Thus, understanding the specific nature and severity of ECF will be helpful in treatment planning for individual patients.

We plan to measure and characterize ECF by using four computerized cognitive tests: the Wisconsin Card Sorting Task (WCST), The Iowa Gambling Task (IGT), a continuous performance, visual working memory task (CPT-WM), and a delay discounting task (DDT). The WSCT and the CPT-CM both involve attention, working memory and strategy, and both are dependent on functions carried out by the lateral surface of the frontal lobes, while the IGT and the DDT both require inhibition and assessment of a behavior's consequences and are thought to depend on the orbital and mesial surfaces of the frontal lobe (Bjork et al., in press).

We have developed computerized versions of the CPT-WM, IGT and DDT. These three tasks have all been used in NIAAA protocol 94-AA-0001, and they successfully discriminate between alcoholic and non-alcoholic individuals, as well as measure an individual's ECF. For the WCST we will use a commercially available computerized version of this task.

Each of these tasks is performed by interacting with a computer. They are all minimal risk procedures and, although demanding, most individuals do not usually consider them unpleasant. The WCST is game-like and measures a person's ability to make strategic changes based on changing circumstances. The IGT simulates a gambling game. In neither the WCST nor the IGT do the participants actually win money. However, both the CPT-WM and the DDT require that the participants be able to win money as part of the task.

The CPT-WM requires subjects to press a button in response to a series of letters displayed on a computer screen. By manipulating the targets it is possible to access an individual's ability to focus attention as well as inhibit inappropriate responses. In the CPT-WM the amount won is based on a participant's performance. The DDT assesses the degree to which a subject devalues a reward as a function of how long the subject must wait to receive it(Mitchell, 1999; Richards et al., 1999). In brief, the participant is presented with a series of choices between receiving either a monetary reward (either in cash or by mailed check as required) at time-points ranging from immediately to up to one year in the future. To enhance the realism of the task, actual reward (selected from a random question) will be delivered. A participant's total winnings in the CPT-WM and DDT typically range from $30.00 - $45.00. The maximum amount possible to win from these two games is $80, but it is very unlikely that a participant can win this much.

C. Bio-medical evaluations are procedures that physically analyze components of a subject's body. They include:
a. Breath alcohol analysis is a state measure of alcohol exposure which is also a clinically important marker of alcohol intoxication and tolerance and is serially used to correlate with signs and symptoms to diagnose the alcohol withdrawal syndrome. It will be done at baseline and each clinic visit during the outpatient treatment phase.
b. Blood test panels to assess physiological functions and screen for organ damage, as well as assessment of the extent of alcohol and drug exposure, including toxicology and biomarkers. The blood tests (with blood volume) include:

i. Complete blood count with differential (CBC with diff) (3 mL, ~ 1 teaspoon). It will be done at baseline and each clinic visit during the outpatient treatment phase.

ii. Chem 20 Panel (Chem 20): Sodium (Na), Potassium (K), Chloride (Cl), Total CO2 (bicarbonate), Creatinine, Glucose, Urea nitrogen (BUN), Albumin, Calcium total, Magnesium total (Mg), Inorganic Phosphorus, Alkaline Phosphatase, ALT/GPT, AST/GOT, Total Bilirubin, Direct Bilirubin, LD, Total Protein, Total CK, Uric Acid, amylase (4 mL, ~1 teaspoon). It will be done at baseline.

iii. Thyroid Screen: Thyroid stimulating hormone (TSH), Free thyroxine (FT4), Tri-iodothyroine (T_3) (3.5 mL, ~1 teaspoon)). It will be done at baseline.

iv. Lipid and essential fatty acid panel: Total Cholesterol, Triglycerides, High-Density lipoprotein (HDL) Cholesterol, Low-Density Lipoprotein (LDL) Cholesterol; Lauric Acid, Myristic Acid, Hexadecenoic Acid, Palmitoleic Acid, Palmitic Acid, g-Linolenic Acid, a-Linolenic Acid, Linoleic Acid, Oleic Acid, Vaccenic Acid, Stearic Acid, EPA C20, Eicosapentaenoic Acid, Arachidonic Acid, Mead Acid, Homo-g-Linolenic Acid, Arachidic Acid, DHA, Docosahexaenoic Acid, DPA, DTA, C22 5W6, 5W3, C22, 4W6, Docosenoic Acid, Nervonic Acid, Triene Tetraene Ratio, Total Saturated Acid, Total Monounsaturated Acid, Total Polyunsaturated Acid, Omega 3, Omega 6, Fatty Acids (6.0 mL, 1.2 teaspoons). It will be done at baseline. The specimen will be stored in the LCTS in the laboratory of Dr. Markus Heilig."

v. Viral Markers Protocol Screen: Hepatitis B surface antigen (HBsAg), Hepatitis C Virus antibody (anti-HCV), Human Immunodeficiency Virus 1 and 2 (anti-HIV) (8 mL, 1.5 teaspoons). It will be done at baseline.

vi. Trace mineral panel: Trace mineral panel and vitamin analysis: Vitamin C, and vitamins B_{12}, homocysteine and methylmalonic acid, copper and ceruloplasmin, iron studies (serum iron, transferrin saturation, ferritin) and zinc (25.5 (5.2 teaspoons). It will be done at baseline.

vii. Biomarkers: Currently, there is no clear standard set of blood and body fluid tests that clearly indicates relapse to alcohol use. However, guidelines are emerging (Litten and Fertig, 2003).

1. Hepatic Panel (blood, 3.5 mL, ~1 teaspoon). It will be done each clinic visit during the outpatient treatment phase.
2. Gammaglutamyl-transpeptidase (GGT) (blood, 3.5 mL, ~1 teaspoon) (Conigrave et al., 2003). It will be done at baseline and each clinic visit during the outpatient treatment phase.
3. Carbohydrate-deficient transferrin (CDT) (blood, 2.5 mL, ½ teaspoon) (Javors and Johnson, 2003). It will be done at baseline and each clinic visit during the outpatient treatment phase.
4. The ratio of 5-hydroxyindolacetic acid (5-HIAA) to 5-hydroxytryptophol (5HTOL) (urine) (Beck and Helander, 2003). It will be done at baseline and each clinic visit during the outpatient treatment phase.
5. Ethyl glucuronide (urine) (Wurst et al., 2003). It will be done at baseline and each clinic visit during the outpatient treatment phase.
6. During the baseline phase, two additional tubes of blood (20 mL) will be collected up to a maximum of five times (100 mL). During the outpatient follow-up phase, 20 mL will be drawn at weeks 2, 4, 8 and 12 (or end of therapy) (80 mL). Various blood components (plasma, serum and intracellular proteins) will be assayed using experimental tests being developed as biomarkers of alcohol exposure and organ damage. The specimens will be stored in the NIAAA LCTS in the laboratory of Dr. Markus Heilig. The total amount of blood for the experimental biomarkers is 180 mL (less than 37 teaspoons).

c. Urine tests
    i. Urinalysis to screen for renal and genitourinary abnormalities will be done at baseline and during follow-up as clinically indicated.
    ii. Urine drug screens
        1. The Qualitative (DLM) tests for benzodiazepines, cocaine, methamphetamines, opiates and tetrahydrocannabinol (THC). It provides a result within hours. It will be done at baseline and each clinic visit during the outpatient treatment phase.
        2. The Drug Profile #1 tests for amphetamines, barbiturates, benzodiazepines, cocaine, lysergic acid diethylamine, opiates, phencyclidine and THC. It has greater sensitivity than the DLM screen and takes about five days to complete. It will be done at baseline and each clinic visit during the outpatient treatment phase.
    iii. Pregnancy test. Some medications which may be indicated during routine treatment of alcohol related problems are inappropriate for pregnant subjects. For this reason, pregnancy tests will be done at baseline and as clinically indicated thereafter.
    iv. Biomarkers (see above)
        1. Ratio of 5-HIAA to 5-HTOL (see above)
        2. Ethyl glucuronide (see above)

d. Other procedures used to screen for medical diseases and abnormalities.
    i. An electrocardiogram (ECG) is a minimal risk procedure that allows detection of cardiac rhythm and structural problems that may be associated with alcoholism. It will be performed at baseline.
    ii. The chest x-ray is a minimal risk procedure to allow detection of cardiac, bone and pulmonary abnormalities that may be associated with alcoholism and cigarette smoking. It will be performed at baseline.
    iii. An MRI of the brain is a minimal risk procedure which allows the diagnosis of brain damage associated with alcoholism. It will be performed at least 2 weeks after the last drink unless clinically urgent.

e. Blood sample for collection of DNA (leukocytes) for analysis of polymorphisms in gene loci such as the following (other genes known to be involved in brain function and/or alcohol-related organ damage may also be examined):
    1) The 44 base-pair repeated element (SLC6A4) of the serotonin (5-HT) reuptake transporter (promoter),
    2) The T102C polymorphism of the 5-HT2a receptor
    3) The 48 base-pair repeated element in Exon III of the Dopamine DRD4 receptor
    4) The Val/Met polymorphism of the Catechol-O-Methyl-transferase (COMT),
    5) The Taq1 (A1)-defined polymorphism of the dopamine DRD2 receptor,
    6) Alleles associated with Brain Derived Neurotrophic Growth Factor (BDNF).

David Goldman, MD, Chief, NIAAA Laboratory of Neurogenetics in collaboration with LCTS will store and conduct the genetic analysis.

The alcohol use disorder patients will not be asked to give consent for the blood draw for genetic analysis until at least five days after their most recent consumption of alcohol containing beverages. This will correspond to when they are asked to sign the “unimpaired subject consent” version of this protocol (05-AA-0121).

D. Therapy
a. The assessments listed above are considered to be an important component of treatment planning, indicating individual areas of treatment focus and monitoring (Institute of Medicine, 1990; Donovan, 2003).
b. CBI is a recently developed form of therapy for alcohol dependence that draws on several approaches having reasonable evidence of efficacy. It combines three elements:

i. A community reinforcement approach (CRA) that integrates functional analysis of the drinking behavior, behavioral skills training (in project MATCH called Cognitive Behavioral Coping Skills Therapy, or CBT) and family involvement. Cognitive-behavioral coping skills therapy (CBT) is an effective treatment approach that basically focuses on the training of interpersonal and self-management skills (Monti et al., 1989; Kadden et al., 1992; Miller et al., 1995).

ii. Motivational interviewing and assessment feedback (in Project MATCH called Motivational Enhancement Therapy, or MET). MET is a systematic intervention approach based on the principles of motivational psychology and designed to produce rapid internally motivated change (Miller et al., 1992; Miller et al., 1995).

iii. CBI will be conducted, as in Project COMBINE, in 4 phases: the first involves motivational interviewing and subject assessment (generally 1-2 sessions); phase 2, a functional analysis, held jointly with a supportive significant other when available, to identify problems in drinking behavior, skills and resources to target specific areas where improvement is need (1-2 sessions); phase 3, cognitive behavioral skills group therapy (10 sessions). These will be scheduled over a 12 to 16 week time period (The COMBINE Study Research Group, 2003b). This arm would be the standard treatment for patients not participating in pharmacotherapy trials (protocols) for relapse prevention.

iv. All CBI sessions will be conducted by trained NIH staff: nurses from the CC and/or counselors from the NIAAA. Phase 1 and 2 will be conducted at the CC. The current plan is that Phase 3 will be conducted at the CC. However, there is initial planning for CBI treatment to be conducted at other local facilities that may be more accessible to outpatients, as it has been shown that one of the most important variables in retention in alcoholism treatment programs is proximity of the treatment to a client's domicile. Also, concern has been raised that the current security circumstances at the NIH's Bethesda facility may be a barrier to treatment retention in our program (personal communication, T. K. Li, Director, NIAAA, October 2004). In each such case where an outside facility may be considered as a treatment site, the plan would be brought to the IRB for discussion, approval and amendment to this protocol as stipulated.

c. Medical Management (MM) is a manual-based behavioral intervention consisting of brief, structured counseling sessions to provide strategies for medication adherence and to support abstinence through education and referral to support groups (Pettinati et al., 2004). Follow-up sessions are used to assess drinking status, overall functioning, medication adherence, and any side effects. It is an adaptation of the BRENDA approach (biopsychosocial assessment, reporting the assessment results, empathetic understanding, identifying patient needs and priorities, and matching needs to treatment options, and adjusting advice to patient response- the "BRENDA" approach) developed and used at the Center for Addiction Studies at the University of Pennsylvania Health System (http://www.uphs.upenn.edu/trc/index.html).

d. Under this protocol, pharmacological treatment to prevent relapse will be offered only using medications which are approved for this indication (naltrexone and acamprosate). Although Antabuse is approved, it will not be offered because of unacceptable safety risks. Other treatment options for relapse prevention may be offered if the patient additionally and separately consents to participation in treatment trial protocols aimed at evaluating novel candidate treatments. These will then be described in the respective clinical trial protocol.

e. Assignment of subjects to CBI or MM will not be random. Instead, assignment to treatment will be based on (a) whether they are in a clinical trial of medication(s) for relapse prevention or (Bobo et al.) whether they are taking an approved medication for relapse prevention, such as naltrexone or acamprosate, as follows:

i. People who participate in approved LCTS drug studies to prevent relapse will be treated with MM. A single treatment keeps the study manageable and allows direct assessment of the drug's effect.
ii. The people who are not on drug studies, but are taking naltrexone or acamprosate off-study (as they could/should in the real-world) will be offered CBI or MM.
iii. Anyone not taking a drug for relapse prevention will be offered CBI.

f. During the outpatient treatment phase participants may experience medical, psychiatric, or psychosocial problems. These clinical situations would be handled by trained staff (nurse/counselors, physicians, clinical psychologists, and/or social workers) in according to the standard of care appropriate for the situation.

5. Inclusion and exclusion criteria

a. Inclusion

1) Age greater than 18 years old.
2) Are seeking help for alcohol drinking-related problems.

b. Exclusion

1) People who present with complicated medical problems requiring intensive medical or diagnostic management, such as:
    a. Hypertensive emergency
    b. Serious GI bleeding
    c. Major organ or body system dysfunction such as decompensated liver disease, renal failure, myocardial ischemia, congestive heart failure or cerebrovascular disease, major endocrine problems such as uncontrolled diabetes, pancreatic or thyroid disease.
2) People who are infected with the Human Immunodeficiency Virus (HIV).
3) Serious neuro-psychiatric conditions which impair judgment or cognitive function to an extent that precludes them from providing informed consent, such as acute psychosis or severe dementia (incompetent individuals).
4) People who are unlikely or unable to complete the treatment program because they become or are likely to be incarcerated while on the protocol.
5) People who are required to receive treatment by a court of law or who are involuntarily committed to treatment.

6. Monitoring research participants and criteria for withdrawal of participants from the study

Significant events in the natural history of alcohol abuse and dependency can have very serious consequences; they include severe depression, harm to self or others, cognitive impairment, medical illness and serious legal problems. Such events do have relevance for monitoring and withdrawal of participants from this protocol, despite the fact that it is they would very unlikely be related to therapy or procedures performed in the protocol.

During the hospitalization treatment phase, patients are kept on a secured unit under close nurse monitoring. Alcohol withdrawal severity is monitored with frequent vital signs (VS) monitoring and CIWA-Ar scoring. In general, during the first few days after stopping alcohol, VS monitoring and CIWA-Ar scoring is done hourly or every 2-4 hours until the scores are consistently below a range of 5-7. They may also be done on an "as indicated" basis, at the discretion of the healthcare team.

During the outpatient treatment phase, participants will be examined in clinic (as scheduled above) by nurses and physicians with training and experience in the addictions who will specifically review symptoms of depression, intent to harm self or others and a history of complications such as serious legal or domestic problems. The healthcare professionals will also review the mood ratings scales from CPRS-S-A. Participants will be examined in greater detail for cognitive impairment and medical illness if either is suggested based on history and/or routine exam.

Withdrawal criteria

- Participants who become incarcerated or who perpetrate harm to self or others.
- Non-compliance with therapy: Guidelines and strategies for handling absences from therapy sessions, lateness, lapse (first instance of drinking) and relapse (first episode of sustained heavy drinking) to alcohol and/or drug use have been established and will be used in the context of CBI (Miller, 2004). For participants who miss appointments, efforts will be made to establish phone or other contact to enquire about the reasons for the absence(s) and to encourage them to return to treatment. Indeed, therapists are trained to use non-compliance itself as an area of focus for treatment, to the extent that the subject stays engaged. Absolute non-compliance, for example exhibited by extensive alcohol or drug use, and repeated absence from therapy will constitute grounds for withdrawal from the protocol. This will be at the discretion of the therapists in consultation with the Principal Investigator.
- Participants may withdraw from the protocol at any time for any reason. If they do, NIAAA and/or CC staff will make efforts to ensure their safety and well being.

The following participants will be considered stopped:

- Those who are withdrawn
- Those who decide to seek treatment referral elsewhere after the detoxification and assessment period
- Those who successfully complete the outpatient course of therapy

7. Analysis of the study

To assess performance of the program, statistics will be cumulatively kept regarding consent, screening, enrollment, and withdrawal/stop, including breakdown by timeline period: inpatient/stabilization, baseline and outpatient treatment. Specific participant outcomes to be measured include attendance at counseling sessions, drop-out, lapse, relapse, and follow-up ASI scores. For administrative and regulatory purposes, information such as the demographic composition of the cohort, co-morbid conditions and resource utilization (inpatient and outpatient visits, duration of stay, etc.) will be kept. This descriptive data will be also used in other protocols of relapse prevention, alcohol withdrawal and other studies in which patients may co-participate. The MRI scans collected under this protocol may be grouped together with the MRI's collected under other descriptive protocols assessing the effects of heavy alcohol use on the recovering brain and correlating them with outcomes.

8. Data Management Plan

Currently, data is collected by paper and entered into application-specific data tables. However, a web-based clinical research data management system is currently under development at the LCTS. The ultimate goal of this system is to permit point-of-observation data collection through user-friendly applications. Additionally, standard operating procedures and documentation for system use, data collection, auditing, disaster recovery and security are also being developed.

In summary the system consists of a relational database server, an object-oriented middle-tier (business model) and a variety of front-end applications. The front-end applications are designed to validate out-of-range values and missing key variables at the time of collection. Data will be entered by trained personnel such as investigators and staff who are directly engaged in and familiar with clinical activities. Self-report data will be audited at the time of capture. Also, data entry personnel without clinical research training may be employed at times to enter data from paper-based forms. Access to the system will be through authentication mechanisms established for all NIH systems (currently Microsoft® Active Directory). Access to data entry screens and reporting modules will be role-based, with oversight of the assignment of roles made by the principal investigator and the NIAAA Clinical Director.

Data from the CC information system, supplied through agreement with the Division of Clinical Research Informatics, is directly imported to the relational database; it includes: biographic and demographic data, medical record numbers, CC visits, protocol participation and laboratory test results.

9. Human subjects protection

A. Rationale for subject selection

Alcohol problems occur in both men and women across all cultures. Thus, participation in our program of detoxification, assessment and treatment of alcohol problems will be open to all qualified people who can be accommodated. Individuals are recruited primarily from the Washington, D.C. metropolitan area through standing newspaper advertisements. A copy of the currently running ad is attached to this protocol (Figure). Any changes to it will be subject to IRB approval. Individuals will also be recruited through outreach to healthcare organizations, particularly those that see patients with alcohol and drug problems throughout Northern Virginia, Maryland, West Virginia and the Washington, D.C. and Baltimore metropolitan areas and elsewhere in the US. Increased efforts will be made to recruit Hispanics. This will take the form of contacts with treatment programs that serve Hispanic populations, as well as through advertising in Spanish language newspapers. Language interpreters are available for non-English speaking participants. The intramural research program is primarily focused on alcohol-related problems in adults, thus patients must be at least 18 years of age to be enrolled. Furthermore, NIH CC policy forbids minors (individuals under age 18) to be housed on the same inpatient unit with adults (over age 18).

This research is covered by a Confidentiality Certificate, issued by the Public Health Service under the authority of 42 U.S.C. 241 (d). Under this certificate, NIAAA is authorized to protect the privacy of the participants engaged in research by withholding the subject's name and other identifying information from all persons not connected to this research, except under the circumstances specified in the consent form under the section: Information on Confidentiality.

B. Evaluation of Benefits and Risks/Discomforts

1. Benefits

To individual participants:

1) A thorough medical and psychiatric screening examination, including dental and gynecological examinations, as indicated, may prevent long-term illness and deterioration of quality of life and identify treatable conditions.
2) Supervised medical withdrawal from alcohol can prevent the risk of seizures, delirium tremens and other neurological and medical complications. It may also help prevent the development of long-term CNS damage.
3) CBT and MET- as in CBI- and brief interventional and medication management approaches- as used in MM- among other counseling approaches and brief interventions, and have been shown to have a specific beneficial effect on improving outcome in alcohol dependency (Miller et al., 1995; Carroll, 1996; Project MATCH Research Group, 1998; Burtscheidt et al., 2001).

Benefit to NIAAA/CC program's mission:

1) Improved treatment program performance through feedback and formal analysis of outcomes of therapy and program subject factors (dimensions of assessments)
2) Basis for clinical training of health professionals in the field of addiction medicine, including a fellowship program for physicians
3) Consistent recruiting basis for alcohol clinical research program

Benefit to community and society

1) Source of referral and treatment for people with alcohol problems that impact negatively on community and society.
2) Promote the research mission of a national health initiative (NIAAA).
3) Competently trained addiction medicine specialists

2. Risks/Discomforts

This research protocol authorizes only routine medical care and proposes no experimental therapies and, indeed, carries no more risk than a conventional alcoholism treatment program. It falls under the minimal risk category.

Bodily and Psychological

Psychological discomfort may derive from a variety of sources, for example during the assessment period or in therapy as patients are challenged to discuss personally sensitive issues. To the extent that they are appreciated by the patient themselves, the feelings themselves may become the subject of therapeutic focus and a target of further monitoring, if they are persistent and compelling. Also, some of the results of blood tests may at first create anxiety, such as a positive HIV test. However, anxiogenic these test results may be, they never-the-less provide the important assurance of safety of all participants and healthcare workers involved in the program. Furthermore, they are a responsible form of medical care and widely practiced in the community, and are thus essential.

Physical discomfort mainly would derive from the alcohol withdrawal experience, if it occurs, which is treated as described above. Phlebotomy entails no serious risk and is a relatively minor discomfort, except in individuals (roughly 5% of the population) who faint in response to a phlebotomy or anticipation thereof. A total of up to 364 mL (74 teaspoons) will be done over the course of the baseline evaluation (up to 178 mL, 36 teaspoons) and outpatient therapy (186 mL, 38 teaspoons). Some of the findings from these tests (for example, anti-HCV antibody (+) results) may require other tests for further clinical assessment. This research blood volume will be included in calculations in any additional protocols in which the subject might participate to ensure maximum allowable limits of blood draws will not be exceeded.

Since the CPT-WM and DDT neurocognitive tasks require the use of monetary compensation as a way of motivating subjects, efforts will be made to reduce the risk that alcoholic subjects will use the money they are paid to buy alcoholic beverages. Specifically, the risk of using the money to buy alcoholic beverages will be discussed with each alcoholic participant by the alcohol treatment unit staff. If an alcoholic subject reports that he or she expects that his/her earnings might be used to buy alcoholic beverages, the subject will be urged to make other arrangements for the disposition of the money (e.g., have a relative hold it).

Confidentiality and information technology standards are in place at the intramural programs of the NIH campus, including the NIAAA/LCTS to protect electronic repositories of patient data. It is reasonably expected that these safeguards will protect participants' medical and personal health information, ensuring their privacy.

C. Consent process

The informed consent process will take place at the NIH Clinical (Research) Center in Bethesda, Maryland. It will follow the policies and procedures as described in Manual Transmittal Sheet M77-2 (rev.), 7 March 2003, "Informed Consent", from the Medical Administrative Series of The Clinical Center. In summary, this policy states that informed consent begins at the time of recruitment of patients, involves oral discussions of the protocol with the potential participant, the signature of an IRB-approved consent document, and ongoing discussion and education about the protocol for the duration of their participation.

The determination of impairment is made by the admitting physician and the primary nurse on the basis of clinical examination, including factors such as the level of intoxication using breath alcohol and urine drug testing; severity of withdrawal (the CIWA has 5 parameters of CNS function); neurologic, psychiatric and other functioning. As a matter of standard practice, all subjects, regardless of the clinical setting (inpatient or outpatient), sign the Impaired version of the consent at the time of their initial protocol evaluation visit. After an extended period of hourly observation, assessment and treatment they sign the Unimpaired version. Practically speaking, for inpatients, this is usually sometime after hospital day 4; for outpatients, this is at the time of their second protocol visit (usually at least 1 week after the initial visit), provided they are not impaired on clinical grounds (see above).

Either the Principal Investigator or an Associate Investigator will conduct the consent process in accordance with CC policy. The investigator will first explain that this protocol is primarily designed to allow patients to be treated at the NIH for alcohol problems, including alcohol withdrawal and outpatient counseling. Also, they will be told of the nature of the research mission at NIAAA and that during their participation in this protocol they may be asked about their willingness to participate in other research studies. Furthermore, the investigator will explain tests (pen-and-paper type and biomedical procedures) that are required and the information that will be sought, and roughly how much time they may take. They will be told of the fact that some of the biomedical tests that are performed may have serious implications about their future prognosis (life expectancy and well-being) and insurability, such as HIV infection. They will further be told that this information will be stored in the CC hospital information system as well as a secure centralized computer system maintained by the NIAAA staff. They will also be informed of the NIH confidentiality policy. Finally, they will be told of their option to voluntarily withdrawal from further participation in the protocol at any time. Signing the consent form will constitute enrollment. In keeping with standard good practice, the protocol will be re-explained to participants after admission to the program, particularly after the treatment for the alcohol withdrawal syndrome, if it occurs, to reassess their understanding of the nature of the protocol and treatment plan.

10. Adverse Event (AE) Reporting
Adverse events will be reported in accordance with Federal and NIH requirements.

11. Subject Reimbursement Schedule

Compensation

Payment of all participants will conform to Clinical Center Schedules. Subjects will receive payment for the MRI Structural Scan as follows:

Activity Testing	Time (+ICUs)	Cost
MRI Structural Scan*	1 hour (1)	20.00 (10.00)

12. Data and Safety Monitoring
Data and safety will be monitored by the Principal investigator. The medical staff, including other PI's, the head nurse for the inpatient unit and outpatient clinic and the Clinical Director, will review adverse events and safety issues on a quarterly basis.

Timeline for Training and Natural History Protocol

Phases with usual duration in parentheses

1) Phone intake/screening (1-2 weeks)
2) Detox/psychosocial stabilization (if necessary) (2-5 days)
3) Baseline assessments (1-4 weeks)
    a. Blood and urine tests
    b. ECG, CXR
    c. Interviews/scales, pencil and paper tests
    d. MRI (or may be done during outpatient follow-up)
    e. Neurocognitive tests
    f. Biomarkers
    g. Genetics
4) Inpatient stabilization, evaluation with treatment (1-30 + days)
5) Outpatient follow-up (12 weeks):

Week	**If in CBI group**	**MD visit, if on medication**	**Blood tests, urine drug screen, rating scales**
1	✓	✓	✓
2	✓	✓	✓
3	✓		
4	✓	✓	✓
5	✓		
6	✓		
7	✓		
8	✓	✓	✓
9			
10	✓		
11			
12	✓	✓	✓

MEDICAL RECORD	**CONSENT TO PARTICIPATE IN A CLINICAL RESEARCH STUDY** • Adult Patient or • Parent, for Minor Patient

INSTITUTE: National Institute on Alcohol Abuse and Alcoholism

STUDY NUMBER: 98-AA-0009 PRINCIPAL INVESTIGATOR: Daniel Hommer, M.D.

STUDY TITLE: Screening Evaluation for NIAAA Protocols

Latest IRB Review: Continuing Review 9/4/04
Latest Amendment Approved: Amend E 5/7/04
Consent A

INTRODUCTION

We invite you to take part in a research study at the National Institutes of Health (NIH).

First, we want you to know that:

Taking part in NIH research is entirely voluntary.

You may choose not to take part, or you may withdraw from the study at any time. In either case, you will not lose any benefits to which you are otherwise entitled. However, to receive care at the NIH, you must be taking part in a study or be under evaluation for study participation.

You may receive no benefit from taking part. The research may give us knowledge that may help people in the future.

Second, some people have personal, religious or ethical beliefs that may limit the kinds of medical or research treatments they would want to receive (such as blood transfusions). If you have such beliefs, please discuss them with your NIH doctors or research team before you agree to the study.

Now we will describe this research study. Before you decide to take part, please take as much time as you need to ask any questions and discuss this study with anyone at NIH, or with family, friends or your personal physician or other health professional.

Consent A: Medical Evaluation Only

Signing this consent form means that you are requesting admission to the inpatient treatment unit of the National Institute on Alcohol Abuse and Alcoholism (NIAAA) for the purpose of treatment of Alcoholism.

You will not have to make any decisions about being in a specific research study right now. After you have been in the hospital for at least 48 hours we will ask you to sign a consent form describing your participation in a more complete evaluation designed to find out which research studies you are eligible for.

Right now, you are going to have an exam to find out if you are medically ill and if you need any immediate medical attention.

MEDICAL RECORD	**CONTINUATION SHEET for either:** **NIH 2514-1, Consent to Participate in A Clinical Research Study** **NIH 2514-2, Minor Patient's Assent to Participate In A Clinical Research Study**

STUDY NUMBER: 98-AA-0009 CONTINUATION: page 2 of 4 pages

This exam will include the following:

1) The nurse and doctor will ask you questions about physical and mental health. You will be asked in detail about your alcohol and drug use.
2) You will have a physical exam.
3) You will have blood tests to determine the general state of your health and nutrition. These will include tests for hepatitis types B and C which are viral infections of the liver.
4) You will have one or more at breath alcohol tests to find out how much alcohol is in your body.
5) You will have an electrocardiogram (ECG) to examine the health of your heart.
6) If you are a women you will have a urine test to determine if you are pregnant.
7) You will have an X-ray of your chest to look for evidence of tuberculosis.
8) Your urine will be tested for illicit drugs.

You will not have a blood test for HIV (the AIDS virus) now unless you have symptoms suggesting you may have AIDS.

The results of this exam will become part of your medical record. Part of your medical record may have the results of the urine tests for illicit drugs and blood tests for hepatitis viruses. Your medical record can only be released with your written agreement. Be aware that insurance companies may require you to release these records and may not give you insurance if you refuse.

Some of this information may be used for research. However, none of the results of this exam will be used for research until you read and sign another consent form explaining this research. When this exam is completed, any abnormal findings will be explained to you.

We are doing this exam to find out if you need immediate medical help. Sometimes we find out something that prevents us from offering you inpatient treatment here at the NIH. For example some acute medical conditions such as bleeding ulcer or pneumonia might exclude you from all our research studies. If we cannot offer you care here, but you need medical or psychiatric help, we will send you to another hospital for the treatment you need.

Risks and Discomforts

It is possible that the exam and questions may uncover uncomfortable feelings that may get you upset. Potentially sensitive information related to history of drug use or psychiatric illness may also be uncovered. It is possible that we may discover previously undiagnosed medical or psychiatric conditions. If any of this happens, we will provide you support and if necessary, make every effort to refer you for treatment if you are not admitted to NIH. If you have a serious life threatening condition, you will be stabilized in our clinic or hospital before transferring you another facility for treatment.

Getting your blood drawn may hurt a little bit. There is also a small risk of infection or a bruise.

If there is something you do not understand, please ask the doctor or the nurse to explain it to you.

MEDICAL RECORD	**CONTINUATION SHEET for either:** **NIH 2514-1, Consent to Participate in A Clinical Research Study** **NIH 2514-2, Minor Patient's Assent to Participate In A Clinical Research Study**

STUDY NUMBER: 98-AA-0009 CONTINUATION: page 3 of 4 pages

Benefits

You will receive a medial evaluation and you will be told of the results.

Information on Confidentiality

This research is covered by a Confidentiality Certificate, issued by the Public Health Service under the authority of 42 U.S.C. 241(d). Under this certificate, NIAAA is authorized to protect your privacy by withholding your name and other identifying characteristics from all persons not connected to this research, except under the following circumstances: 1) where you consent in writing for disclosure of your medical records; 2) where authorized persons of the United States Department of Health and Human Services request such information for audit or program evaluation or other investigation; 3) where release is required under the Federal Food, Drug and Cosmetic Act or its implementing regulations; 4) where NIAAA researchers identify any evidence of child or spouse abuse which endangers a third party and that is required to be reported under state law; or 5) where NIAAA researchers have reason to believe a protocol subject has a real and specific intention to inflict injury upon a specific victim or group of victims. This means that unless one or more of these circumstances listed above apply, the NIAAA researchers may not be compelled in any Federal, State, or local civil, criminal, administrative, legislative or other proceeding to identify you.

MEDICAL RECORD	CONSENT TO PARTICIPATE IN A CLINICAL RESEARCH STUDY . Adult Patient or . Parent, for Minor Patient

STUDY NUMBER: 98-AA-0009 CONTINUATION: page 4 of 4 pages

OTHER PERTINENT INFORMATION

1. Confidentiality. When results of an NIH research study are reported in medical journals or at scientific meetings, the people who take part are not named and identified. In most cases, the NIH will not release any information about your research involvement without your written permission. However, if you sign a release of information form, for example, for an insurance company, the NIH will give the insurance company information from your medical record. This information might affect (either favorably or unfavorably) the willingness of the insurance company to sell you insurance.

The Federal Privacy Act protects the confidentiality of your NIH medical records. However, you should know that the Act allows release of some information from your medical record without your permission, for example, if it is required by the Food and Drug Administration (FDA), members of Congress, law enforcement officials, or other authorized people.

2. Policy Regarding Research-Related Injuries. The Clinical Center will provide short-term medical care for any injury resulting from your participation in research here. In general, no long-term medical care or financial compensation for research-related injuries will be provided by the National Institutes of Health, the Clinical Center, or the Federal Government. However, you have the right to pursue legal remedy if you believe that your injury justifies such action.

3. Payments. The amount of payment to research volunteers is guided by the National Institutes of Health policies. In general, patients are not paid for taking part in research studies at the National Institutes of Health.

4. Problems or Questions. If you have any problems or questions about this study, or about your rights as a research participant, or about any research-related injury, contact the Principal Investigator, Daniel Hommer, MD; Building 10, Room 6S240, Telephone: 301-496-8996.. Other researchers you may call are: David T. George, MD Telephone: 301-496-9705 and Joseph R. Hibbeln, MD, 301-435-4028.

You may also call the Clinical Center Patient Representative at 301-496-2626.

5. Consent Document. Please keep a copy of this document in case you want to read it again.

COMPLETE APPROPRIATE ITEM(S) BELOW:

A. Adult Patient's Consent

I have read the explanation about this study and have been given the opportunity to discuss it and to ask questions. I hereby consent to take part in this study.

Signature of Adult Patient/Legal Representative _____ Date _____

B. Parent's Permission for Minor Patient.

I have read the explanation about this study and have been given the opportunity to discuss it and to ask questions. I hereby give permission for my child to take part in this study.
(Attach NIH 2514-2, Minor's Assent, if applicable.)

Signature of Parent(s)/Guardian _____ Date _____

C. Child's Verbal Assent (If Applicable)

The information in the above consent was described to my child and my child agrees to participate in the study.

Signature of Parent(s)/Guardian _____ Date _____

THIS CONSENT DOCUMENT HAS BEEN APPROVED FOR USE FROM OCTOBER 2, 2004 THROUGH OCTOBER 2, 2005.

Signature of Investigator _____ Date _____ Signature of Witness _____ Date _____

PATIENT IDENTIFICATION	CONSENT TO PARTICIPATE IN A CLINICAL RESEARCH STUDY (Continuation Sheet) • Adult Patient or • Parent, for Minor Patient NIH-2514-1 (5-98) P.A.: 09-25-0099 **FAX TO: (301) 480-3126** File in Section 4: Protocol Consent

Appendix I

Clinical Institute Withdrawal Assessment of Alcohol Scale, Revised (CIWA-Ar)

Patient:_____ Date: _____ Time: _____
(24 hour clock, midnight = 00:00)
Pulse or heart rate, taken for one minute:_____ Blood pressure:_____

NAUSEA AND VOMITING -- Ask "Do you feel sick to your stomach? Have you vomited?"

0 no nausea and no vomiting
1 mild nausea with no vomiting
2
3
4 intermittent nausea with dry heaves
5
6
7 constant nausea, frequent dry heaves and vomiting

TACTILE DISTURBANCES -- Ask "Have you any itching, pins and needles sensations, any burning, any numbness, or do you feel bugs crawling on or under your skin?"

0 none
1 very mild itching, pins and needles, burning or numbness
2 mild itching, pins and needles, burning or numbness
3 moderate itching, pins and needles, burning or numbness
4 moderately severe hallucinations
5 severe hallucinations
6 extremely severe hallucinations
7 continuous hallucinations

TREMOR -- Arms extended and fingers spread apart.

0 no tremor
1 not visible, but can be felt fingertip to fingertip
2
3
4 moderate, with patient's arms extended
5
6
7 severe, even with arms not extended

AUDITORY DISTURBANCES -- Ask "Are you more aware of sounds around you? Are they harsh? Do they frighten you? Are you hearing anything that is disturbing to you? Are you hearing things you know are not there?"

0 not present
1 very mild harshness or ability to frighten
2 mild harshness or ability to frighten

3 moderate harshness or ability to frighten
4 moderately severe hallucinations
5 severe hallucinations
6 extremely severe hallucinations
7 continuous hallucinations

PAROXYSMAL SWEATS –

0 no sweat visible
1 barely perceptible sweating, palms moist
2
3
4 beads of sweat obvious on forehead
5
6
7 drenching sweats

VISUAL DISTURBANCES -- Ask "Does the light appear to be too bright? Is its color different? Does it hurt your eyes? Are you seeing anything that is disturbing to you? Are you seeing things you know are not there?"

0 not present
1 very mild sensitivity
2 mild sensitivity
3 moderate sensitivity
4 moderately severe hallucinations
5 severe hallucinations
6 extremely severe hallucinations
7 continuous hallucinations

ANXIETY -- Ask "Do you feel nervous?"

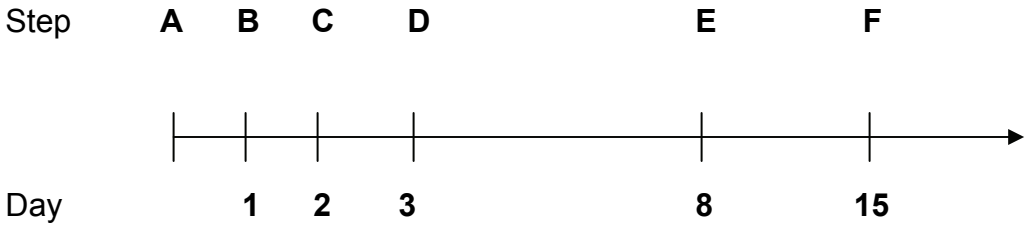
0 no anxiety, at ease
1 mild anxious
2
3
4 moderately anxious, or guarded, so anxiety is inferred
5
6
7 equivalent to acute panic states as seen in severe delirium or acute schizophrenic reactions

HEADACHE, FULLNESS IN HEAD -- Ask "Does your head feel different? Does it feel like there is a band around your head?" Do not rate for dizziness or lightheadedness. Otherwise, rate severity.

0 not present
1 very mild
2 mild
3 moderate
4 moderately severe
5 severe
6 very severe
7 extremely severe

Appendix J

Activty Flow Sheet – Clinical Study #2



Step A: Potential subject contact with NIAAA; Interview and assess whether patient would like to participate. Informed Consent Form discussion, comprehension, and signing; Physical and psychiatric examination including DSM-IV/SCID-I and Drugs of Abuse testing; Inclusion and exclusion criteria evaluation of patient.

Step B: Admission for detoxification and stabilization at NIAAA/CRC. BrAC evaluation; Baseline sampling will be performed including: Blood biological sampling, CIWA-AR, and vital signs, urine Drugs of Abuse testing.

Step C: Day 2 after admission. BrAC evaluation; Baseline sampling will be performed including: blood biological sampling, CIWA-AR, and vital signs, urine Drugs of Abuse testing; Biological testing for biomarkers performed in the AM, *before* smoking and eating. FTND administration to volunteer for categorization into subgroup (NS, LS, HS).

Steps D-F: Days 3, 8 and 15 after admission. Same as step C. Alcohol TLFB, Food Inventory administration only once any time after sobriety.

Appendix K

Patient Summary – Clinical Study #2

Sample #	Sample ID	SS	GEN	ADMIT_DRUG SC	BrAC	BrAC level	ETH	WEIGHT	AGE	ACAMP	other drgs
1	BJ 426574	HS	F	cocaine	N	0.00	W	48.8	33.0	y	dexamethasone
2	GL 429351	HS	F	benzo	N	0.00	W	44.1	57.0	n	folic acid, cetirizine
3	MA 413368	HS	F	cocaine	N	0.00	W	63.1	40.0	n	fluoxetine, naltrexone, diazepam, cyclobenazeprine, nicotine
4	BA 387068	HS	F	none	Y	0.48	W	69.6	40.0	y	diazepam, mag sulfate, dexamethasone, naltrexone
5	AJ 134479	HS	F	none	Y	0.15	W	70.0	35.0	y	dexamethasone
6	PR 434767	HS	F	cocaine	Y	0.07	B	52.1	40.0	y	folic acid, clonidine, HCTZ, diltiazem, fluconazole
7	MN 428274	HS	M	none	N	0.00	W	87.2	33.0	y	dexamethasone, nicotine
8	AC 430856	HS	M	none	N	0.00	W	85.3	52.0	n	thiamine
9	SP 138958	HS	M	benzo	N	0.00	W	105.0	56.0	n	varenicline, diazepam, thiamine, HCTZ
10	MC 424155	HS	M	none	Y	0.05	W	92.0	53.0	n	nicotine
11	SC 336280	HS	M	none	Y	0.18	W	89.4	28.0	y	lorazepam
12	RJ 433869	HS	M	none	Y	0.06	W	78.0	39.0	y	dexamethasone, Alum Hydr/Mag, ranitidine, sertraline, trazodone, na
13	MA 405373	LS	F	none	N	0.00	W	63.4	34.0	n	nicotine, expt drug (NK1 antag)
15	CT 429363	LS	F	none	N	0.00	W	75.2	45.0	n	venlfaxine,
16	SS 433876	LS	F	none	N	0.00	W	70.0	31.0	n	sertraline, oxycodone, nicotine
17	WN 423181	LS	F	THC	Y	0.11	B	99.2	28.0	y	diazepam, dexamethasone
18	MS 424158	LS	F	none	Y	0.31	B	76.6	50.0	n	amoxicillin, HCTZ, levofloxacin
19	RA 423267	LS	M	cocaine	N	0.00	U	89.5	41.0	n	ketoconazole, diphenhydramine,
20	CM 428250	LS	M	none	N	0.00	W	87.9	58.0	n	clonidine, enalapril,
21	BH 431163	LS	M	benzo	N	0.00	W	91.2	45.0	n	thiamine
22	SM 427078	LS	M	none	Y	0.30	W	116.0	29.0	y	diazepam, cholecalciferol, diphenhydramine
23	DK 343165	LS	M	none	Y	0.10	B	100.0	43.0	n	thiamine, cephalexin
24	BD 433377	LS	M	THC	Y	0.10	B	75.0	30.0	y	naltrexone, dexamethasone, cholecalciferol
25	BA 394144	NS	F	none	N	0.00	W	74.0	56.0	n	alum hydrox/mag, pseuephedrine, NK1 antag
26	TC 429955	NS	F	none	N	0.00	W	80.0	53.0	n	thiamine, dyphenhtdramine
27	ML 431173	NS	F	none	N	0.00	W	54.0	32.0	n	diazepam, trazodone
28	BK 432873	NS	F	none	N	0.00	W	63.7	32.0	n	dydonine lozenge, pseudoephedrine,
29	RA 326261	NS	F	amphetamines	Y	0.29	W	47.0	46.0	n	diazepam, trazodone
30	TJ 432467	NS	F	none	Y	0.08	B	69.8	40.0	n	HCTZ, sertraline, diazepam, diphenhydramine
31	AO 426871	NS	M	none	N	0.00	H	78.7	36.0	n	thiamine, ranitidine
32	GP 427076	NS	M	none	N	0.00	H	98.9	31.0	n	thiamine
33	CR 181466	NS	M	benzo	N	0.00	W	98.8	41.0	n	pseudoephednine, naltraxone,
34	LK 432070	NS	M	benzo	N	0.00	B	72	38	n	diazepam, thiamine, fluoxetine, quetiapine
35	RE 432150	NS	M	none	N	0.00	U	71.8	46.0	n	naltrexone, omeprazole
36	GR 433260	NS	M	benzo	N	0.00	W	103.0	48.0	n	diazepam, atenolol, HCTZ

Appendix L

S-PLUS® ANCOVA and Linear model outputs – Clinical Study #1

Table L-1: ANOVA for main effects of SS and GEN on Log H

```
*** Analysis of Variance Model ***

Short Output:
Call:
   aov(formula = log.H ~ SS + GEN + visit + SS:GEN, data =
       SAL.BC..Final.Bioanalysis.Data.VCU.Smoking.031808, na.action =
       na.exclude)

Terms:
                      SS      GEN    visit   SS:GEN Residuals
 Sum of Squares  7.40497  0.02159  0.71119  1.34362  16.63132
Deg. of Freedom        2        1        1        2        75

Residual standard error: 0.4709044
Estimated effects may be unbalanced

          Df Sum of Sq  Mean Sq  F Value     Pr(F)
       SS  2   7.40497 3.702483 16.69658 0.0000010
      GEN  1   0.02159 0.021590  0.09736 0.7558877
    visit  1   0.71119 0.711188  3.20715 0.0773521
   SS:GEN  2   1.34362 0.671812  3.02958 0.0742898
Residuals 75  16.63132 0.221751

Residual standard error: 0.4832 on 77 degrees of freedom
Multiple R-Squared: 0.3116
F-statistic: 8.715 on 4 and 77 degrees of freedom, the p-value is 7.41e-006

95 % simultaneous confidence intervals for specified
linear combinations, by the Scheffe method

critical point: 2.86
response variable: log.H
rank used for Scheffe method: 3

intervals excluding 0 are flagged by '****'

      Estimate Std.Error Lower Bound Upper Bound
HS-LS   -0.032     0.143      -0.440       0.376
HS-NS    0.571     0.127       0.209       0.933 ****
LS-NS    0.603     0.127       0.241       0.965 ****
```

Table L-2: ANOVA for main effects of SS and GEN on Log NH

```
*** Analysis of Variance Model ***

Short Output:
Call:
   aov(formula = log.NH ~ SS + GEN + visit + SS:GEN, data =
       SAL.BC..Final.Bioanalysis.Data.VCU.Smoking.031808, na.action =
       na.exclude)

Terms:
                      SS      GEN    visit   SS:GEN Residuals
 Sum of Squares 13.94415  0.35342  0.01848  1.90789  12.54758
Deg. of Freedom        2        1        1        2        75

Residual standard error: 0.4090246
Estimated effects may be unbalanced

          Df Sum of Sq  Mean Sq  F Value     Pr(F)
       SS  2  13.94415 6.972076 41.67382 0.0000000
      GEN  1   0.35342 0.353417  2.11246 0.1502757
    visit  1   0.01848 0.018483  0.11048 0.7405308
   SS:GEN  2   1.90789 0.953946  2.70197 0.0649521
Residuals 75  12.54758 0.167301

Residual standard error: 0.4333 on 77 degrees of freedom
Multiple R-Squared: 0.4976
F-statistic: 19.06 on 4 and 77 degrees of freedom, the p-value is 6.246e-011

95 % simultaneous confidence intervals for specified
linear combinations, by the Scheffe method

critical point: 2.86
response variable: log.NH
rank used for Scheffe method: 3

intervals excluding 0 are flagged by '****'

      Estimate Std.Error Lower Bound Upper Bound
HS-LS    0.276     0.124     -0.0778       0.631
HS-NS    0.924     0.110      0.6100       1.240 ****
LS-NS    0.648     0.110      0.3330       0.962 ****
```

Table L-3: Linear Model for effects of FTND on Log H

```
*** Linear Model ***

Call: lm(formula = log.H ~ GEN + FTND + GEN:FTND, data =
      SAL.BC..Final.Bioanalysis.Data.VCU.Smoking.032908, na.action =
      na.exclude)
Residuals:
    Min      1Q  Median     3Q    Max
 -1.212 -0.3671 0.02479 0.4088 0.9894

Coefficients:
               Value Std. Error  t value Pr(>|t|)
(Intercept)   1.0875   0.0974    11.1606   0.0000
        GEN   0.2575   0.1425     1.8074   0.0746
       FTND   0.1561   0.0310     5.0431   0.0000
   GEN:FTND  -0.1241   0.0414    -2.9971   0.0037

Residual standard error: 0.4991 on 78 degrees of freedom
Multiple R-Squared: 0.2558
F-statistic: 8.939 on 3 and 78 degrees of freedom, the p-value is 0.00003681
```

Table L-4: Linear Model for effects of #cig/day on Log H

```
*** Linear Model ***

Call: lm(formula = log.H ~ .cig.day + GEN + .cig.day:GEN, data =
      SAL.BC..Final.Bioanalysis.Data.VCU.Smoking.032908, na.action =
      na.exclude)
Residuals:
    Min      1Q  Median     3Q   Max
 -1.206 -0.3661 0.03123 0.3959 1.015

Coefficients:
               Value Std. Error  t value Pr(>|t|)
 (Intercept)   1.0810   0.0967    11.1744   0.0000
    .cig.day   0.0342   0.0067     5.1395   0.0000
         GEN   0.2159   0.1431     1.5088   0.1354
.cig.day:GEN  -0.0149   0.0126    -1.1823   0.2407

Residual standard error: 0.4924 on 78 degrees of freedom
Multiple R-Squared: 0.2757
F-statistic: 9.896 on 3 and 78 degrees of freedom, the p-value is 0.00001328
```

Table L-5: Linear Model for effects of FTND on Log NH

```
*** Linear Model ***

Call: lm(formula = log.NH ~ FTND + GEN + FTND:GEN, data =
      SAL.BC..Final.Bioanalysis.Data.VCU.Smoking.032908, na.action =
      na.exclude)
Residuals:
    Min      1Q Median     3Q    Max
 -1.054 -0.3148 0.1162 0.2901 0.7051

Coefficients:
               Value Std. Error  t value Pr(>|t|)
(Intercept)   1.1142   0.0862    12.9225   0.0000
       FTND   0.2006   0.0274     7.3253   0.0000
        GEN   0.2987   0.1261     2.3691   0.0203
   FTND:GEN  -0.1082   0.0367    -2.9514   0.0042

Residual standard error: 0.4417 on 78 degrees of freedom
Multiple R-Squared: 0.4712
F-statistic: 23.16 on 3 and 78 degrees of freedom, the p-value is 8.026e-011
```

Table L-6: Linear Model for effects of #cig/day on Log NH

```
*** Linear Model ***

Call: lm(formula = log.NH ~ .cig.day + GEN + .cig.day:GEN, data =
      SAL.BC..Final.Bioanalysis.Data.VCU.Smoking.032908, na.action =
      na.exclude)
Residuals:
    Min      1Q  Median     3Q    Max
 -1.042 -0.2874 0.07357 0.2893 0.8194

Coefficients:
                Value Std. Error  t value Pr(>|t|)
 (Intercept)   1.1027   0.0842    13.0910   0.0000
    .cig.day   0.0443   0.0058     7.6449   0.0000
         GEN   0.2711   0.1246     2.1756   0.0326
.cig.day:GEN  -0.0041   0.0110    -0.3731   0.7101

Residual standard error: 0.4288 on 78 degrees of freedom
Multiple R-Squared: 0.5016
F-statistic: 26.16 on 3 and 78 degrees of freedom, the p-value is 8.201e-012
```

Table L-7: ANCOVA for SS and GEN on Log H, Food and AAI as Covariates

```
*** Analysis of Variance Model ***

Short Output:
Call:
   aov(formula = log.H ~ SS + GEN + visit + Food.H + AAI, data =
       SAL.BC..Final.Bioanalysis.Data.VCU.Smoking.032908, na.action =
       na.exclude)

Terms:
                       SS      GEN    visit   Food.H      AAI Residuals
 Sum of Squares  7.40497  0.02159  0.71119  0.41445  0.37278  17.18772
Deg. of Freedom        2        1        1        1        1        75

Residual standard error: 0.4787166
Estimated effects may be unbalanced

          Df Sum of Sq  Mean Sq  F Value     Pr(F)
      SS   2   7.40497 3.702483 16.15609 0.0000015
     GEN   1   0.02159 0.021590  0.09421 0.7597453
   visit   1   0.71119 0.711188  3.10333 0.0822077
  Food.H   1   0.41445 0.414448  1.80848 0.1827439
     AAI   1   0.37278 0.372784  1.62667 0.2061005
Residuals 75  17.18772 0.229170
```

Table L-8: ANCOVA for SS and GEN on Log NH, Food and AAI as Covariates

```
*** Analysis of Variance Model ***

Short Output:
Call:
   aov(formula = log.NH ~ SS + GEN + visit + Food.NH + AAI, data =
       SAL.BC..Final.Bioanalysis.Data.VCU.Smoking.032908, na.action =
       na.exclude)

Terms:
                       SS      GEN    visit  Food.NH      AAI Residuals
 Sum of Squares 13.94415  0.35342  0.01848  0.28137  0.14743  14.02668
Deg. of Freedom        2        1        1        1        1        75

Residual standard error: 0.4324608
Estimated effects may be unbalanced

          Df Sum of Sq  Mean Sq  F Value     Pr(F)
      SS   2  13.94415 6.972076 37.27937 0.0000000
     GEN   1   0.35342 0.353417  1.88971 0.1733284
   visit   1   0.01848 0.018483  0.09883 0.7541161
  Food.NH  1   0.28137 0.281367  1.50445 0.2238240
     AAI   1   0.14743 0.147430  5.78830 0.0774522
Residuals 75  14.02668 0.187022
```

Table L-9: ANOVA for main effects of SS and GEN on Log S-SAL

```
*** Analysis of Variance Model ***

Short Output:
Call:
   aov(formula = log.SAL ~ SS + GEN + visit + SS:GEN, data =
       SAL.BC..Final.Bioanalysis.Data.VCU.Smoking.031808, na.action =
       na.exclude)

Terms:
                      SS      GEN    visit   SS:GEN Residuals
 Sum of Squares  7.40497  0.02159  0.71119  1.34362  16.63132
Deg. of Freedom        2        1        1        2        75

Residual standard error: 0.4709044
Estimated effects may be unbalanced

            Df Sum of Sq  Mean Sq  F Value     Pr(F)
       SS   2   7.89131 3.945656 15.52240 0.0000012
      GEN   1   0.14529 0.145293  0.57159 0.4512744
    visit   1   0.00779 0.007790 0.017859 0.8940485
   SS:GEN   2   1.52140 0.760699  2.99263 0.0543319
 Residuals 75  32.71512 0.436202

Multiple R-Squared: 0.1921
F-statistic: 3.402 on 5 and 75 degrees of freedom, the p-value is 0.007939

95 % simultaneous confidence intervals for specified
linear combinations, by the Scheffe method

critical point: 2.8393
response variable: log.S.SAL
rank used for Scheffe method: 3

intervals excluding 0 are flagged by '****'

      Estimate Std.Error Lower Bound Upper Bound
HS-LS    0.267     0.128     -0.0969       0.632
HS-NS    0.623     0.116      0.2930       0.952 ****
LS-NS    0.355     0.116      0.0252       0.685 ****
```

Table L-10: ANOVA for main effects of SS and GEN on Log R-SAL

```
*** Analysis of Variance Model ***

Short Output:
Call:
   aov(formula = log.R.SAL ~ SS + GEN + visit + SS:GEN, data =
       SAL.BC..Final.Bioanalysis.Data.VCU.Smoking.032908, na.action =
       na.exclude)

Response: LOG R-SAL

          Df Sum of Sq  Mean Sq  F Value     Pr(F)
       SS   2   9.62605 4.813024 13.97085 0.0000040
      GEN   1   0.01798 0.017981  0.05219 0.8197207
    visit   1   0.00047 0.000468 0.000777 0.9778414
   SS:GEN   2   1.41383 0.706913  2.05197 0.1334615
Residuals  75  37.20651 0.344505

Multiple R-Squared: 0.2571
F-statistic: 9.156 on 3 and 75 degrees of freedom, the p-value is 0.00001845

95 % simultaneous confidence intervals for specified
linear combinations, by the Scheffe method

critical point: 2.8401
response variable: log.R.SAL
rank used for Scheffe method: 3

intervals excluding 0 are flagged by '****'

      Estimate Std.Error Lower Bound Upper Bound
HS-LS    0.233     0.147     -0.1840       0.650
HS-NS    0.676     0.133      0.2990       1.050 ****
LS-NS    0.443     0.133      0.0656       0.821 ****
```

Table L-11: ANOVA for main effects of SS and GEN on Log DA

```
*** Analysis of Variance Model ***

Short Output:
Call:
   aov(formula = log.DA ~ SS + GEN + visit + SS:GEN, data =
       SAL.BC..Final.Bioanalysis.Data.VCU.Smoking.032908, na.action =
       na.exclude)

Terms:
                      SS      GEN    visit   SS:GEN Residuals
 Sum of Squares 1.165686 0.019696 0.000109 1.426734  7.412780
Deg. of Freedom        2        1        1        2        75

Residual standard error: 0.3143836
Estimated effects may be unbalanced

          Df Sum of Sq   Mean Sq  F Value     Pr(F)
       SS  2  1.165686 0.5828430 5.897008 0.0041824
      GEN  1  0.019696 0.0196963 0.199280 0.6565887
    visit  1  0.000109 0.0001090 0.001103 0.9735986
   SS:GEN  2  1.426734 0.7133668 7.217604 0.0213590
Residuals 75  7.412780 0.0988371

R-Squared:  0.2656
F-statistic: 5.277 on 2 and 75 degrees of freedom, the p-value is 0.0002244

95 % simultaneous confidence intervals for specified
linear combinations, by the Scheffe method

critical point: 2.86
response variable: log.DA
rank used for Scheffe method: 3

intervals excluding 0 are flagged by '****'

      Estimate Std.Error Lower Bound Upper Bound
HS-LS   0.1980    0.0952     -0.0742       0.470
HS-NS   0.2650    0.0845      0.0238       0.507 ****
LS-NS   0.0674    0.0845     -0.1740       0.309
```

Table L-12: Linear Model for effects of FTND on Log S-SAL

```
*** Linear Model ***

Call: lm(formula = log.S.SAL ~ FTND + GEN + FTND:GEN, data =
       SAL.BC..Final.Bioanalysis.Data.VCU.Smoking.032908, na.action =
       na.exclude)
Residuals:
    Min      1Q   Median     3Q   Max
 -1.152 -0.5319 -0.08436 0.5098 1.389

Coefficients:
               Value Std. Error  t value Pr(>|t|)
(Intercept)   1.7735   0.1288    13.7686   0.0000
       FTND   0.1235   0.0409     3.0171   0.0034
        GEN   0.6218   0.1883     3.3014   0.0015
   FTND:GEN  -0.1668   0.0548    -3.0472   0.0032

Residual standard error: 0.6598 on 78 degrees of freedom
Multiple R-Squared: 0.1521
F-statistic: 4.664 on 3 and 78 degrees of freedom, the p-value is 0.004743
```

Table L-13: Linear Model for effects of #cig/day on Log S-SAL

```
*** Linear Model ***

Call: lm(formula = log.S.SAL ~ .cig.day + GEN + .cig.day:GEN, data =
       SAL.BC..Final.Bioanalysis.Data.VCU.Smoking.032908, na.action =
       na.exclude)
Residuals:
    Min      1Q  Median     3Q   Max
 -1.058 -0.5184 -0.1093 0.5255 1.483

Coefficients:
                Value Std. Error  t value Pr(>|t|)
 (Intercept)   1.7999   0.1325    13.5873   0.0000
    .cig.day   0.0237   0.0091     2.5953   0.0113
         GEN   0.5011   0.1960     2.5568   0.0125
.cig.day:GEN  -0.0250   0.0173    -1.4457   0.1523

Residual standard error: 0.6743 on 78 degrees of freedom
Multiple R-Squared: 0.1143
F-statistic: 3.354 on 3 and 78 degrees of freedom, the p-value is 0.02307
```

Table L-14: Linear Model for effects of FTND on Log R-SAL

```
*** Linear Model ***

Call: lm(formula = log.R.SAL ~ FTND + GEN + FTND:GEN, data =
      SAL.BC..Final.Bioanalysis.Data.VCU.Smoking.032908, na.action =
      na.exclude)
Residuals:
   Min      1Q  Median     3Q   Max
 -1.67 -0.5535 -0.1323 0.6083 1.506

Coefficients:
              Value Std. Error  t value Pr(>|t|)
(Intercept)   1.8309   0.1512    12.1058   0.0000
       FTND   0.1216   0.0480     2.5308   0.0134
        GEN   0.5504   0.2211     2.4887   0.0150
   FTND:GEN  -0.1620   0.0643    -2.5200   0.0138

Residual standard error: 0.7747 on 78 degrees of freedom
Multiple R-Squared: 0.1016
F-statistic: 2.94 on 3 and 78 degrees of freedom, the p-value is 0.03829
```

Table L-15: Linear Model for effects of #cig/day on Log R-SAL

```
*** Linear Model ***

Call: lm(formula = log.R.SAL ~ .cig.day + GEN + .cig.day:GEN, data =
      SAL.BC..Final.Bioanalysis.Data.VCU.Smoking.032908, na.action =
      na.exclude)
Residuals:
    Min      1Q  Median     3Q   Max
 -1.583 -0.6324 -0.1439 0.6064 1.593

Coefficients:
               Value Std. Error  t value Pr(>|t|)
 (Intercept)   1.8738   0.1552    12.0761   0.0000
    .cig.day   0.0215   0.0107     2.0136   0.0475
         GEN   0.4203   0.2295     1.8312   0.0709
.cig.day:GEN  -0.0228   0.0202    -1.1279   0.2628

Residual standard error: 0.7899 on 78 degrees of freedom
Multiple R-Squared: 0.06611
F-statistic: 1.841 on 3 and 78 degrees of freedom, the p-value is 0.1467
```

Table L-16: Linear Model for effects of FTND on Log DA

```
*** Linear Model ***

Call: lm(formula = log.DA ~ FTND + GEN + FTND:GEN, data =
       SAL.BC..Final.Bioanalysis.Data.VCU.Smoking.032908, na.action =
       na.exclude)
Residuals:
    Min      1Q  Median    3Q    Max
 -1.491 -0.1326 0.01949 0.167 0.6027

Coefficients:
               Value Std. Error  t value Pr(>|t|)
(Intercept)   0.7331   0.0590    12.4207   0.0000
       FTND   0.1049   0.0188     5.5961   0.0000
        GEN   0.2546   0.0863     2.9499   0.0042
   FTND:GEN  -0.1122   0.0251    -4.4708   0.0000

Residual standard error: 0.3023 on 78 degrees of freedom
Multiple R-Squared: 0.2888
F-statistic: 10.56 on 3 and 78 degrees of freedom, the p-value is 6.65e-006
```

Table L-17: Linear Model for effects of #cig/day on Log DA

```
*** Linear Model ***

Call: lm(formula = log.DA ~ .cig.day + GEN + .cig.day:GEN, data =
       SAL.BC..Final.Bioanalysis.Data.VCU.Smoking.032908, na.action =
       na.exclude)
Residuals:
    Min      1Q    Median     3Q    Max
 -1.488 -0.1701 -0.008683 0.2211 0.6238

Coefficients:
                Value Std. Error  t value Pr(>|t|)
 (Intercept)   0.7519   0.0618    12.1760   0.0000
    .cig.day   0.0205   0.0042     4.8250   0.0000
         GEN   0.2147   0.0914     2.3501   0.0213
.cig.day:GEN  -0.0199   0.0081    -2.4692   0.0157

Residual standard error: 0.3144 on 78 degrees of freedom
Multiple R-Squared: 0.2311
F-statistic: 7.815 on 3 and 78 degrees of freedom, the p-value is 0.0001258
```

Table L-18: ANCOVA for SS and GEN on Log S-SAL, Food and AAI as Covariates

```
*** Analysis of Variance Model ***

Short Output:
Call:
   aov(formula = log.S.SAL ~ SS + GEN + visit + Food.SAL + AAI, data =
       SAL.BC..Final.Bioanalysis.Data.VCU.Smoking.032908, na.action =
       na.exclude)

Terms:
                      SS      GEN    visit Food.SAL      AAI Residuals
 Sum of Squares  1.06763  1.54763  0.00779  0.01073  0.80364  36.60837
Deg. of Freedom        2        1        1        1        1        75

Residual standard error: 0.6986498
Estimated effects may be unbalanced

          Df Sum of Sq  Mean Sq  F Value     Pr(F)
       SS  2   1.06763 0.533813 1.093630 0.3402800
      GEN  1   1.54763 1.547630 3.170647 0.0790223
    visit  1   0.00779 0.007790 0.015959 0.8998087
 Food.SAL  1   0.01073 0.010733 0.021989 0.8825146
      AAI  1   0.80364 0.803638 1.646422 0.2033975
Residuals 75  36.60837 0.488112
```

Table L-19: ANCOVA for SS and GEN on Log R-SAL, Food and AAI as Covariates

```
*** Analysis of Variance Model ***

Short Output:
Call:
   aov(formula = log.R.SAL ~ SS + GEN + visit + Food.SAL + AAI, data =
       SAL.BC..Final.Bioanalysis.Data.VCU.Smoking.032908, na.action =
       na.exclude)

Terms:
                      SS      GEN    visit Food.SAL      AAI Residuals
 Sum of Squares  1.01355  0.94390  0.00047  0.00001  2.06821  48.08055
Deg. of Freedom        2        1        1        1        1        75

Residual standard error: 0.8006709
Estimated effects may be unbalanced

          Df Sum of Sq  Mean Sq  F Value     Pr(F)
       SS  2   1.01355 0.506773 0.790506 0.4573579
      GEN  1   0.94390 0.943902 1.472376 0.2287788
    visit  1   0.00047 0.000468 0.000731 0.9785085
 Food.SAL  1   0.00001 0.000013 0.000020 0.9964389
      AAI  1   2.06821 2.068209 3.226163 0.0764974
Residuals 75  48.08055 0.641074
```

Appendix M

Individual concentration-time profiles – Clinical Study #2

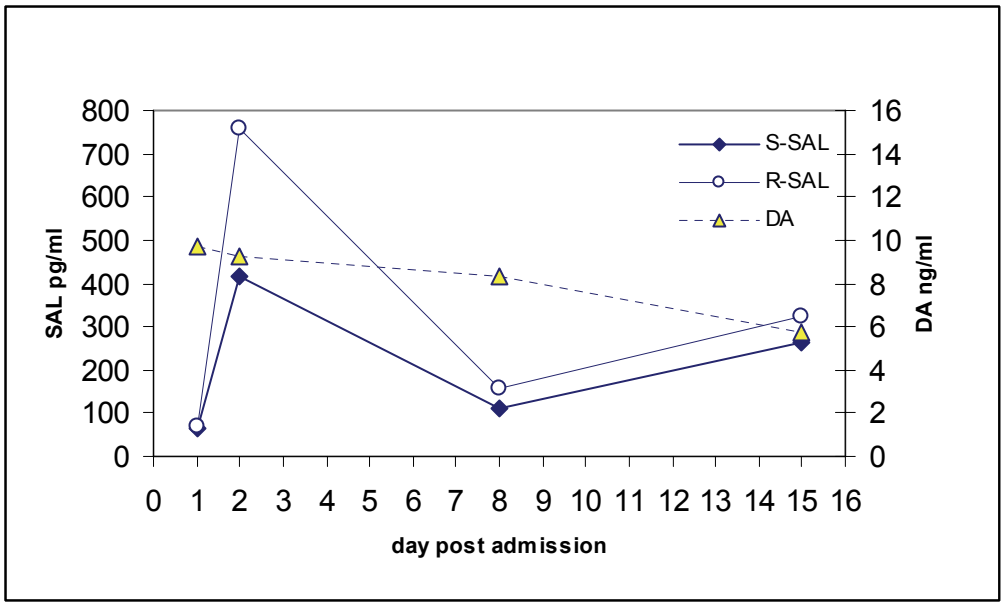


Figure M-1: S/R-SAL (pg/ml) and DA (ng/ml) concentration vs. time profile for Subject 1 (HS, F)

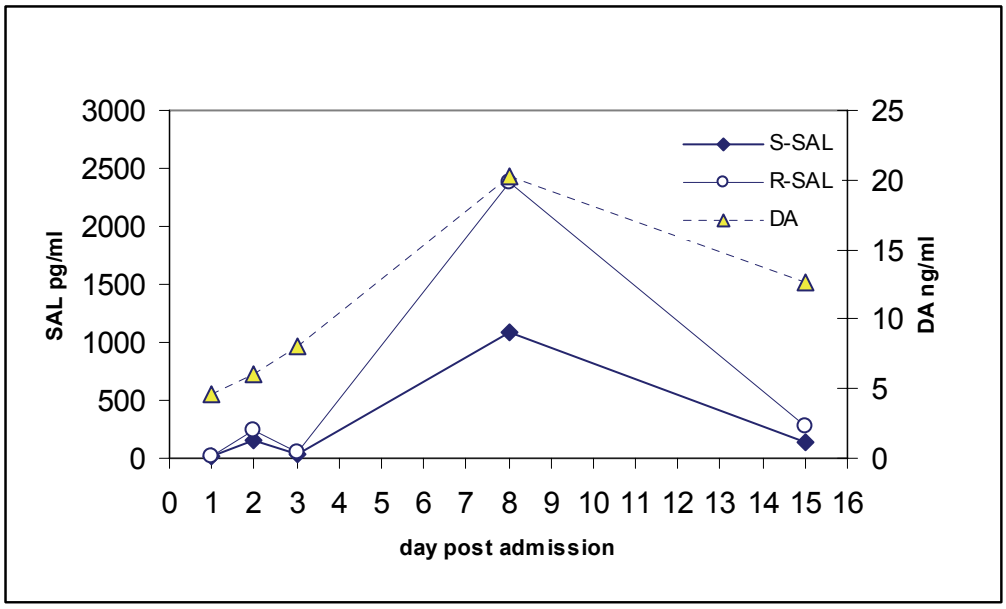


Figure M-2: S/R-SAL (pg/ml) and DA (ng/ml) concentration vs. time profile for Subject 2 (HS, F)

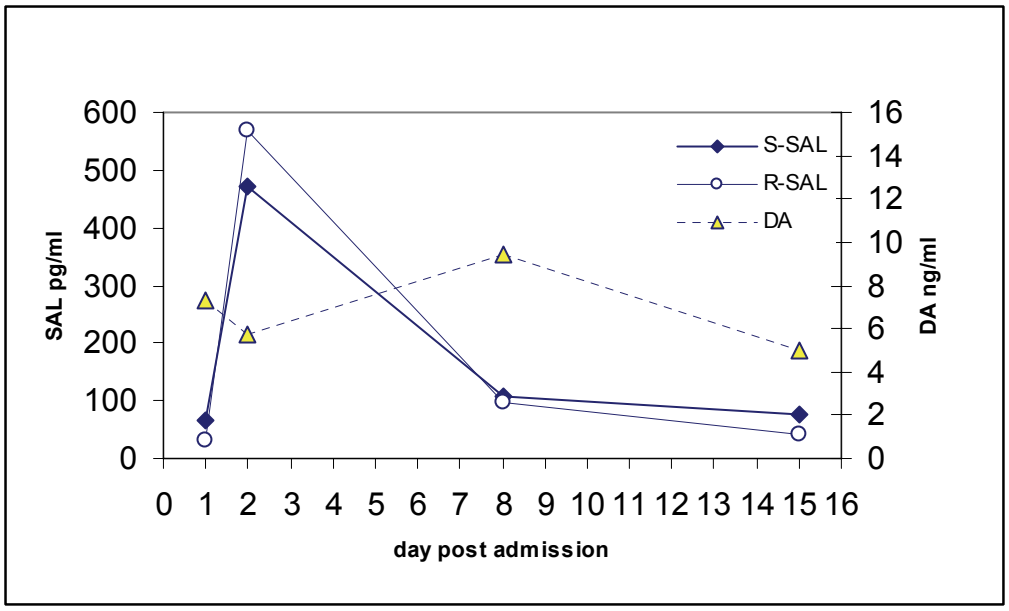


Figure M-3: S/R-SAL (pg/ml) and DA (ng/ml) concentration vs. time profile for Subject 3 (HS, F)

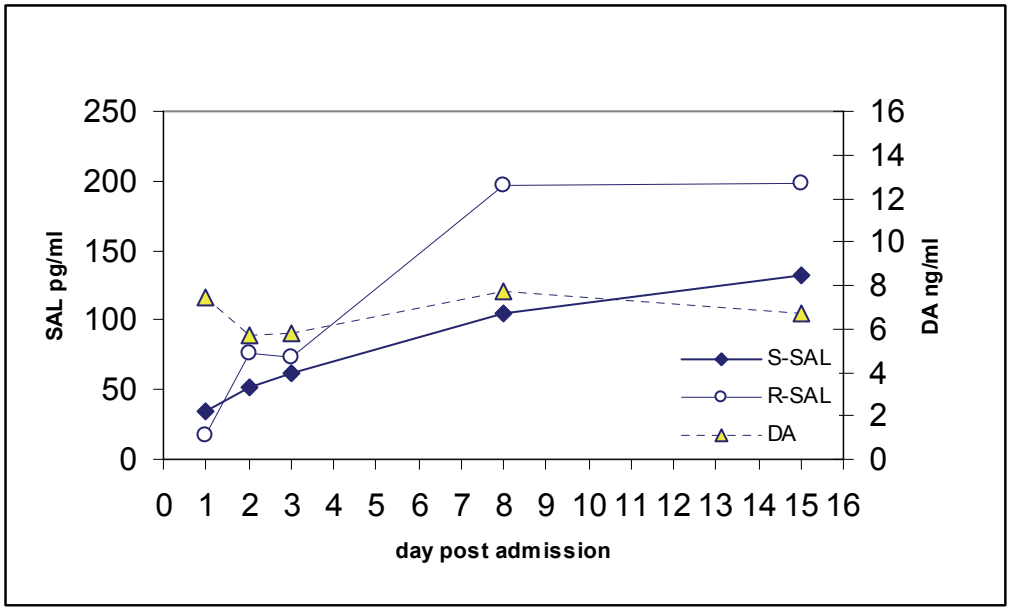


Figure M-4: S/R-SAL (pg/ml) and DA (ng/ml) concentration vs. time profile for Subject 4 (HS, F)

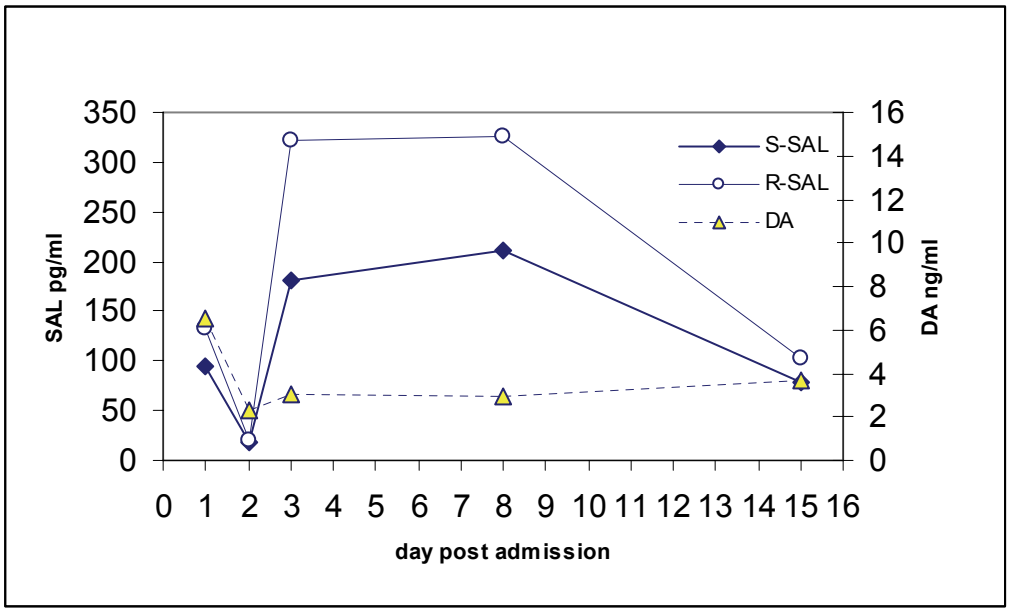


Figure M-5: S/R-SAL (pg/ml) and DA (ng/ml) concentration vs. time profile for Subject 5 (HS, F)

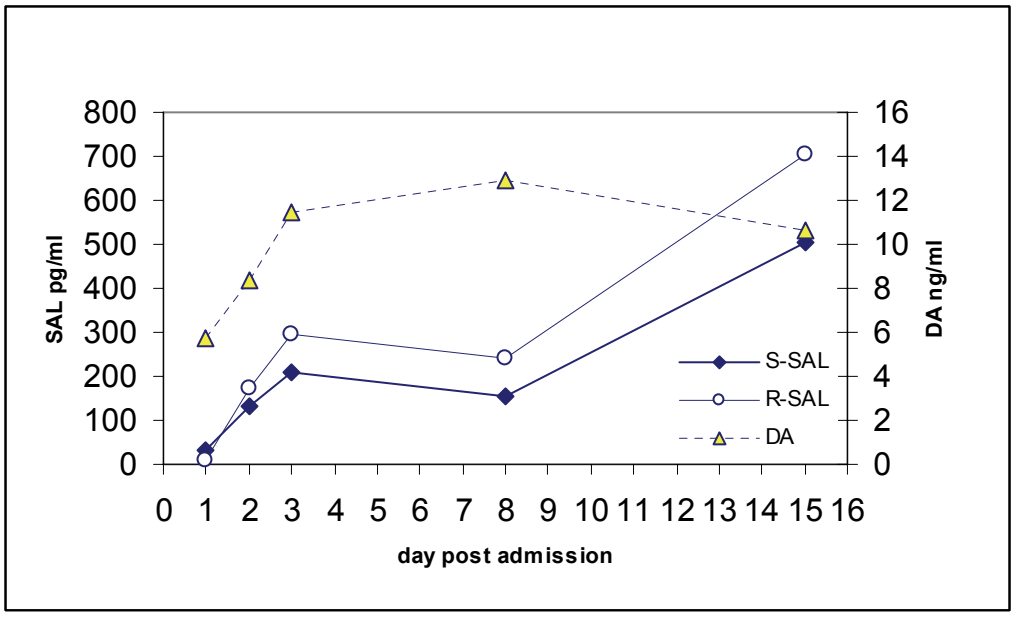


Figure M-6: S/R-SAL (pg/ml) and DA (ng/ml) concentration vs. time profile for Subject 6 (HS, F)

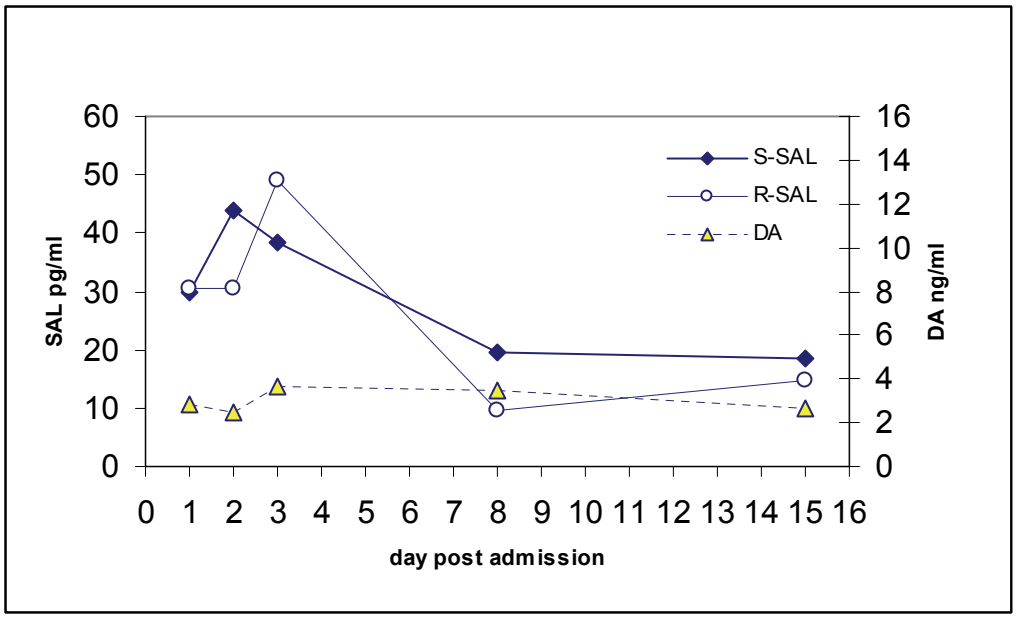


Figure M-7: S/R-SAL (pg/ml) and DA (ng/ml) concentration vs. time profile for Subject 7 (HS, M)

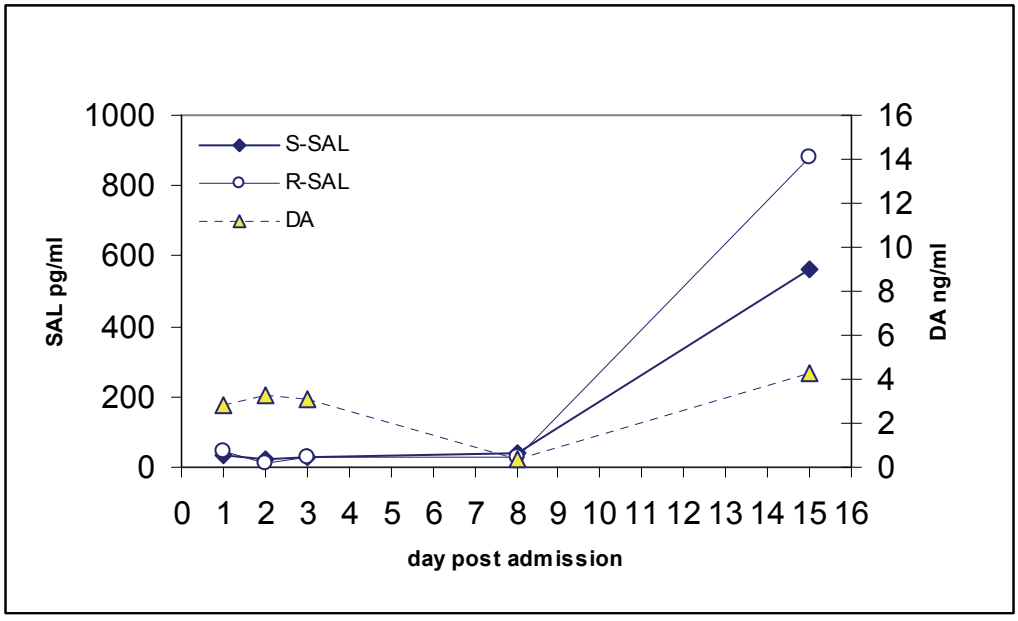


Figure M-8: S/R-SAL (pg/ml) and DA (ng/ml) concentration vs. time profile for Subject 8 (HS, M)

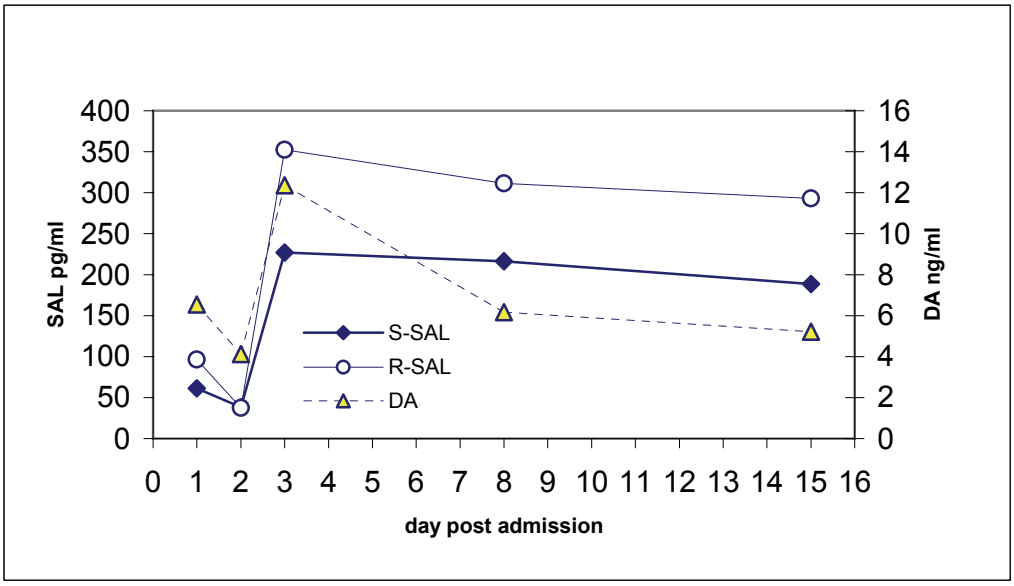


Figure M-9: S/R-SAL (pg/ml) and DA (ng/ml) concentration vs. time profile for Subject 9 (HS, M)

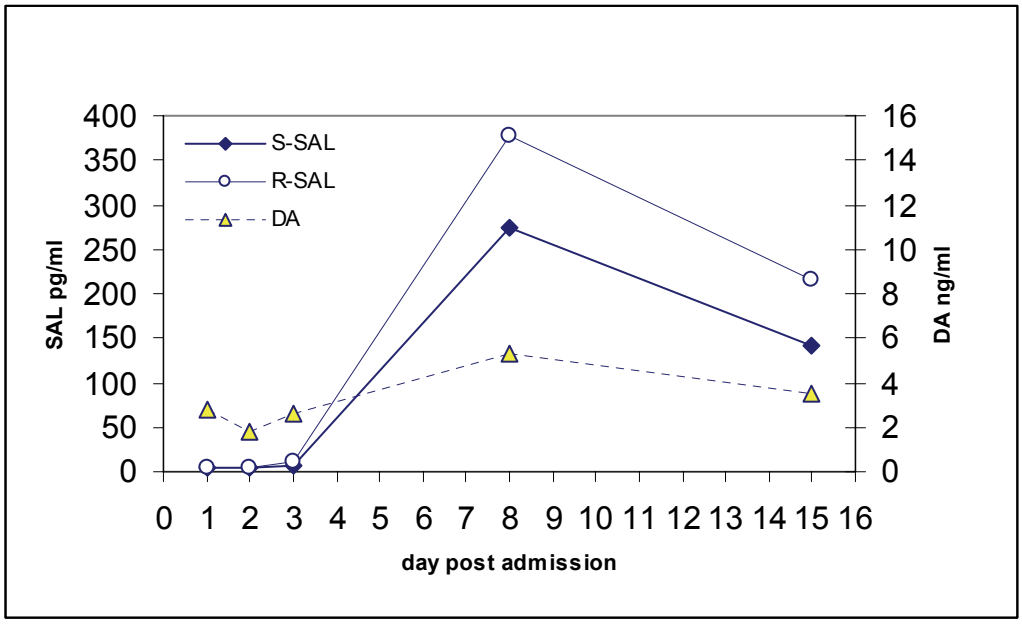


Figure M-10: S/R-SAL (pg/ml) and DA (ng/ml) concentration vs. time profile for Subject 10 (HS, M)

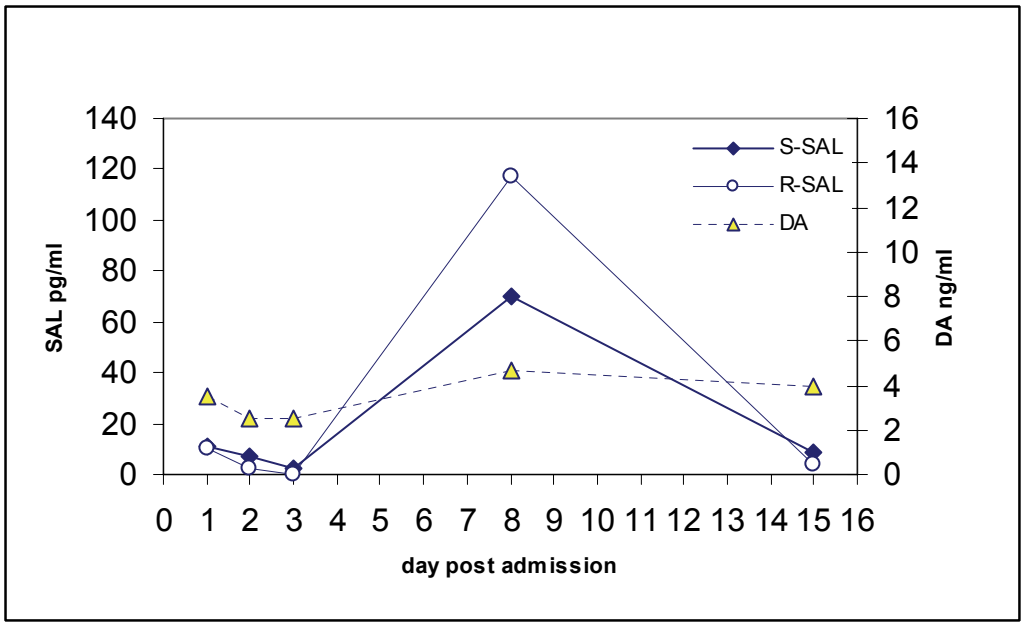


Figure M-11: S/R-SAL (pg/ml) and DA (ng/ml) concentration vs. time profile for Subject 11 (HS, M)

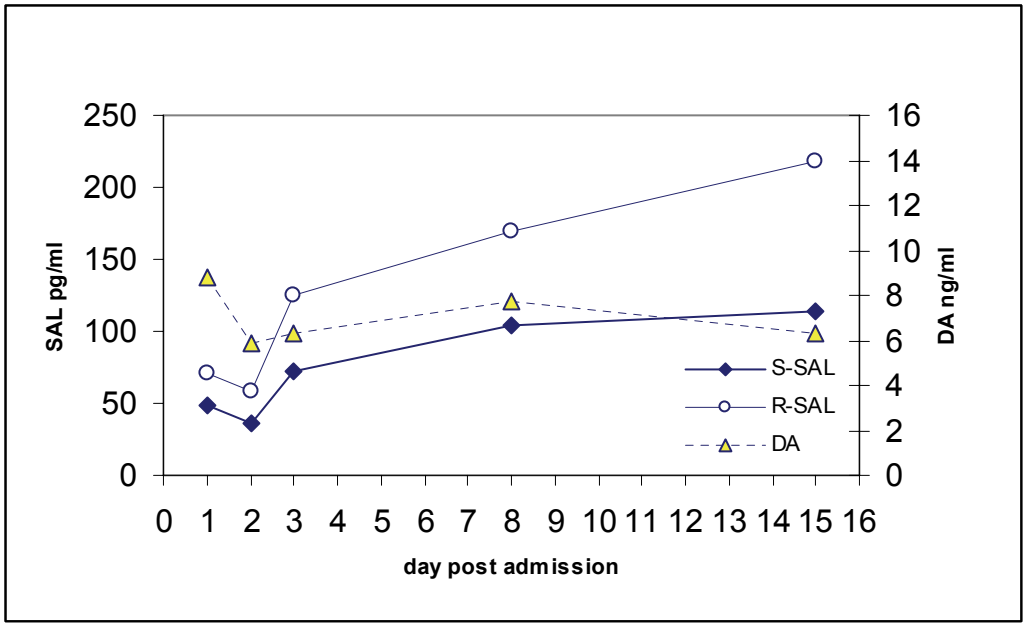


Figure M-12: S/R-SAL (pg/ml) and DA (ng/ml) concentration vs. time profile for Subject 12 (HS, M)

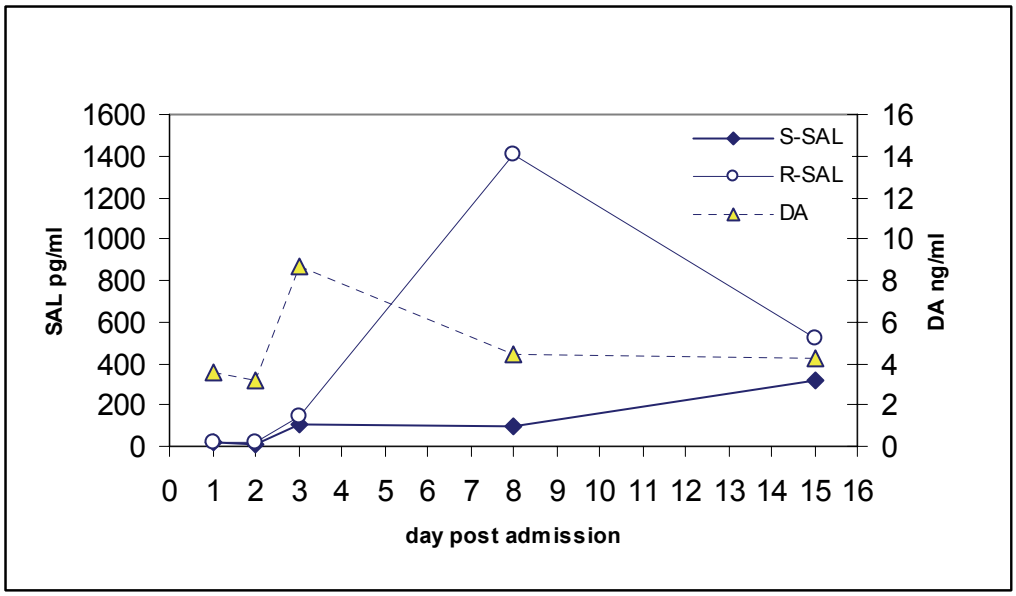


Figure M-13: S/R-SAL (pg/ml) and DA (ng/ml) concentration vs. time profile for Subject 13 (LS, F)

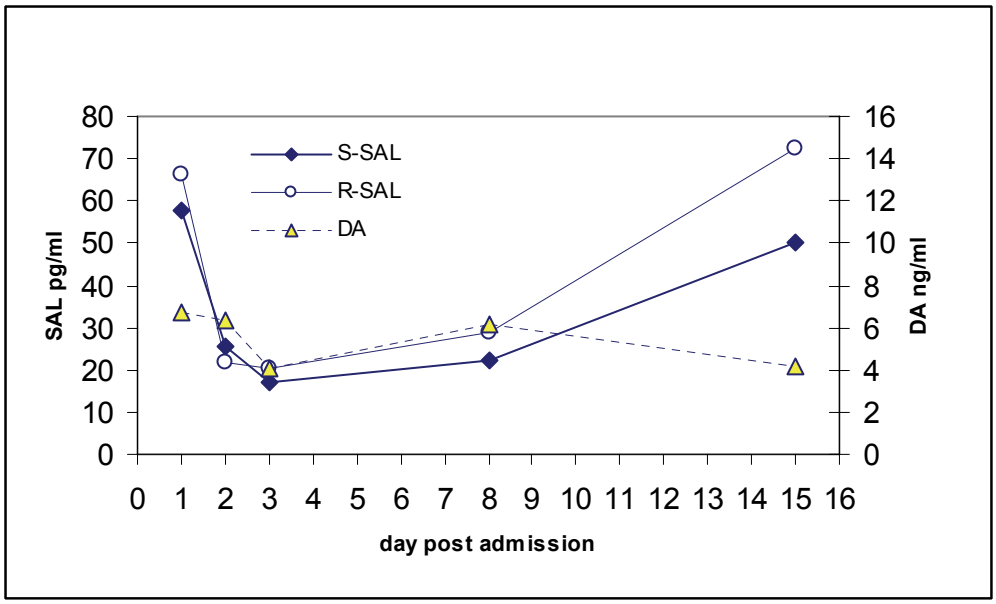


Figure M-14: S/R-SAL (pg/ml) and DA (ng/ml) concentration vs. time profile for Subject 15 (LS, F)

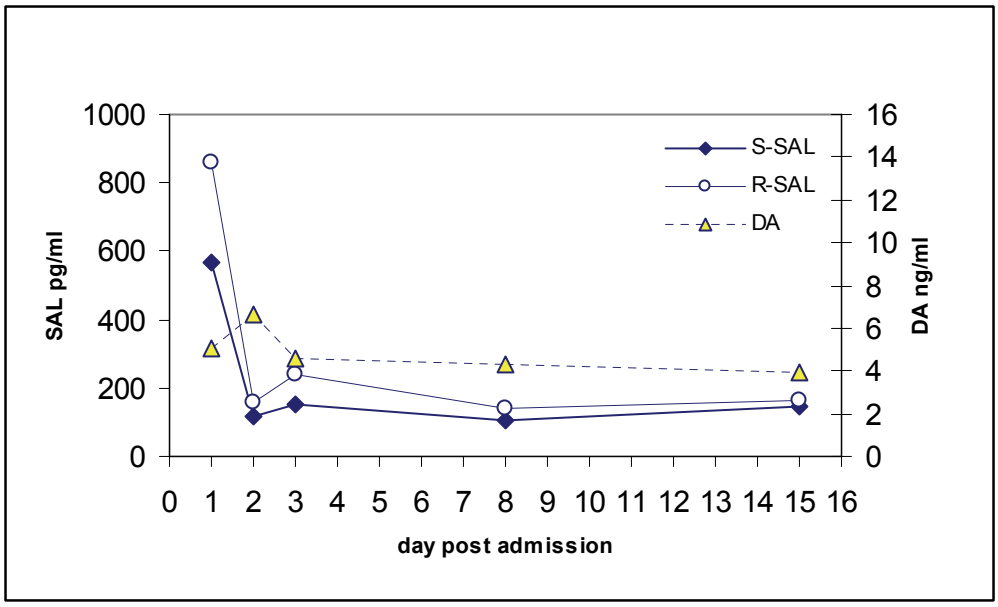


Figure M-15: S/R-SAL (pg/ml) and DA (ng/ml) concentration vs. time profile for Subject 16 (LS, F)

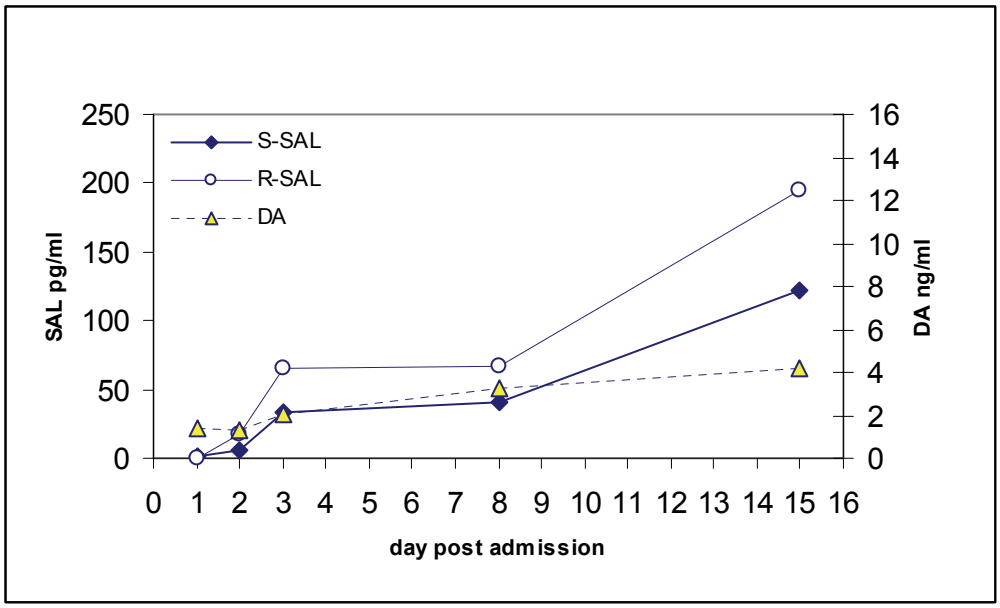


Figure M-16: S/R-SAL (pg/ml) and DA (ng/ml) concentration vs. time profile for Subject 17 (LS, F)

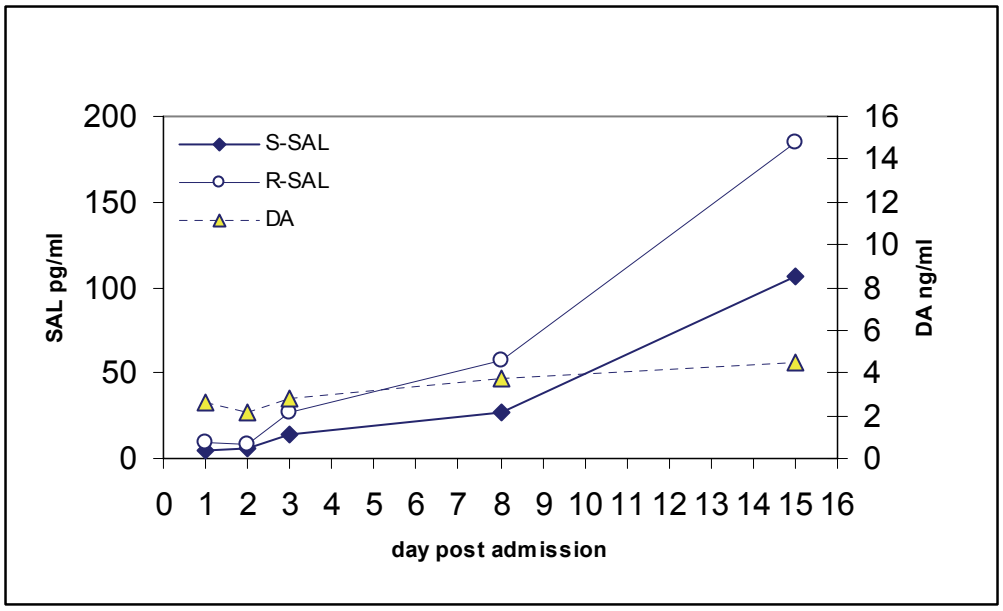


Figure M-17: S/R-SAL (pg/ml) and DA (ng/ml) concentration vs. time profile for Subject 18 (LS, F)

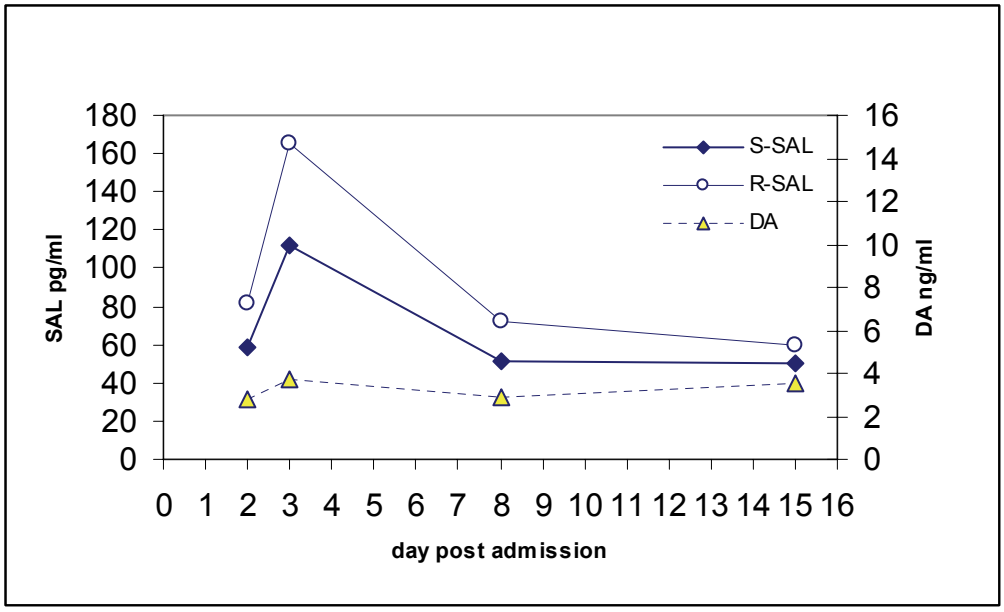


Figure M-18: S/R-SAL (pg/ml) and DA (ng/ml) concentration vs. time profile for Subject 19 (LS, M)

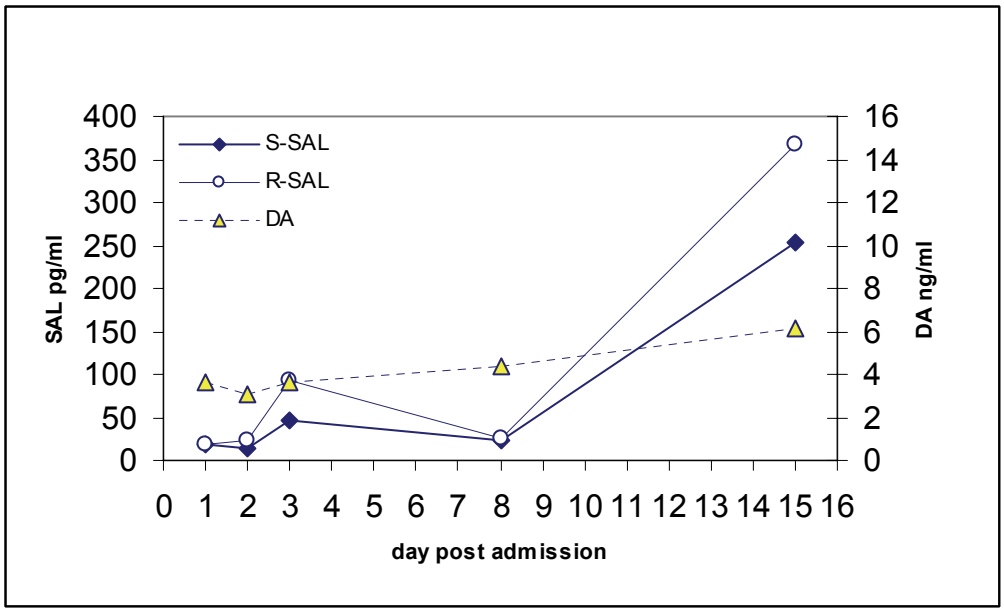


Figure M-19: S/R-SAL (pg/ml) and DA (ng/ml) concentration vs. time profile for Subject 20 (LS, M)

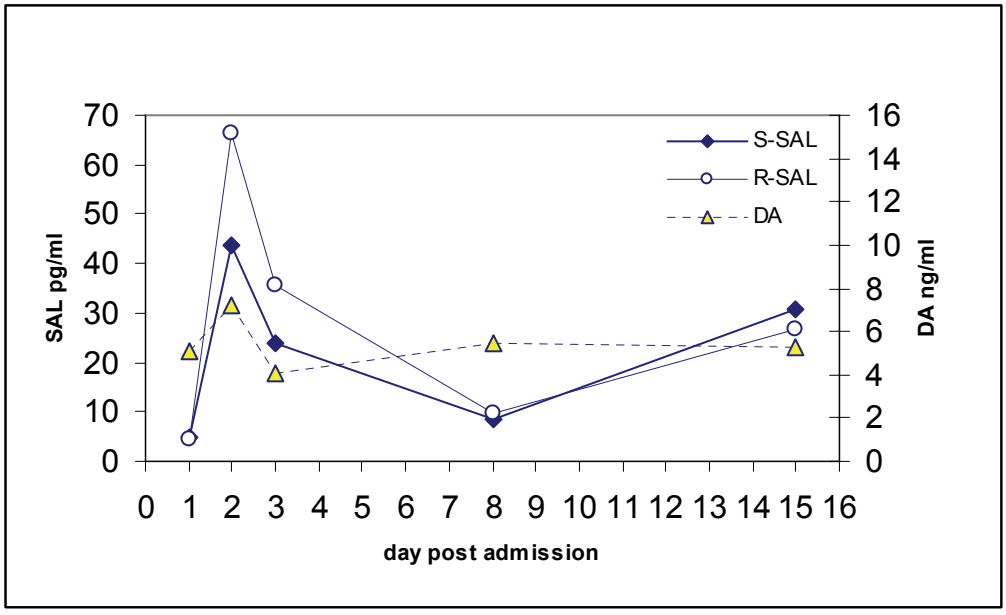


Figure M-20: S/R-SAL (pg/ml) and DA (ng/ml) concentration vs. time profile for Subject 21 (LS, M)

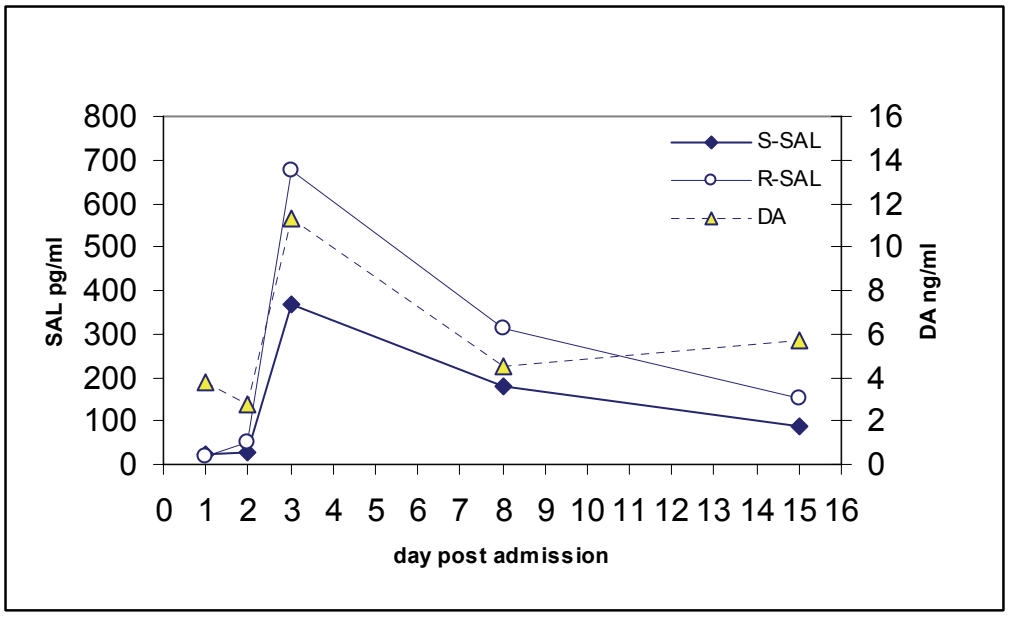


Figure M-21: S/R-SAL (pg/ml) and DA (ng/ml) concentration vs. time profile for Subject 22 (LS, M)

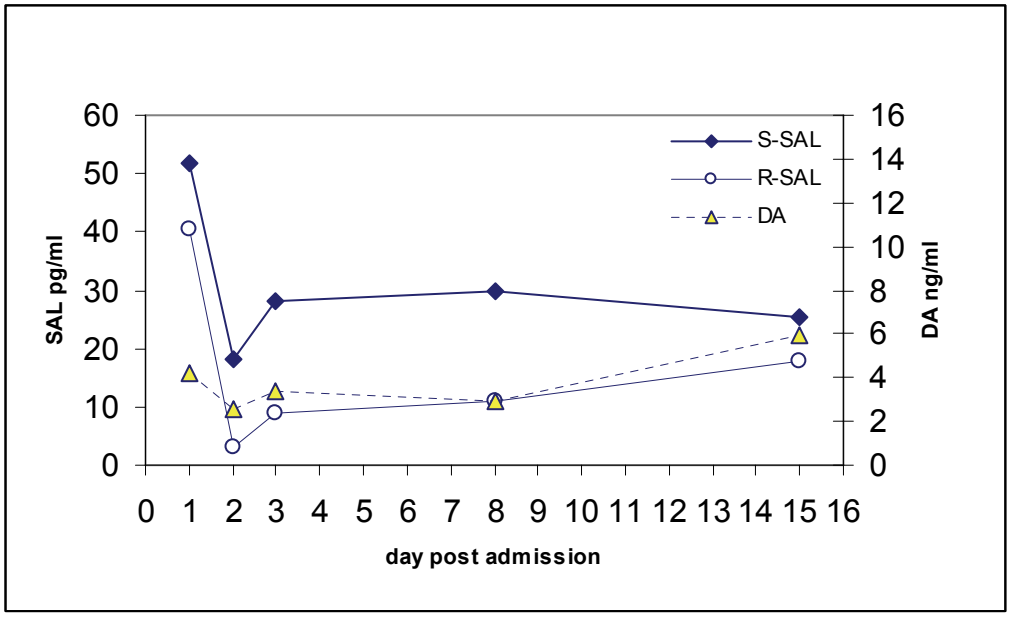


Figure M-22: S/R-SAL (pg/ml) and DA (ng/ml) concentration vs. time profile for Subject 23 (LS, M)

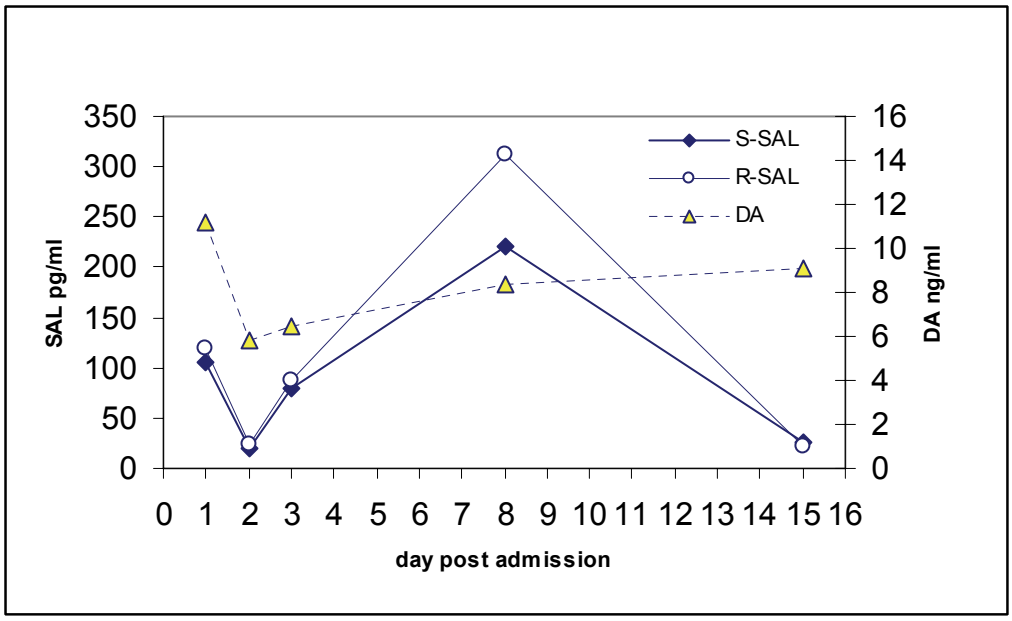


Figure M-23: S/R-SAL (pg/ml) and DA (ng/ml) concentration vs. time profile for Subject 24 (LS, M)

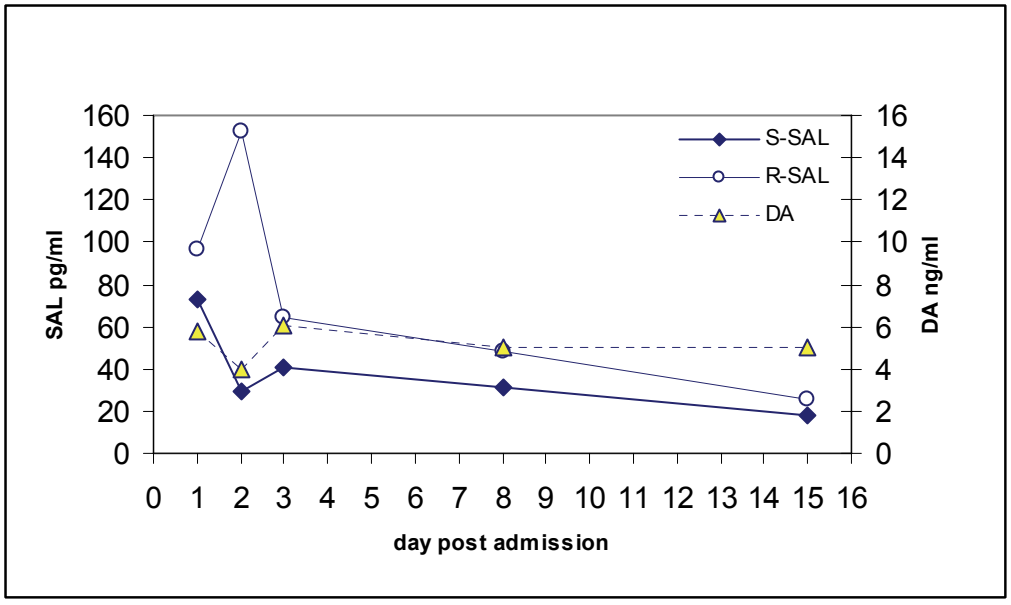


Figure M-24: S/R-SAL (pg/ml) and DA (ng/ml) concentration vs. time profile for Subject 25 (NS, F)

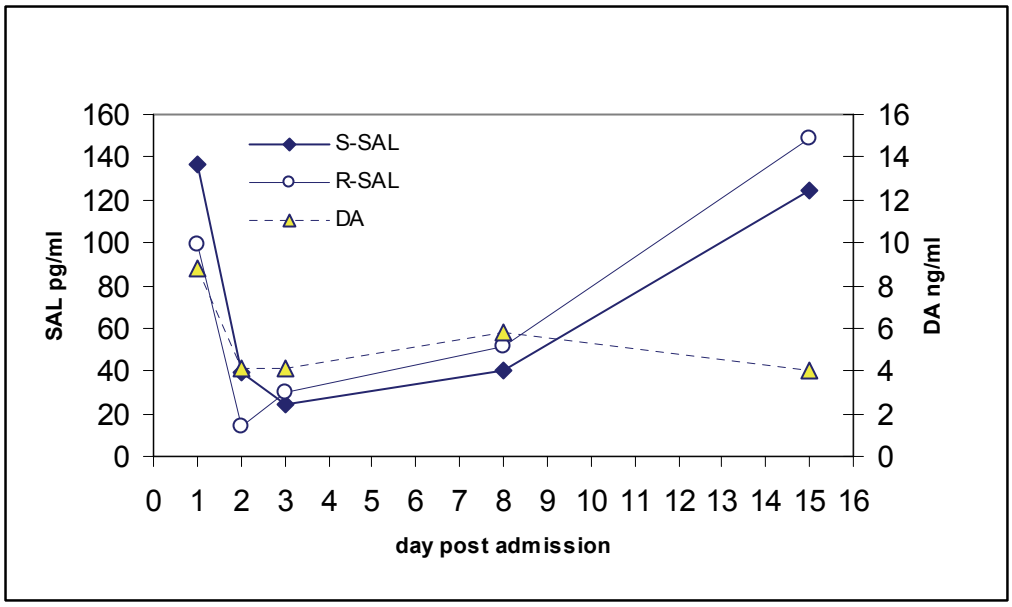


Figure M-25: S/R-SAL (pg/ml) and DA (ng/ml) concentration vs. time profile for Subject 26 (NS, F)

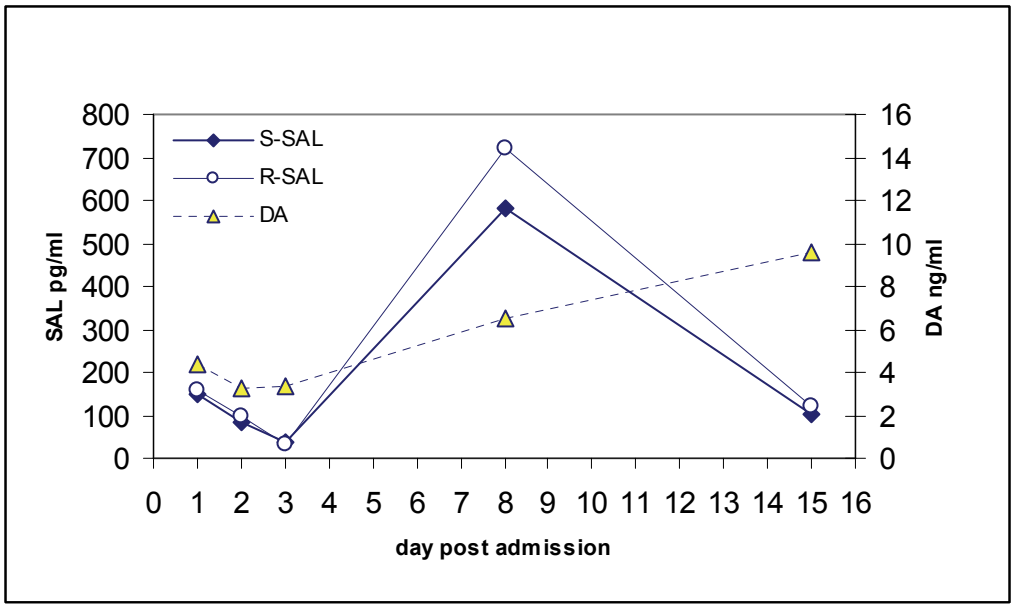


Figure M-26: S/R-SAL (pg/ml) and DA (ng/ml) concentration vs. time profile for Subject 27 (NS, F)

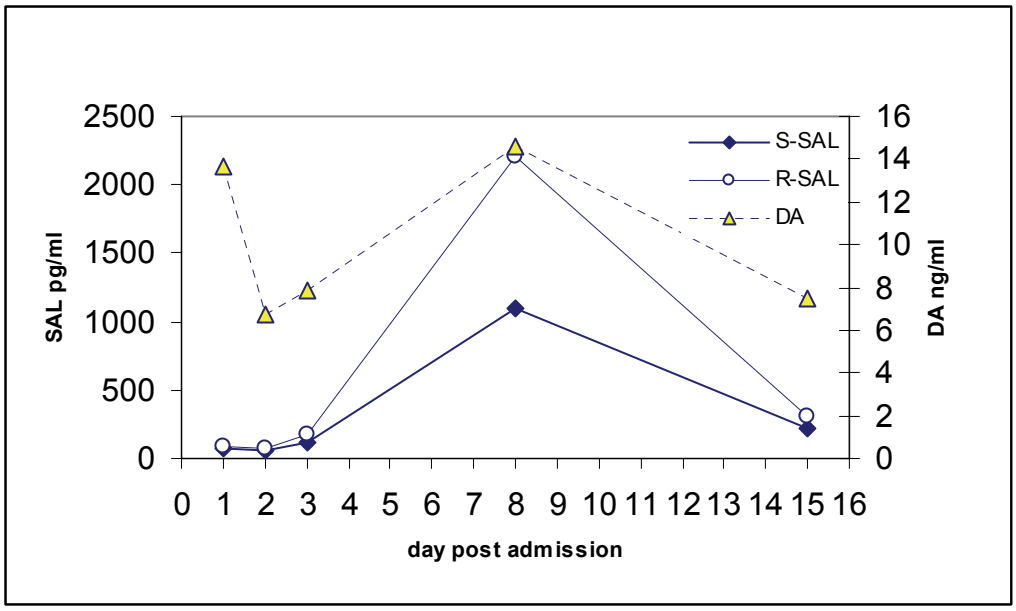


Figure M-27: S/R-SAL (pg/ml) and DA (ng/ml) concentration vs. time profile for Subject 28 (NS, F)

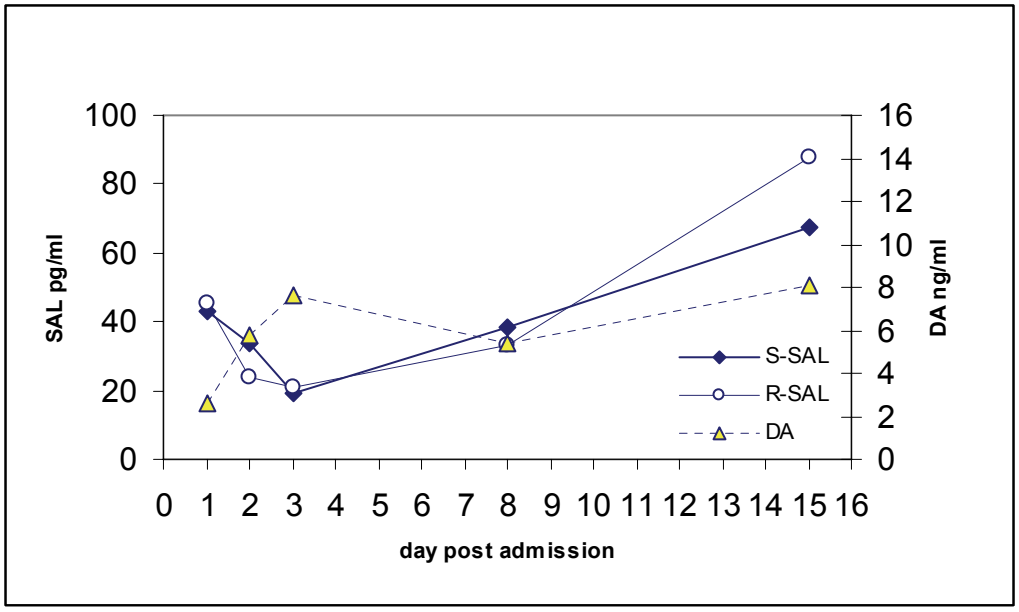


Figure M-28: S/R-SAL (pg/ml) and DA (ng/ml) concentration vs. time profile for Subject 29 (NS, F)

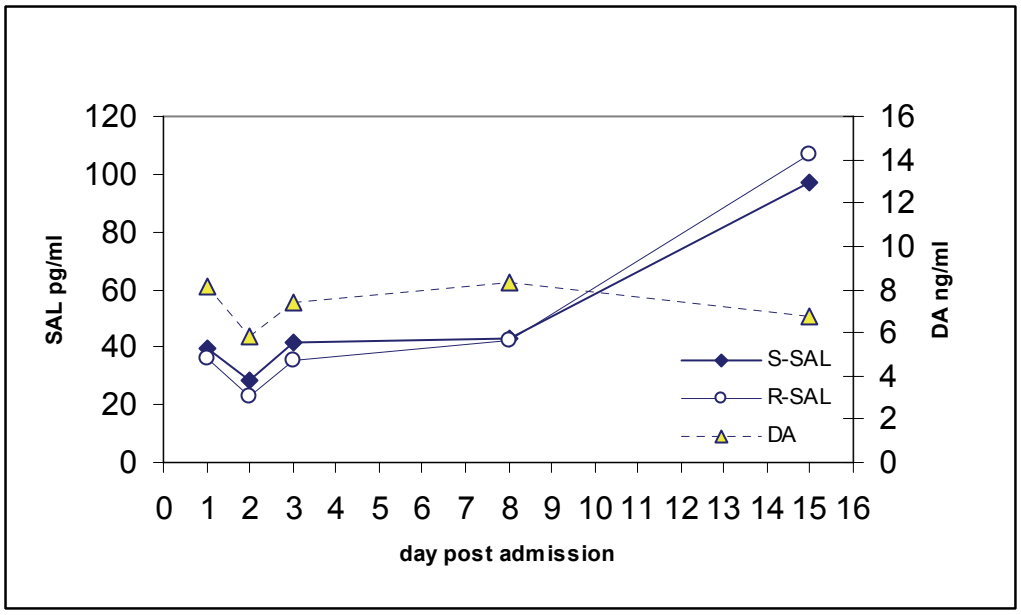


Figure M-29: S/R-SAL (pg/ml) and DA (ng/ml) concentration vs. time profile for Subject 30 (NS, F)

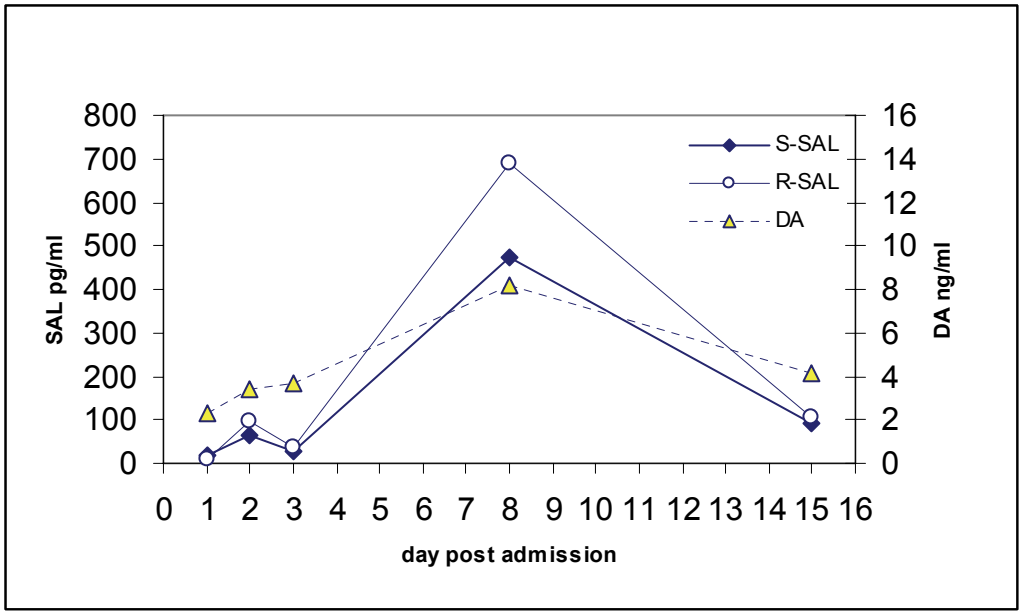


Figure M-30: S/R-SAL (pg/ml) and DA (ng/ml) concentration vs. time profile for Subject 31 (NS, M)

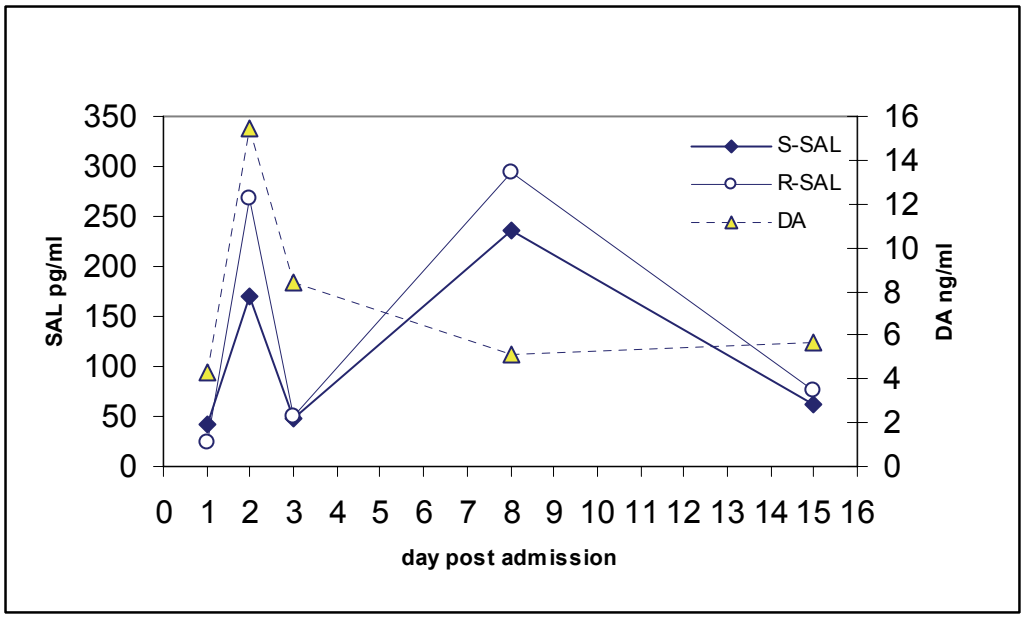


Figure M-31: S/R-SAL (pg/ml) and DA (ng/ml) concentration vs. time profile for Subject 32 (NS, M)

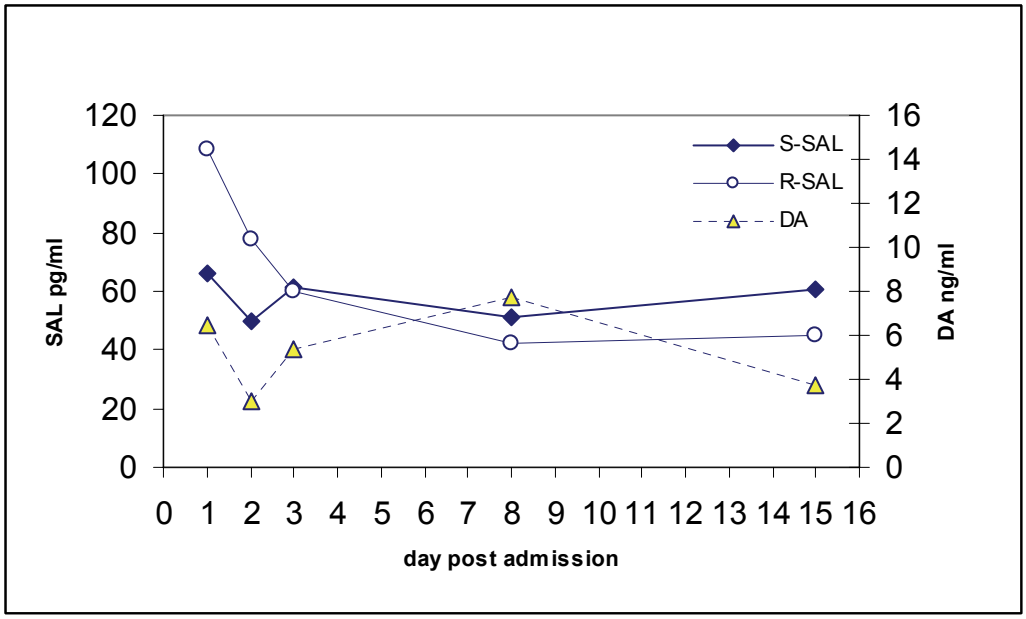


Figure M-32: S/R-SAL (pg/ml) and DA (ng/ml) concentration vs. time profile for Subject 33 (NS, M)

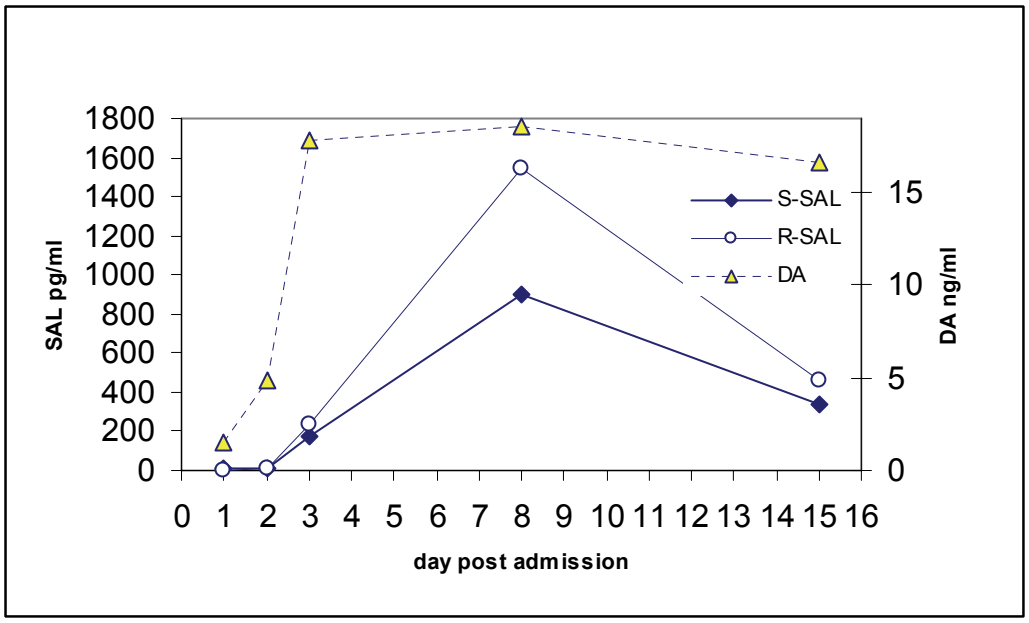


Figure M-33: S/R-SAL (pg/ml) and DA (ng/ml) concentration vs. time profile for Subject 34 (NS, M)

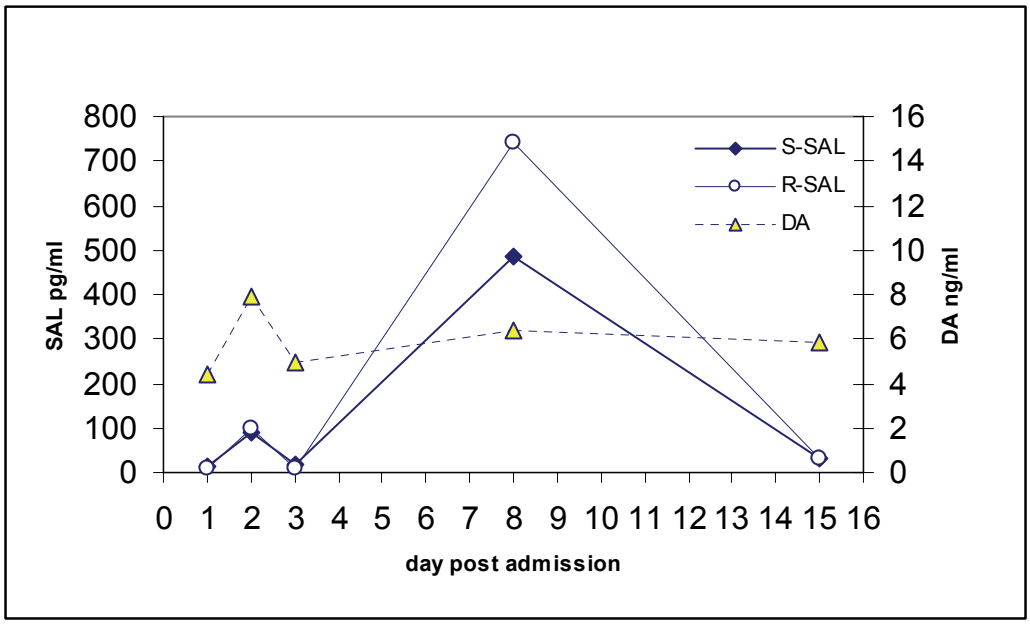


Figure M-34: S/R-SAL (pg/ml) and DA (ng/ml) concentration vs. time profile for Subject 35 (NS, M)

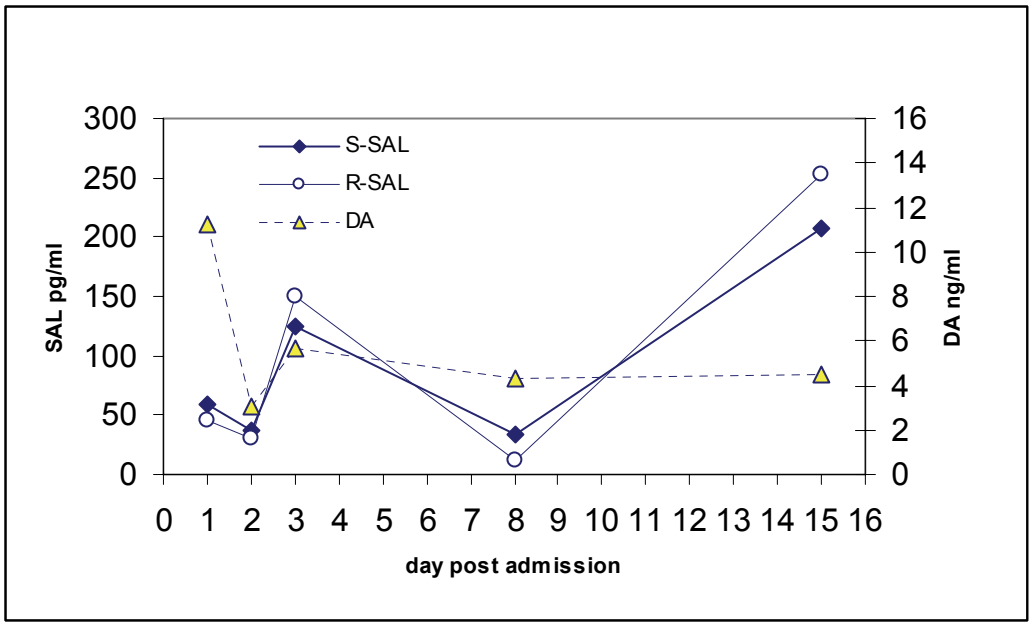


Figure M-35: S/R-SAL (pg/ml) and DA (ng/ml) concentration vs. time profile for Subject 36 (NS, M)

Appendix N

Individual Clinical Endpoint (CIWA-AR) vs. time – Clinical Study #2

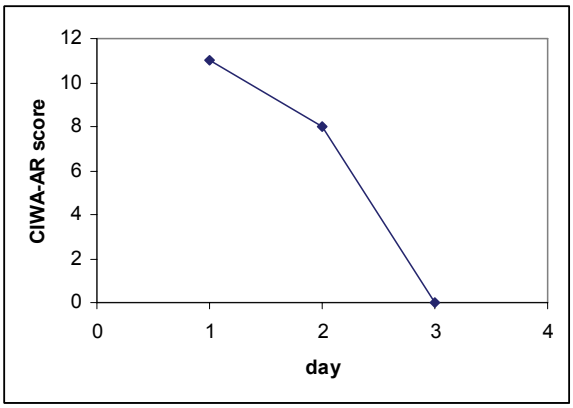


Figure N-1: CIWA-AR vs. time profile for Subject 1 (HS, F)

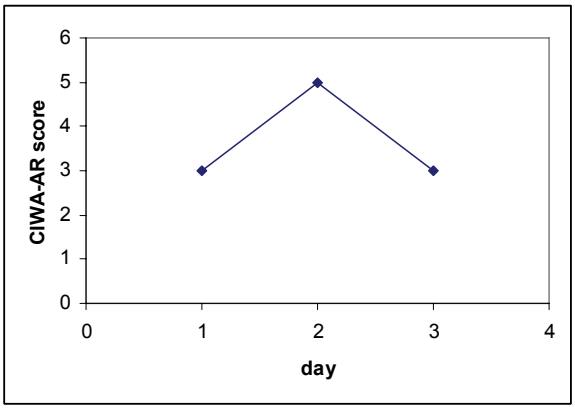


Figure N-2: CIWA-AR vs. time profile for Subject 2 (HS, F)

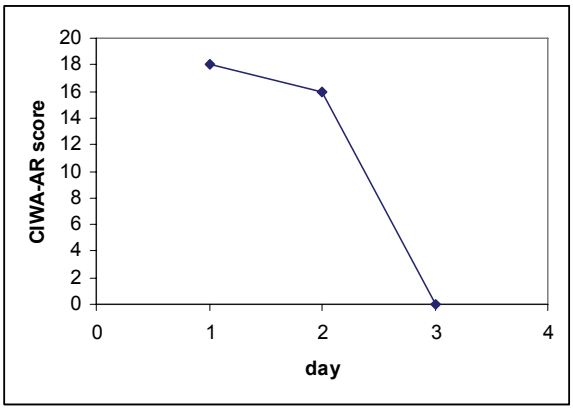


Figure N-3: CIWA-AR vs. time profile for Subject 3 (HS, F)

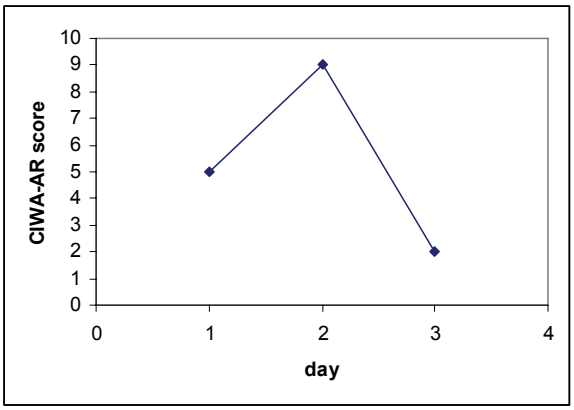


Figure N-4: CIWA-AR vs. time profile for Subject 4 (HS, F)

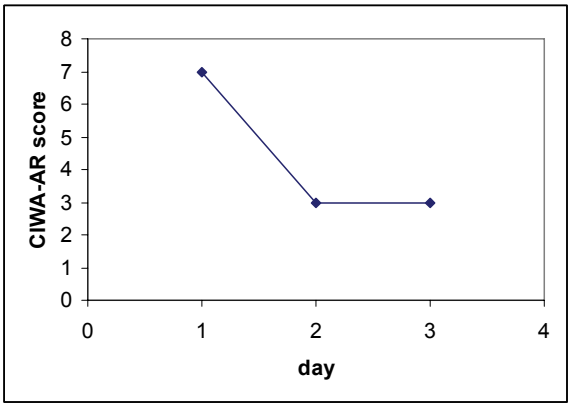


Figure N-5: CIWA-AR vs. time profile for Subject 5 (HS, F)

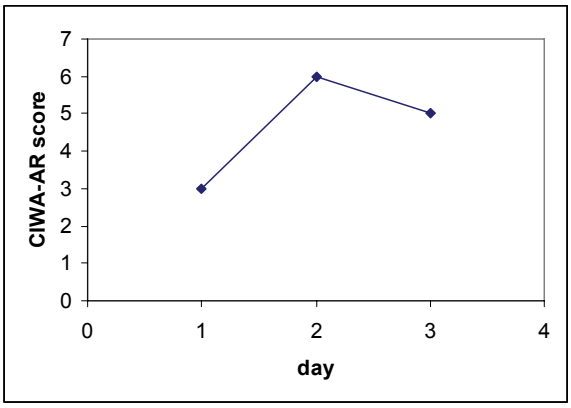


Figure N-6: CIWA-AR vs. time profile for Subject 6 (HS, F)

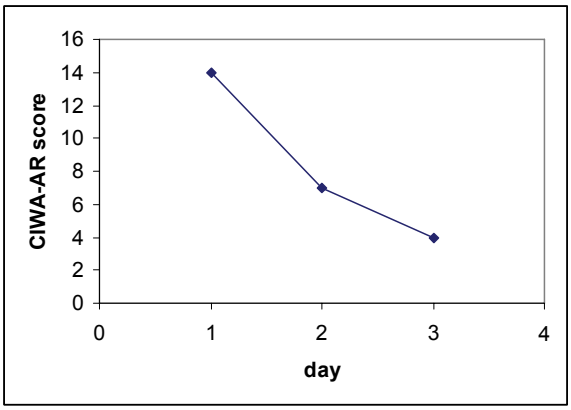


Figure N-7: CIWA-AR vs. time profile for Subject 7 (HS, M)

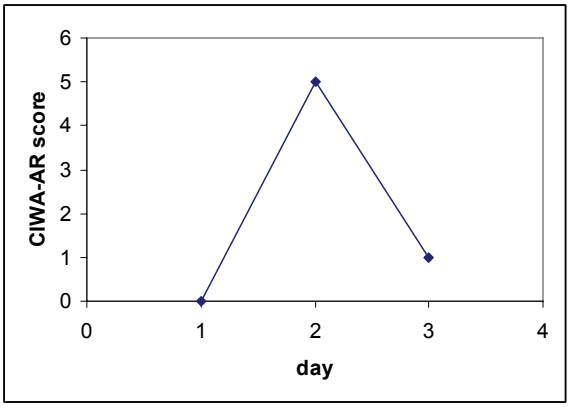


Figure N-8: CIWA-AR vs. time profile for Subject 8 (HS, M)

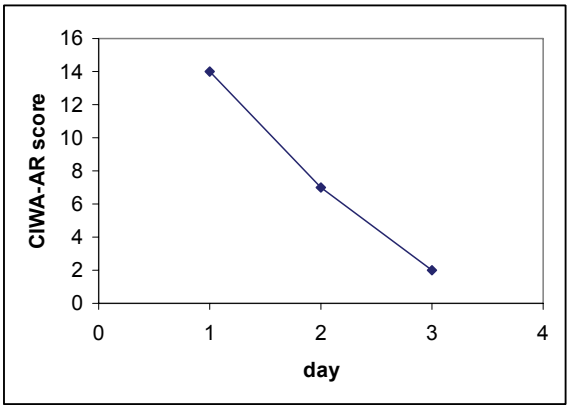


Figure N-9: CIWA-AR vs. time profile for Subject 9 (HS, M)

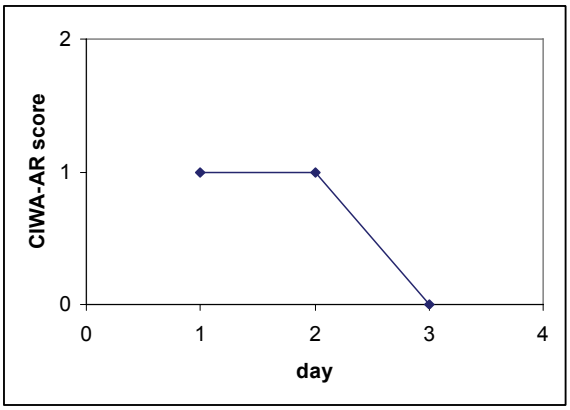


Figure N-10: CIWA-AR vs. time profile for Subject 10 (HS, M)

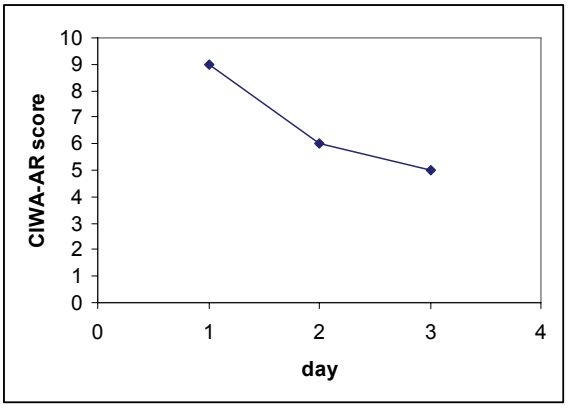


Figure N-11: CIWA-AR vs. time profile for Subject 11 (HS, M)

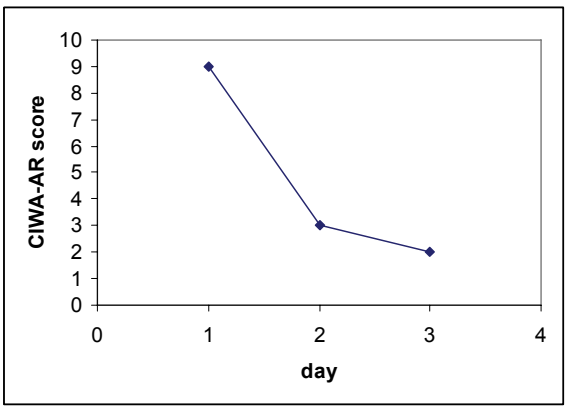


Figure N-12: CIWA-AR vs. time profile for Subject 12 (HS, M)

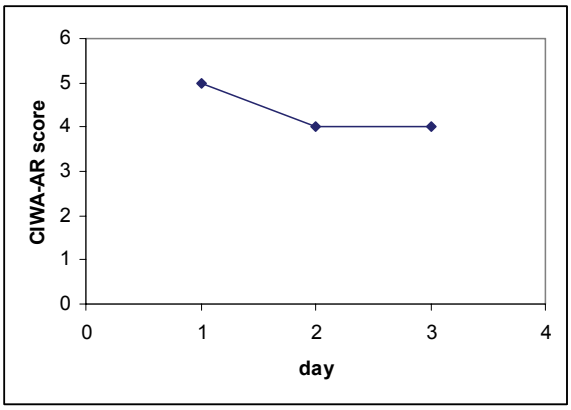


Figure N-13: CIWA-AR vs. time profile for Subject 13 (LS, F)

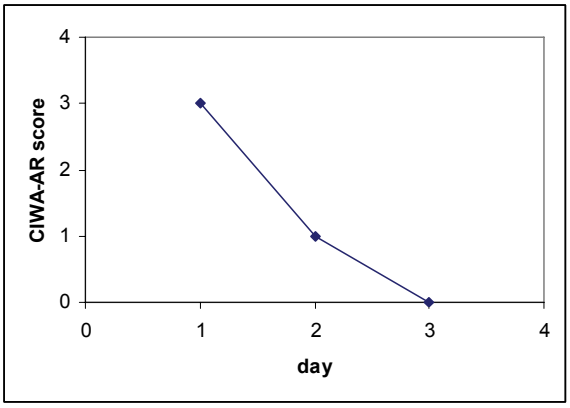


Figure N-14: CIWA-AR vs. time profile for Subject 15 (LS, F)

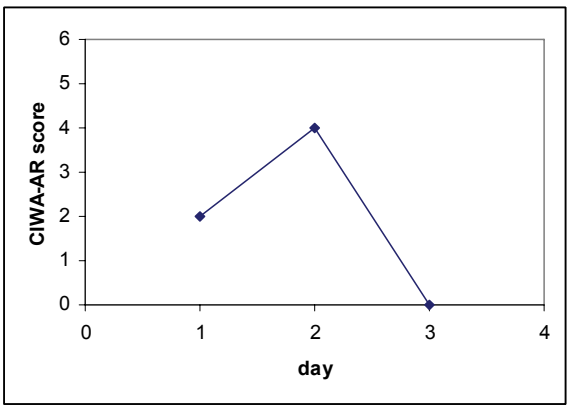


Figure N-15: CIWA-AR vs. time profile for Subject 16 (LS, F)

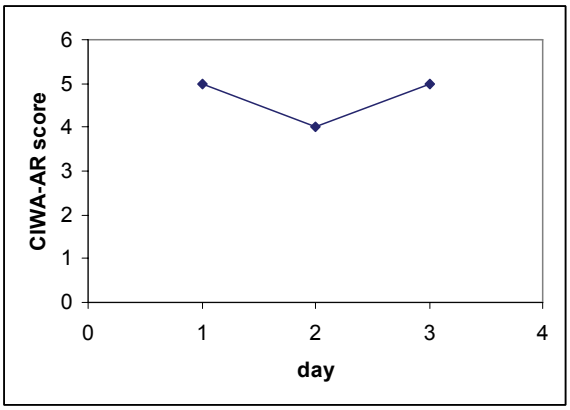


Figure N-16: CIWA-AR vs. time profile for Subject 17 (LS, F)

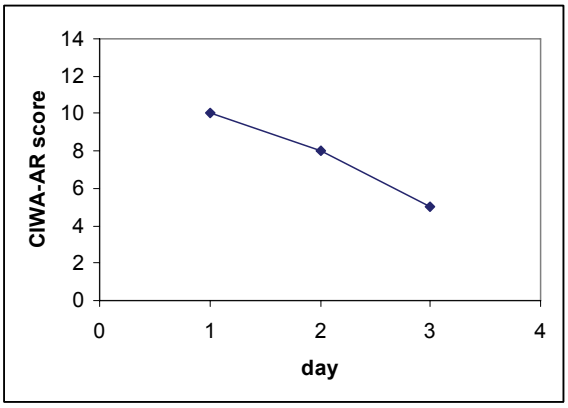


Figure N-17: CIWA-AR vs. time profile for Subject 18 (LS, F)

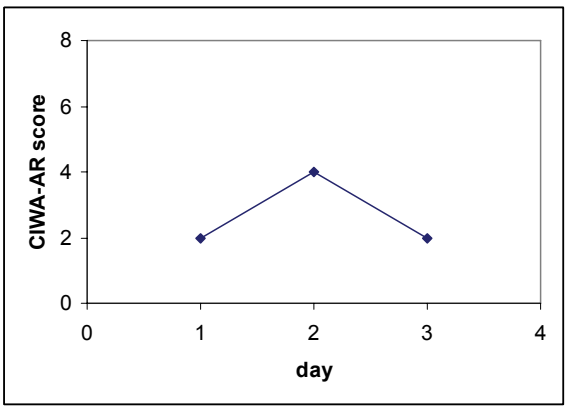


Figure N-18: CIWA-AR vs. time profile for Subject 19 (LS, M)

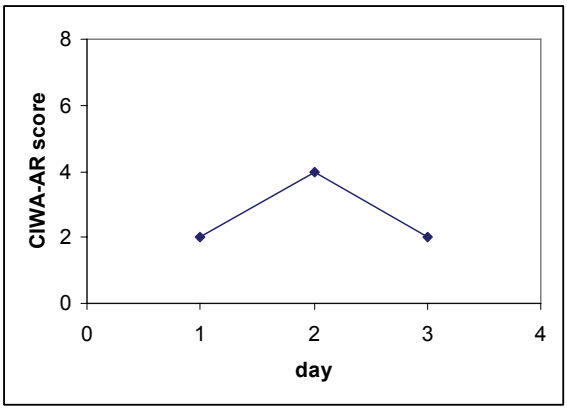


Figure N-19: CIWA-AR vs. time profile for Subject 20 (LS, M)

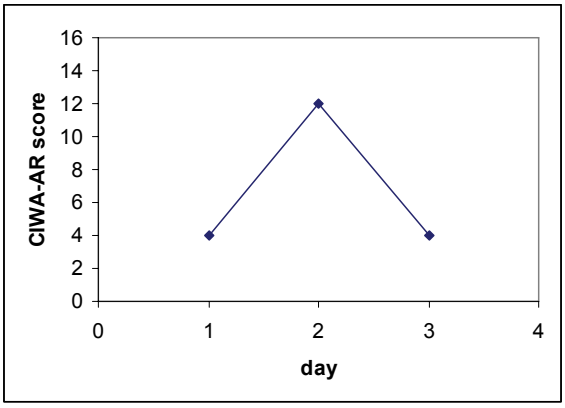


Figure N-20: CIWA-AR vs. time profile for Subject 21 (LS, M)

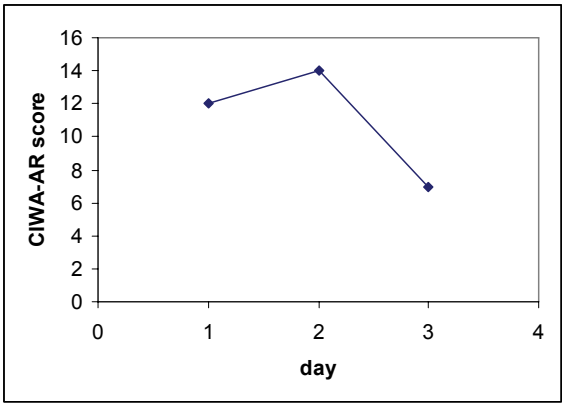


Figure N-21: CIWA-AR vs. time profile for Subject 22 (LS, M)

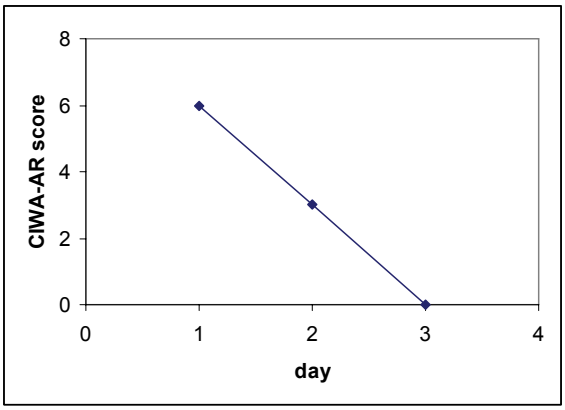


Figure N-22: CIWA-AR vs. time profile for Subject 23 (LS, M)

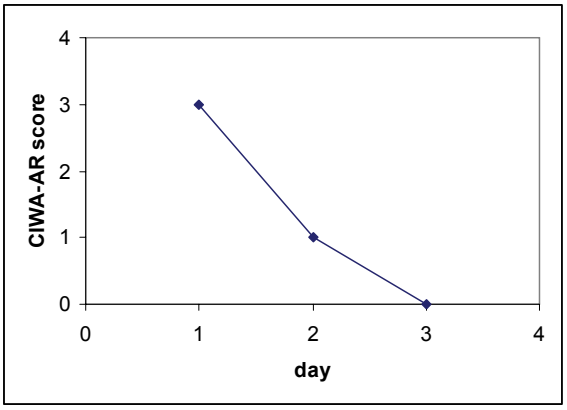


Figure N-23: CIWA-AR vs. time profile for Subject 24 (LS, M)

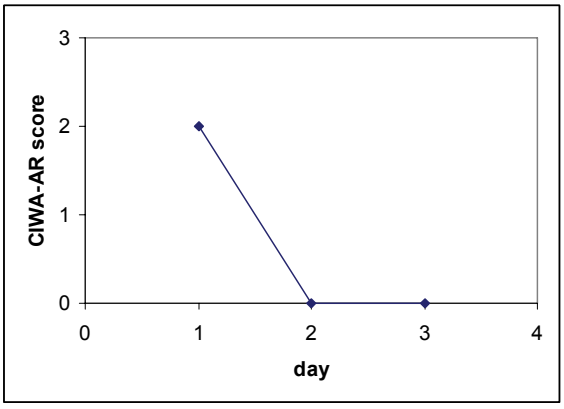


Figure N-24: CIWA-AR vs. time profile for Subject 25 (NS, F)

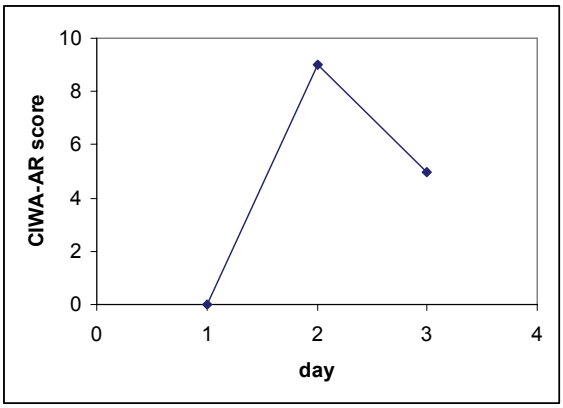


Figure N-25: CIWA-AR vs. time profile for Subject 26 (NS, F)

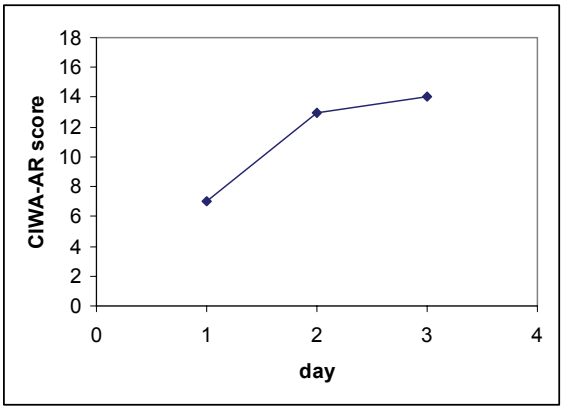


Figure N-26: CIWA-AR vs. time profile for Subject 27 (NS, F)

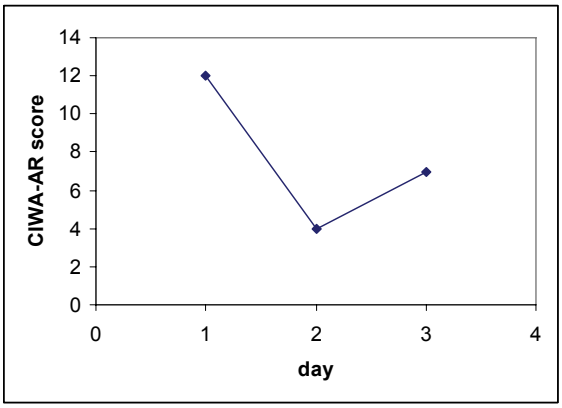


Figure N-27: CIWA-AR vs. time profile for Subject 28 (NS, F)

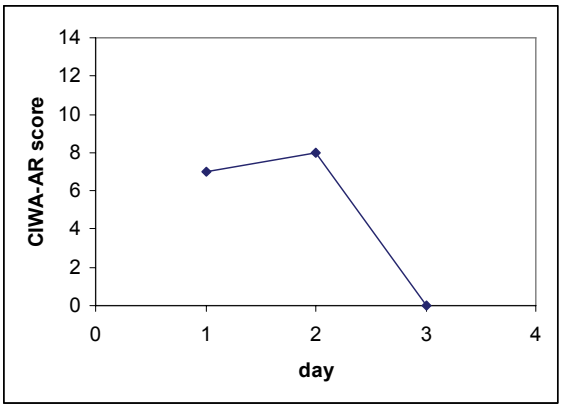


Figure N-28: CIWA-AR vs. time profile for Subject 29 (NS, F)

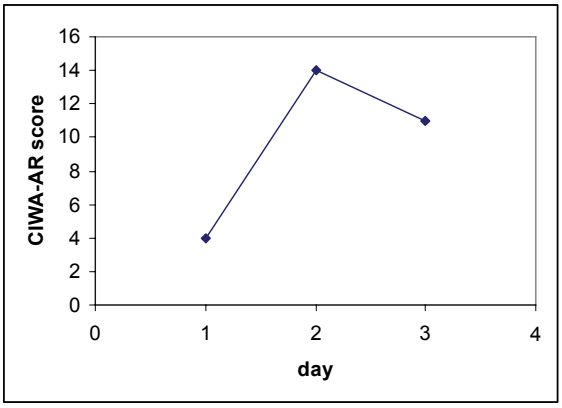


Figure N-29: CIWA-AR vs. time profile for Subject 30 (NS, F)

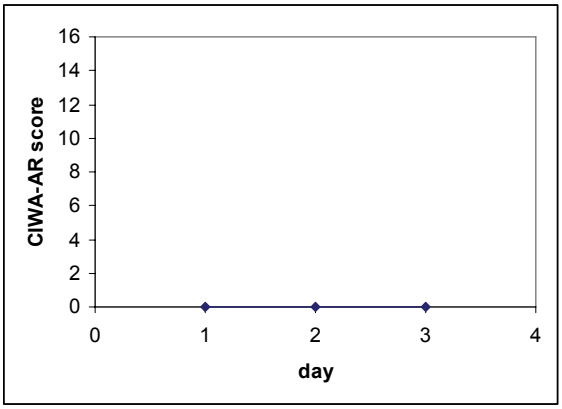


Figure N-30: CIWA-AR vs. time profile for Subject 31 (NS, M)

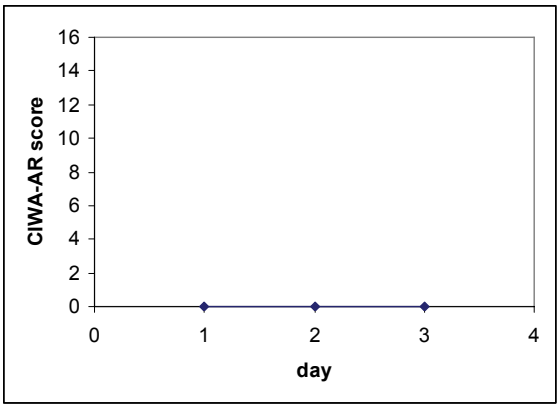


Figure N-31: CIWA-AR vs. time profile for Subject 32 (NS, M)

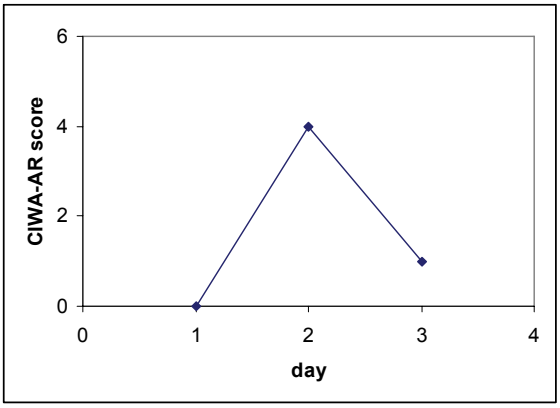


Figure N-32: CIWA-AR vs. time profile for Subject 33 (NS, M)

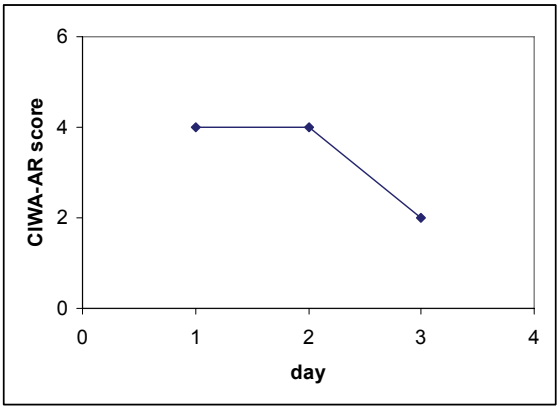


Figure N-33: CIWA-AR vs. time profile for Subject 34 (NS, M)

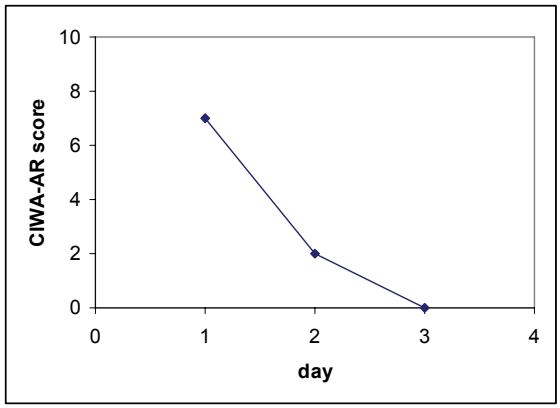


Figure N-34: CIWA-AR vs. time profile for Subject 35 (NS, M)

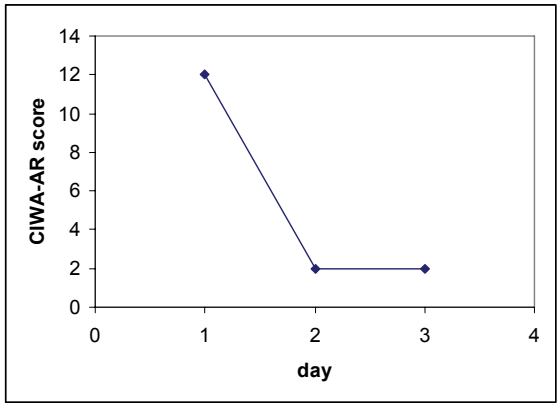


Figure N-35: CIWA-AR vs. time profile for Subject 36 (NS, M)

Appendix O

S-PLUS® ANCOVA and Linear model outputs – Clinical Study #2

Table O-1: Linear model for main effects of time, SS and GEN on Log S-SAL

```
      *** Linear Model ***

Call: lm(formula = log.S.SAL ~ Day + SS + GEN, data =
      NIAAA.d.14.SAL.DA.time.assess.041108, na.action = na.exclude)
Residuals:
    Min      1Q   Median     3Q   Max
 -1.579 -0.2889 -0.01269 0.2943 1.182

Coefficients:
               Value Std. Error  t value Pr(>|t|)
(Intercept)   1.5160   0.1125    13.4803   0.0000
        Day   0.0337   0.0072     4.7046   0.0000
         SS  -0.0125   0.0451    -0.2780   0.7814
        GEN   0.1234   0.0748     1.6497   0.1009

Residual standard error: 0.4895 on 168 degrees of freedom
Multiple R-Squared: 0.128
F-statistic: 8.218 on 3 and 168 degrees of freedom, the p-value is 0.00003901
```

Table O-2: Linear model for main effects of time, SS and GEN on Log R-SAL

```
*** Linear Model ***

Call: lm(formula = log.R.SAL ~ Day + SS + GEN, data =
      NIAAA.d.14.SAL.DA.time.assess.041108, na.action = na.exclude)
Residuals:
    Min      1Q    Median     3Q   Max
 -2.537 -0.4059 0.0004769 0.4087 1.407

Coefficients:
               Value Std. Error  t value Pr(>|t|)
(Intercept)   1.4628   0.1420    10.3005   0.0000
        Day   0.0404   0.0090     4.4732   0.0000
         SS  -0.0031   0.0569    -0.0538   0.9572
        GEN   0.2270   0.0944     2.4043   0.0173

Residual standard error: 0.6182 on 168 degrees of freedom
Multiple R-Squared: 0.1313
F-statistic: 8.467 on 3 and 168 degrees of freedom, the p-value is 0.00002852
```

Table O-3: Linear model for main effects of time, SS and GEN on Log DA

```
      *** Linear Model ***

Call: lm(formula = log.DA ~ Day + SS + GEN, data =
      NIAAA.d.14.SAL.DA.time.assess.041108, na.action = na.exclude)
Residuals:
   Min      1Q   Median     3Q   Max
 -1.14 -0.1661 -0.02412 0.1347 1.046

Coefficients:
               Value Std. Error  t value Pr(>|t|)
(Intercept)   0.7457   0.0615    12.1299   0.0000
        Day   0.0051   0.0039     1.3100   0.1920
         SS  -0.0439   0.0246    -1.7798   0.0769
        GEN   0.0505   0.0409     1.2348   0.2186

Residual standard error: 0.2676 on 168 degrees of freedom
Multiple R-Squared: 0.03693
F-statistic: 2.148 on 3 and 168 degrees of freedom, the p-value is 0.09615
```

Table O-4: ANOVA model for main effects of SS and GEN on Log S-SAL Day 1

```
*** Analysis of Variance Model ***

Short Output:
Call:
   aov(formula = Log.S.SAL.d.1 ~ SS + GEN + SS:GEN, data =
       LOG.SAL.Data.NIAAA.Detox.d1.d15.AVG.041108, na.action = na.exclude)

Terms:
                      SS      GEN   SS:GEN Residuals
 Sum of Squares 0.404638 0.357304 0.531942  7.420785
Deg. of Freedom        2        1        2        29

Residual standard error: 0.5058548
Estimated effects may be unbalanced

          Df Sum of Sq   Mean Sq  F Value     Pr(F)
       SS  2  0.404638 0.2023190 0.790651 0.4630836
      GEN  1  0.357304 0.3573041 1.396324 0.2469397
   SS:GEN  2  0.531942 0.2659710 1.039400 0.3664710
Residuals 29  7.420785 0.2558891

Residual standard error: 0.5059 on 29 degrees of freedom
Multiple R-Squared: 0.1485
F-statistic: 1.011 on 5 and 29 degrees of freedom, the p-value is 0.4289
```

Table O-5: ANOVA model for main effects of SS and GEN on Log R-SAL Day 1

```
*** Analysis of Variance Model ***

Short Output:
Call:
   aov(formula = Log.R.SAL.d.1 ~ SS + GEN + SS:GEN, data =
       LOG.SAL.Data.NIAAA.Detox.d1.d15.AVG.041108, na.action = na.exclude)

Terms:
                      SS      GEN   SS:GEN Residuals
 Sum of Squares 0.078214 0.500541 0.807796  9.586996
Deg. of Freedom        2        1        2        29

Residual standard error: 0.5749662
Estimated effects may be unbalanced

          Df Sum of Sq   Mean Sq  F Value     Pr(F)
       SS  2  0.078214 0.0391069 0.118296 0.8888598
      GEN  1  0.500541 0.5005412 1.514103 0.2284020
   SS:GEN  2  0.807796 0.4038981 1.221764 0.3094337
Residuals 29  9.586996 0.3305861

Residual standard error: 0.575 on 29 degrees of freedom
Multiple R-Squared: 0.1264
F-statistic: 0.8388 on 5 and 29 degrees of freedom, the p-value is 0.5332
```

Table O-6: ANOVA model for main effects of SS and GEN on Log DA Day 1

```
*** Analysis of Variance Model ***

Short Output:
Call:
   aov(formula = Log.DA.d.1 ~ SS + GEN + SS:GEN, data =
       LOG.SAL.Data.NIAAA.Detox.d1.d15.AVG.041108, na.action = na.exclude)

Terms:
                      SS      GEN   SS:GEN Residuals
 Sum of Squares 0.196977 0.195106 0.333961  2.242850
Deg. of Freedom        2        1        2        29

Residual standard error: 0.2781001
Estimated effects may be unbalanced

          Df Sum of Sq   Mean Sq  F Value     Pr(F)
       SS  2  0.196977 0.0984884 1.273453 0.2950514
      GEN  1  0.195106 0.1951058 2.522713 0.1230619
   SS:GEN  2  0.333961 0.1669806 2.159055 0.1336306
Residuals 29  2.242850 0.0773397

Residual standard error: 0.2781 on 29 degrees of freedom
Multiple R-Squared: 0.2446
F-statistic: 1.878 on 5 and 29 degrees of freedom, the p-value is 0.1291
```

Table O-7: ANOVA model for main effects of SS and GEN on Log S-SAL Day 15

```
*** Analysis of Variance Model ***

Short Output:
Call:
   aov(formula = Log.S.SAL.d.15 ~ SS + GEN + SS:GEN, data =
       LOG.SAL.Data.NIAAA.Detox.d1.d15.AVG.041108, na.action = na.exclude)

Terms:
                      SS      GEN   SS:GEN Residuals
 Sum of Squares 0.155235 0.327546 0.309619  5.207807
Deg. of Freedom        2        1        2        29

Residual standard error: 0.4237683
Estimated effects may be unbalanced

          Df Sum of Sq   Mean Sq  F Value     Pr(F)
       SS  2  0.155235 0.0776174 0.432217 0.6531812
      GEN  1  0.327546 0.3275460 1.823960 0.1872881
   SS:GEN  2  0.309619 0.1548093 0.862065 0.4328294
Residuals 29  5.207807 0.1795795

Residual standard error: 0.4238 on 29 degrees of freedom
Multiple R-Squared: 0.1321
F-statistic: 0.8825 on 5 and 29 degrees of freedom, the p-value is 0.5052
```

Table O-8: ANOVA model for main effects of SS and GEN on Log R-SAL Day 15

```
*** Analysis of Variance Model ***

Short Output:
Call:
   aov(formula = Log.R.SAL.d.15 ~ SS + GEN + SS:GEN, data =
       LOG.SAL.Data.NIAAA.Detox.d1.d15.AVG.041108, na.action = na.exclude)

Terms:
                      SS      GEN   SS:GEN Residuals
 Sum of Squares 0.129790 0.616341 0.392646  8.204339
Deg. of Freedom        2        1        2        29

Residual standard error: 0.5318912
Estimated effects may be unbalanced

          Df Sum of Sq   Mean Sq  F Value     Pr(F)
       SS  2  0.129790 0.0648950 0.229385 0.7964508
      GEN  1  0.616341 0.6163408 2.178589 0.1507193
   SS:GEN  2  0.392646 0.1963232 0.693947 0.5077060
Residuals 29  8.204339 0.2829082

Residual standard error: 0.5319 on 29 degrees of freedom
Multiple R-Squared: 0.1219
F-statistic: 0.8051 on 5 and 29 degrees of freedom, the p-value is 0.5554
```

Table O-9: ANOVA model for main effects of SS and GEN on Log DA Day 15

```
*** Analysis of Variance Model ***

Short Output:
Call:
   aov(formula = Log.DA.d.15 ~ SS + GEN + SS:GEN, data =
       LOG.SAL.Data.NIAAA.Detox.d1.d15.AVG.041108, na.action = na.exclude)

Terms:
                       SS       GEN    SS:GEN Residuals
 Sum of Squares 0.0369333 0.0133690 0.1738083 0.7588887
Deg. of Freedom         2         1         2        29

Residual standard error: 0.161767
Estimated effects may be unbalanced

          Df Sum of Sq    Mean Sq  F Value     Pr(F)
       SS  2 0.0369333 0.01846667 0.705681 0.5020544
      GEN  1 0.0133690 0.01336899 0.510880 0.4804731
   SS:GEN  2 0.1738083 0.08690416 3.320936 0.0502740
Residuals 29 0.7588887 0.02616858

Residual standard error: 0.1618 on 29 degrees of freedom
Multiple R-Squared: 0.228
F-statistic: 1.713 on 5 and 29 degrees of freedom, the p-value is 0.1632
```

Table O-10: ANOVA model for main effects of SS and GEN on Log S-SAL GTM

```
*** Analysis of Variance Model ***

Short Output:
Call:
   aov(formula = Av.Log.S.SAL ~ SS + GEN + SS:GEN, data =
       LOG.SAL.Data.NIAAA.Detox.d1.d15.AVG.041108, na.action = na.exclude)

Terms:
                      SS      GEN   SS:GEN Residuals
 Sum of Squares 0.386512 0.084351 0.504868  2.811766
Deg. of Freedom        2        1        2        29

Residual standard error: 0.3113799
Estimated effects may be unbalanced

          Df Sum of Sq   Mean Sq  F Value     Pr(F)
       SS  2  0.386512 0.1932561 1.993205 0.1544943
      GEN  1  0.084351 0.0843511 0.869980 0.3586619
   SS:GEN  2  0.504868 0.2524341 2.603555 0.0912186
Residuals 29  2.811766 0.0969575

Residual standard error: 0.3114 on 29 degrees of freedom
Multiple R-Squared: 0.2576
F-statistic: 2.013 on 5 and 29 degrees of freedom, the p-value is 0.1065
```

Table O-11: ANOVA model for main effects of SS and GEN on Log R-SAL GTM

```
*** Analysis of Variance Model ***

Short Output:
Call:
   aov(formula = Av.Log.R.SAL ~ SS + GEN + SS:GEN, data =
       LOG.SAL.Data.NIAAA.Detox.d1.d15.AVG.041108, na.action = na.exclude)

Terms:
                      SS      GEN   SS:GEN Residuals
 Sum of Squares 0.288904 0.225614 0.557630  4.321198
Deg. of Freedom        2        1        2        29

Residual standard error: 0.386014
Estimated effects may be unbalanced

          Df Sum of Sq   Mean Sq  F Value     Pr(F)
       SS  2  0.288904 0.1444520 0.969432 0.3912530
      GEN  1  0.225614 0.2256137 1.514116 0.2284000
   SS:GEN  2  0.557630 0.2788149 1.871155 0.1720623
Residuals 29  4.321198 0.1490068

Residual standard error: 0.3967 on 31 degrees of freedom
Multiple R-Squared: 0.0954
F-statistic: 1.09 on 3 and 31 degrees of freedom, the p-value is 0.368
```

Table O-12: ANOVA model for main effects of SS and GEN on Log DA GTM

```
*** Analysis of Variance Model ***

Short Output:
Call:
   aov(formula = Av.Log.DA ~ SS + GEN + SS:GEN, data =
       LOG.SAL.Data.NIAAA.Detox.d1.d15.AVG.041108, na.action = na.exclude)

Terms:
                       SS       GEN    SS:GEN Residuals
 Sum of Squares 0.2755988 0.0241072 0.2139152 0.9730832
Deg. of Freedom         2         1         2        29

Residual standard error: 0.1831791
Estimated effects may be unbalanced

          Df Sum of Sq   Mean Sq  F Value     Pr(F)
       SS  2 0.2755988 0.1377994 4.106723 0.0268920
      GEN  1 0.0241072 0.0241072 0.718446 0.4035956
   SS:GEN  2 0.2139152 0.1069576 3.187569 0.0560593
Residuals 29 0.9730832 0.0335546

Residual standard error: 0.1832 on 29 degrees of freedom
Multiple R-Squared: 0.3455
F-statistic: 3.061 on 5 and 29 degrees of freedom, the p-value is 0.0244
```

Table O-13: Linear model: FTND or #cig/day and GEN on Log S-SAL GTM

```
*** Linear Model ***

Call: lm(formula = Av.Log.S.SAL ~ GEN + FTND, data =
      LOG.SAL.Data.NIAAA.Detox.d1.d15.AVG.040508, na.action = na.exclude)
Residuals:
     Min      1Q  Median     3Q    Max
 -0.5998 -0.3068 0.03356 0.2557 0.6058

Coefficients:
              Value Std. Error  t value Pr(>|t|)
(Intercept)   1.9577   0.1057    18.5170   0.0000
        GEN  -0.1054   0.1143    -0.9225   0.3632
       FTND   0.0087   0.0160     0.5434   0.5906

Residual standard error: 0.3379 on 32 degrees of freedom
Multiple R-Squared: 0.03532
F-statistic: 0.5859 on 2 and 32 degrees of freedom, the p-value is 0.5625



*** Linear Model ***

Call: lm(formula = Av.Log.S.SAL ~ GEN + ..cig, data =
      LOG.SAL.Data.NIAAA.Detox.d1.d15.AVG.040508, na.action = na.exclude)
Residuals:
     Min      1Q Median     3Q    Max
 -0.6023 -0.3328 0.0426 0.2562 0.5828

Coefficients:
              Value Std. Error  t value Pr(>|t|)
(Intercept)   1.9837   0.0970    20.4443   0.0000
        GEN  -0.1085   0.1150    -0.9436   0.3525
      ..cig   0.0008   0.0042     0.1987   0.8438

Residual standard error: 0.3392 on 32 degrees of freedom
Multiple R-Squared: 0.02762
F-statistic: 0.4545 on 2 and 32 degrees of freedom, the p-value is 0.6388
```

Table O-14: Linear model: FTND or #cig/day and GEN on Log R-SAL GTM

```
*** Linear Model ***

Call: lm(formula = Av.Log.R.SAL ~ GEN + FTND, data =
      LOG.SAL.Data.NIAAA.Detox.d1.d15.AVG.040508, na.action = na.exclude)
Residuals:
     Min      1Q  Median     3Q    Max
 -0.8089 -0.2907 0.04535 0.2861 0.7036

Coefficients:
              Value Std. Error  t value Pr(>|t|)
(Intercept)   2.1172   0.1248    16.9681   0.0000
        GEN  -0.1659   0.1349    -1.2295   0.2278
       FTND   0.0113   0.0189     0.6010   0.5521

Residual standard error: 0.3988 on 32 degrees of freedom
Multiple R-Squared: 0.05634
F-statistic: 0.9553 on 2 and 32 degrees of freedom, the p-value is 0.3954



*** Linear Model ***

Call: lm(formula = Av.Log.R.SAL ~ GEN + ..cig, data =
      LOG.SAL.Data.NIAAA.Detox.d1.d15.AVG.040508, na.action = na.exclude)
Residuals:
     Min      1Q  Median     3Q    Max
 -0.7832 -0.3127 0.07042 0.2845 0.6785

Coefficients:
              Value Std. Error  t value Pr(>|t|)
(Intercept)   2.1470   0.1146    18.7418   0.0000
        GEN  -0.1705   0.1358    -1.2559   0.2182
      ..cig   0.0014   0.0050     0.2901   0.7736

Residual standard error: 0.4005 on 32 degrees of freedom
Multiple R-Squared: 0.04819
F-statistic: 0.8101 on 2 and 32 degrees of freedom, the p-value is 0.4537
```

Table O-15: Linear model: FTND or #cig/day and GEN on Log DA GTM

```
*** Linear Model ***

Call: lm(formula = Av.Log.DA ~ GEN + FTND, data =
      LOG.SAL.Data.NIAAA.Detox.d1.d15.AVG.040508, na.action = na.exclude)
Residuals:
     Min      1Q   Median      3Q     Max
 -0.4196 -0.124 -0.07247 0.1278 0.4732

Coefficients:
              Value Std. Error  t value Pr(>|t|)
(Intercept)   0.8290   0.0651    12.7259   0.0000
        GEN  -0.0604   0.0704    -0.8580   0.3973
       FTND  -0.0125   0.0099    -1.2687   0.2137

Residual standard error: 0.2082 on 32 degrees of freedom
Multiple R-Squared: 0.06688
F-statistic: 1.147 on 2 and 32 degrees of freedom, the p-value is 0.3304



*** Linear Model ***

Call: lm(formula = Av.Log.DA ~ GEN + ..cig, data =
      LOG.SAL.Data.NIAAA.Detox.d1.d15.AVG.040508, na.action = na.exclude)
Residuals:
     Min      1Q   Median      3Q     Max
 -0.4184 -0.133 -0.04882 0.1359 0.4816

Coefficients:
              Value Std. Error  t value Pr(>|t|)
(Intercept)   0.8207   0.0593    13.8505   0.0000
        GEN  -0.0517   0.0702    -0.7367   0.4667
      ..cig  -0.0036   0.0026    -1.3966   0.1721

Residual standard error: 0.2072 on 32 degrees of freedom
Multiple R-Squared: 0.07625
F-statistic: 1.321 on 2 and 32 degrees of freedom, the p-value is 0.2811
```

Table O-16: Linear model: FTND or #cig/day and GEN on Log S-SAL Day 1

```
*** Linear Model ***

Call: lm(formula = Log.S.SAL.d.1 ~ GEN + FTND, data =
      LOG.SAL.Data.NIAAA.Detox.d1.d15.AVG.040508, na.action = na.exclude)
Residuals:
    Min      1Q Median     3Q   Max
 -1.583 -0.2234 0.1005 0.3107 1.119

Coefficients:
               Value Std. Error  t value Pr(>|t|)
(Intercept)   1.6712   0.1593    10.4925   0.0000
        GEN  -0.2112   0.1722    -1.2263   0.2290
       FTND  -0.0092   0.0241    -0.3832   0.7041

Residual standard error: 0.5091 on 32 degrees of freedom
Multiple R-Squared: 0.04842
F-statistic: 0.8142 on 2 and 32 degrees of freedom, the p-value is 0.452


*** Linear Model ***

Call: lm(formula = Log.S.SAL.d.1 ~ GEN + ..cig, data =
      LOG.SAL.Data.NIAAA.Detox.d1.d15.AVG.040508, na.action = na.exclude)
Residuals:
    Min      1Q Median     3Q   Max
 -1.575 -0.2275 0.1261 0.3069 1.117

Coefficients:
               Value Std. Error  t value Pr(>|t|)
(Intercept)   1.6524   0.1458    11.3348   0.0000
        GEN  -0.2066   0.1728    -1.1960   0.2405
      ..cig  -0.0016   0.0063    -0.2565   0.7992

Residual standard error: 0.5097 on 32 degrees of freedom
Multiple R-Squared: 0.04602
F-statistic: 0.7718 on 2 and 32 degrees of freedom, the p-value is 0.4706
```

Table O-17: Linear model: FTND or #cig/day and GEN on Log R-SAL Day 1

```
*** Linear Model ***

Call: lm(formula = Log.R.SAL.d.1 ~ GEN + FTND, data =
      LOG.SAL.Data.NIAAA.Detox.d1.d15.AVG.040508, na.action = na.exclude)
Residuals:
    Min      1Q  Median      3Q   Max
 -1.408 -0.4111 0.01098 0.3415 1.314

Coefficients:
             Value Std. Error t value Pr(>|t|)
(Intercept)  1.6481  0.1787     9.2217  0.0000
        GEN -0.2430  0.1932    -1.2574  0.2177
       FTND -0.0069  0.0270    -0.2548  0.8005

Residual standard error: 0.5712 on 32 degrees of freedom
Multiple R-Squared: 0.0485
F-statistic: 0.8155 on 2 and 32 degrees of freedom, the p-value is 0.4514



*** Linear Model ***

Call: lm(formula = Log.R.SAL.d.1 ~ GEN + ..cig, data =
      LOG.SAL.Data.NIAAA.Detox.d1.d15.AVG.040508, na.action = na.exclude)
Residuals:
    Min      1Q  Median      3Q   Max
 -1.403 -0.4092 0.02338 0.3539 1.312

Coefficients:
              Value Std. Error  t value Pr(>|t|)
(Intercept)   1.6357   0.1635    10.0073   0.0000
        GEN  -0.2393   0.1937    -1.2356   0.2256
      ..cig  -0.0013   0.0071    -0.1889   0.8514

Residual standard error: 0.5715 on 32 degrees of freedom
Multiple R-Squared: 0.04763
F-statistic: 0.8002 on 2 and 32 degrees of freedom, the p-value is 0.458
```

Table O-18: Linear model: FTND or #cig/day and GEN on Log DA Day 1

```
*** Linear Model ***

Call: lm(formula = Log.DA.d.1 ~ GEN + FTND, data =
      LOG.SAL.Data.NIAAA.Detox.d1.d15.AVG.040508, na.action = na.exclude)
Residuals:
     Min      1Q   Median    3Q   Max
 -0.6615 -0.1592 -0.01597 0.112 1.002

Coefficients:
             Value Std. Error t value Pr(>|t|)
(Intercept)  0.8020  0.0917     8.7459  0.0000
        GEN -0.1560  0.0992    -1.5733  0.1255
       FTND -0.0047  0.0139    -0.3363  0.7389

Residual standard error: 0.2931 on 32 degrees of freedom
Multiple R-Squared: 0.07417
F-statistic: 1.282 on 2 and 32 degrees of freedom, the p-value is 0.2914


*** Linear Model ***

Call: lm(formula = Log.DA.d.1 ~ GEN + ..cig, data =
      LOG.SAL.Data.NIAAA.Detox.d1.d15.AVG.040508, na.action = na.exclude)
Residuals:
     Min      1Q   Median    3Q   Max
 -0.6591 -0.1536 -0.01238 0.121 1.008

Coefficients:
             Value Std. Error t value Pr(>|t|)
(Intercept)  0.7956  0.0839     9.4876  0.0000
        GEN -0.1532  0.0994    -1.5418  0.1330
      ..cig -0.0011  0.0036    -0.2952  0.7698

Residual standard error: 0.2932 on 32 degrees of freedom
Multiple R-Squared: 0.07343
F-statistic: 1.268 on 2 and 32 degrees of freedom, the p-value is 0.2952
```

Table O-19: Linear model: FTND or #cig/day and GEN on Log S-SAL Day 15

```
*** Linear Model ***

Call: lm(formula = Log.S.SAL.d.15 ~ GEN + FTND, data =
      LOG.SAL.Data.NIAAA.Detox.d1.d15.AVG.040508, na.action = na.exclude)
Residuals:
     Min      1Q  Median     3Q    Max
 -0.9397 -0.2655 0.01889 0.2384 0.8188

Coefficients:
              Value Std. Error  t value Pr(>|t|)
(Intercept)   1.9870   0.1296    15.3343   0.0000
        GEN  -0.1942   0.1401    -1.3863   0.1752
       FTND   0.0195   0.0196     0.9957   0.3269

Residual standard error: 0.4141 on 32 degrees of freedom
Multiple R-Squared: 0.08527
F-statistic: 1.492 on 2 and 32 degrees of freedom, the p-value is 0.2402



*** Linear Model ***

Call: lm(formula = Log.S.SAL.d.15 ~ GEN + ..cig, data =
      LOG.SAL.Data.NIAAA.Detox.d1.d15.AVG.040508, na.action = na.exclude)
Residuals:
     Min     1Q    Median     3Q    Max
 -0.9868 -0.215 -0.001007 0.2376 0.7486

Coefficients:
              Value Std. Error  t value Pr(>|t|)
(Intercept)   2.0075   0.1185    16.9349   0.0000
        GEN  -0.2067   0.1405    -1.4715   0.1509
      ..cig   0.0050   0.0051     0.9703   0.3392

Residual standard error: 0.4145 on 32 degrees of freedom
Multiple R-Squared: 0.08389
F-statistic: 1.465 on 2 and 32 degrees of freedom, the p-value is 0.2461
```

Table O-20: Linear model: FTND or #cig/day and GEN on Log R-SAL Day 15

```
*** Linear Model ***

Call: lm(formula = Log.R.SAL.d.15 ~ GEN + FTND, data =
      LOG.SAL.Data.NIAAA.Detox.d1.d15.AVG.040508, na.action = na.exclude)
Residuals:
    Min      1Q  Median      3Q     Max
 -1.339 -0.2579 0.04198 0.3555 0.9668

Coefficients:
              Value Std. Error  t value Pr(>|t|)
(Intercept)   2.1039   0.1616   13.0219   0.0000
        GEN  -0.2651   0.1747   -1.5172   0.1390
       FTND   0.0200   0.0244    0.8191   0.4188

Residual standard error: 0.5164 on 32 degrees of freedom
Multiple R-Squared: 0.08666
F-statistic: 1.518 on 2 and 32 degrees of freedom, the p-value is 0.2345



*** Linear Model ***

Call: lm(formula = Log.R.SAL.d.15 ~ GEN + ..cig, data =
      LOG.SAL.Data.NIAAA.Detox.d1.d15.AVG.040508, na.action = na.exclude)
Residuals:
    Min     1Q  Median     3Q    Max
 -1.395 -0.267 0.04639 0.3433 0.8795

Coefficients:
              Value Std. Error  t value Pr(>|t|)
(Intercept)   2.1178   0.1474   14.3662   0.0000
        GEN  -0.2789   0.1747   -1.5967   0.1202
      ..cig   0.0057   0.0064    0.8924   0.3789

Residual standard error: 0.5154 on 32 degrees of freedom
Multiple R-Squared: 0.09015
F-statistic: 1.585 on 2 and 32 degrees of freedom, the p-value is 0.2206
```

Table O-21: Linear model: FTND or #cig/day and GEN on Log DA Day 15

```
*** Linear Model ***

Call: lm(formula = Log.DA.d.15 ~ GEN + FTND, data =
      LOG.SAL.Data.NIAAA.Detox.d1.d15.AVG.040508, na.action = na.exclude)
Residuals:
     Min      1Q   Median     3Q    Max
 -0.2844 -0.1342 -0.02744 0.0747 0.4999

Coefficients:
              Value Std. Error  t value Pr(>|t|)
(Intercept)   0.7631   0.0544    14.0380   0.0000
        GEN  -0.0416   0.0588    -0.7075   0.4844
       FTND  -0.0023   0.0082    -0.2783   0.7826

Residual standard error: 0.1737 on 32 degrees of freedom
Multiple R-Squared: 0.01746
F-statistic: 0.2843 on 2 and 32 degrees of freedom, the p-value is 0.7544



*** Linear Model ***

Call: lm(formula = Log.DA.d.15 ~ GEN + ..cig, data =
      LOG.SAL.Data.NIAAA.Detox.d1.d15.AVG.040508, na.action = na.exclude)
Residuals:
    Min      1Q  Median      3Q    Max
 -0.268 -0.1356 -0.0329 0.06635 0.4918

Coefficients:
              Value Std. Error  t value Pr(>|t|)
(Intercept)   0.7685   0.0495    15.5263   0.0000
        GEN  -0.0390   0.0587    -0.6641   0.5114
      ..cig  -0.0012   0.0021    -0.5723   0.5711

Residual standard error: 0.1731 on 32 degrees of freedom
Multiple R-Squared: 0.02506
F-statistic: 0.4113 on 2 and 32 degrees of freedom, the p-value is 0.6662
```

Table O-22: Linear model: TLFB on Log S-SAL GTM

```
*** Linear Model ***

Call: lm(formula = Av.Log.S.SAL ~ TLFB..90., data = COV.ANALYSIS.41208,
na.action = na.exclude)

Residuals:
     Min      1Q  Median     3Q    Max
 -0.6332 -0.2766 0.05041 0.2253 0.6245

Coefficients:
               Value Std. Error  t value Pr(>|t|)
(Intercept)   2.0715   0.1014    20.4327   0.0000
  TLFB..90.  -0.0001   0.0001    -1.5593   0.1285

Residual standard error: 0.327 on 33 degrees of freedom
Multiple R-Squared: 0.06862
F-statistic: 2.431 on 1 and 33 degrees of freedom, the p-value is 0.1285
```

Table O-23: Linear model: TLFB on Log R-SAL GTM

```
*** Linear Model ***

Call: lm(formula = Av.Log.R.SAL ~ TLFB..90., data = COV.ANALYSIS.41208,
na.action = na.exclude)

Residuals:
     Min      1Q  Median    3Q    Max
 -0.7282 -0.2831 0.04199 0.271 0.6894

Coefficients:
               Value Std. Error  t value Pr(>|t|)
(Intercept)   2.2199   0.1219    18.2181   0.0000
  TLFB..90.  -0.0001   0.0001    -1.3868   0.1748

Residual standard error: 0.393 on 33 degrees of freedom
Multiple R-Squared: 0.05507
F-statistic: 1.923 on 1 and 33 degrees of freedom, the p-value is 0.1748
```

Table O-24: Linear model: TLFB on Log DA GTM

```
*** Linear Model ***

Call: lm(formula = Av.Log.DA ~ TLFB..90., data = COV.ANALYSIS.41208, na.action
= na.exclude)

Residuals:
     Min      1Q   Median      3Q     Max
 -0.3673 -0.132 -0.03049 0.1246 0.5372

Coefficients:
               Value Std. Error  t value Pr(>|t|)
(Intercept)   0.7751   0.0656    11.8238   0.0000
  TLFB..90.   0.0000   0.0001    -0.5137   0.6109

Residual standard error: 0.2114 on 33 degrees of freedom
Multiple R-Squared: 0.007932
F-statistic: 0.2638 on 1 and 33 degrees of freedom, the p-value is 0.6109
```

Table O-25: Linear model: ACAMP on Log S-SAL GTM

```
*** Linear Model ***

Call: lm(formula = Av.Log.S.SAL ~ ACAMP, data = COV.ANALYSIS.41208, na.action
= na.exclude)

Residuals:
     Min      1Q  Median      3Q     Max
 -0.6062 -0.2748 0.01026 0.2228 0.5399

Coefficients:
              Value Std. Error t value Pr(>|t|)
(Intercept)  1.8986  0.1068    17.7754  0.0000
      ACAMP  0.0565  0.1264     0.4471  0.6577

Residual standard error: 0.3378 on 33 degrees of freedom
Multiple R-Squared: 0.00602
F-statistic: 0.1999 on 1 and 33 degrees of freedom, the p-value is 0.6577
```

Table O-26: Linear model: ACAMP on Log R-SAL GTM

```
*** Linear Model ***

Call: lm(formula = Av.Log.R.SAL ~ ACAMP, data = COV.ANALYSIS.41208, na.action
= na.exclude)

Residuals:
     Min      1Q   Median    3Q    Max
 -0.8786 -0.2586 0.002235 0.257 0.6849

Coefficients:
             Value Std. Error t value Pr(>|t|)
(Intercept)  2.0512  0.1277    16.0601  0.0000
      ACAMP  0.0379  0.1511     0.2509  0.8035

Residual standard error: 0.4039 on 33 degrees of freedom
Multiple R-Squared: 0.001904
F-statistic: 0.06294 on 1 and 33 degrees of freedom, the p-value is 0.8035
```

Table O-27: Linear model: ACAMP on Log R-SAL GTM

```
*** Linear Model ***

Call: lm(formula = Av.Log.DA ~ ACAMP, data = COV.ANALYSIS.41208, na.action =
       na.exclude)

Residuals:
     Min      1Q  Median     3Q    Max
 -0.3362 -0.1452 -0.0249 0.1158 0.5449

Coefficients:
             Value Std. Error t value Pr(>|t|)
(Intercept)  0.7207  0.0669    10.7719  0.0000
      ACAMP  0.0367  0.0792     0.4631  0.6463

Residual standard error: 0.2116 on 33 degrees of freedom
Multiple R-Squared: 0.006458
F-statistic: 0.2145 on 1 and 33 degrees of freedom, the p-value is 0.6463
```

Table O-28: Regression and ANCOVA: TLFB on Log S-SAL Day 1

```
*** Linear Model ***

Call: lm(formula = Log.S.SAL.d.1 ~ TLFB..90., data = COV.ANALYSIS.41208,
na.action
       = na.exclude)
Residuals:
    Min     1Q Median     3Q  Max
 -1.489 -0.31 0.1064 0.296 1.08

Coefficients:
               Value Std. Error  t value Pr(>|t|)
(Intercept)   1.7201   0.1541    11.1623   0.0000
  TLFB..90.  -0.0002   0.0001    -1.5114   0.1402

Residual standard error: 0.497 on 33 degrees of freedom
Multiple R-Squared: 0.06474
F-statistic: 2.284 on 1 and 33 degrees of freedom, the p-value is 0.1402


*** Analysis of Variance Model ***

Short Output:
Call:
   aov(formula = Log.S.SAL.d.1 ~ SS + GEN + SS:GEN + TLFB..90., data =
       COV.ANALYSIS.41208, na.action = na.exclude)

Terms:
                      SS      GEN TLFB..90.   SS:GEN Residuals
 Sum of Squares 0.404638 0.357304  0.174893 0.597973  7.179861
Deg. of Freedom        2        1         1        2        28

Residual standard error: 0.5063829
Estimated effects may be unbalanced

          Df Sum of Sq   Mean Sq  F Value     Pr(F)
       SS  2  0.404638 0.2023190 0.789003 0.4641389
      GEN  1  0.357304 0.3573041 1.393413 0.2477607
TLFB..90.  1  0.174893 0.1748931 0.682048 0.4158642
   SS:GEN  2  0.597973 0.2989863 1.165986 0.3262898
Residuals 28  7.179861 0.2564236
```

Table O-29: Regression and ANCOVA: TLFB on Log R-SAL Day 1

```
*** Linear Model ***

Call: lm(formula = Log.R.SAL.d.1 ~ TLFB..90., data = COV.ANALYSIS.41208,
na.action
       = na.exclude)
Residuals:
    Min      1Q Median     3Q   Max
 -1.745 -0.2703 0.1181 0.4162 1.292

Coefficients:
             Value Std. Error t value Pr(>|t|)
(Intercept)  1.6936  0.1843     9.1893  0.0000
  TLFB..90. -0.0002  0.0002    -1.3689  0.1803

Residual standard error: 0.5944 on 33 degrees of freedom
Multiple R-Squared: 0.05374
F-statistic: 1.874 on 1 and 33 degrees of freedom, the p-value is 0.1803



*** Analysis of Variance Model ***

Short Output:
Call:
   aov(formula = Log.R.SAL.d.1 ~ SS + GEN + SS:GEN + TLFB..90., data =
       COV.ANALYSIS.41208, na.action = na.exclude)

Terms:
                      SS      GEN TLFB..90.   SS:GEN Residuals
 Sum of Squares  0.13377  0.38933   0.31112  1.15898  10.32713
Deg. of Freedom        2        1         1        2        28

Residual standard error: 0.6073106
Estimated effects may be unbalanced

          Df Sum of Sq   Mean Sq  F Value     Pr(F)
       SS  2   0.13377 0.0668843 0.181344 0.8351204
      GEN  1   0.38933 0.3893300 1.055592 0.3130170
TLFB..90.  1   0.31112 0.3111247 0.843554 0.3662269
   SS:GEN  2   1.15898 0.5794899 1.571174 0.2255764
Residuals 28  10.32713 0.3688261
```

Table O-30: Regression and ANCOVA: TLFB on Log DA Day 1

```
*** Linear Model ***

Call: lm(formula = Log.DA.d.1 ~ TLFB..90., data = COV.ANALYSIS.41208,
na.action =
       na.exclude)
Residuals:
     Min      1Q   Median     3Q    Max
 -0.5607 -0.1329 -0.01419 0.1573 0.4198

Coefficients:
              Value Std. Error t value Pr(>|t|)
(Intercept)  0.7313  0.0743     9.8376  0.0000
  TLFB..90.  0.0000  0.0001    -0.7656  0.4494

Residual standard error: 0.2397 on 33 degrees of freedom
Multiple R-Squared: 0.01745
F-statistic: 0.5861 on 1 and 33 degrees of freedom, the p-value is 0.4494
```

Table O-31: Regression and ANCOVA: BrAC on Log S-SAL Day 1

```
*** Linear Model ***

Call: lm(formula = Log.S.SAL.d.1 ~ BrAC, data = COV.ANALYSIS.41208, na.action
= na.exclude)

Residuals:
    Min      1Q Median     3Q   Max
 -1.275 -0.3314 0.1402 0.2413 1.122

Coefficients:
              Value Std. Error  t value Pr(>|t|)
(Intercept)   1.6316   0.1052    15.5050   0.0000
      BrAC  -0.2874   0.1727    -1.6647   0.1054

Residual standard error: 0.4936 on 33 degrees of freedom
Multiple R-Squared: 0.07747
F-statistic: 2.771 on 1 and 33 degrees of freedom, the p-value is 0.1054



*** Analysis of Variance Model ***

Short Output:
Call:
   aov(formula = Log.S.SAL.d.1 ~ SS + GEN + BrAC + SS:BrAC, data =
       COV.ANALYSIS.41208, na.action = na.exclude)

Terms:
                      SS      GEN     BrAC  SS:BrAC Residuals
 Sum of Squares 0.404638 0.357304 0.563675 0.055005  7.334047
Deg. of Freedom        2        1        1        2        28

Residual standard error: 0.5117912
Estimated effects may be unbalanced

          Df Sum of Sq   Mean Sq  F Value     Pr(F)
       SS  2  0.404638 0.2023190 0.772416 0.4714887
      GEN  1  0.357304 0.3573041 1.364119 0.2526742
     BrAC  1  0.563675 0.5636746 2.152003 0.1535270
  SS:BrAC  2  0.055005 0.0275026 0.105000 0.9006774
Residuals 28  7.334047 0.2619302
```

Table O-32: Regression and ANCOVA: BrAC on Log R-SAL Day 1

```
*** Linear Model ***

Call: lm(formula = Log.R.SAL.d.1 ~ BrAC, data = COV.ANALYSIS.41208, na.action
= na.exclude)

Residuals:
    Min      1Q  Median      3Q   Max
 -1.515 -0.3028 0.05519 0.3786 1.338

Coefficients:
              Value Std. Error  t value Pr(>|t|)
(Intercept)   1.5964   0.1261    12.6584   0.0000
      BrAC   -0.3077   0.2069    -1.4870   0.1465

Residual standard error: 0.5915 on 33 degrees of freedom
Multiple R-Squared: 0.0628
F-statistic: 2.211 on 1 and 33 degrees of freedom, the p-value is 0.1465



*** Analysis of Variance Model ***

Short Output:
Call:
   aov(formula = Log.R.SAL.d.1 ~ SS + GEN + BrAC + SS:BrAC, data =
       COV.ANALYSIS.41208, na.action = na.exclude)

Terms:
                      SS      GEN     BrAC  SS:BrAC Residuals
 Sum of Squares  0.13377  0.38933  0.78504  0.15329  10.85890
Deg. of Freedom        2        1        1        2        28

Residual standard error: 0.6227502
Estimated effects may be unbalanced

          Df Sum of Sq   Mean Sq  F Value     Pr(F)
       SS  2   0.13377 0.0668843 0.172463 0.8424764
      GEN  1   0.38933 0.3893300 1.003899 0.3249496
     BrAC  1   0.78504 0.7850445 2.024261 0.1658507
  SS:BrAC  2   0.15329 0.0766467 0.197636 0.8218036
Residuals 28  10.85890 0.3878178
```

Table O-33: Regression and ANCOVA: BrAC on Log DA Day 1

```
*** Linear Model ***

Call: lm(formula = Log.DA.d.1 ~ BrAC, data = COV.ANALYSIS.41208, na.action =
      na.exclude)
Residuals:
     Min      1Q   Median     3Q    Max
 -0.5407 -0.1342 -0.03163 0.1595 0.4436

Coefficients:
              Value Std. Error  t value Pr(>|t|)
(Intercept)   0.6905   0.0515    13.4012   0.0000
       BrAC  -0.0186   0.0845    -0.2205   0.8268

Residual standard error: 0.2417 on 33 degrees of freedom
Multiple R-Squared: 0.001471
F-statistic: 0.04863 on 1 and 33 degrees of freedom, the p-value is 0.8268


*** Analysis of Variance Model ***

Short Output:
Call:
   aov(formula = Log.DA.d.1 ~ SS + GEN + BrAC + SS:BrAC, data =
       COV.ANALYSIS.41208, na.action = na.exclude)

Terms:
                      SS      GEN     BrAC  SS:BrAC Residuals
 Sum of Squares 0.114532 0.107844 0.001429 0.011235  1.695221
Deg. of Freedom        2        1        1        2        28

Residual standard error: 0.2460561
Estimated effects may be unbalanced

          Df Sum of Sq   Mean Sq  F Value     Pr(F)
       SS  2  0.114532 0.0572659 0.945863 0.4004042
      GEN  1  0.107844 0.1078437 1.781256 0.1927473
     BrAC  1  0.001429 0.0014292 0.023606 0.8789927
  SS:BrAC  2  0.011235 0.0056176 0.092786 0.9116676
Residuals 28  1.695221 0.0605436
```

Table O-34: Regression and ANCOVA: Admit Drug of Abuse on Log S-SAL Day 1

```
*** Linear Model ***

Call: lm(formula = Log.S.SAL.d.1 ~ Admit.DOA, data = COV.ANALYSIS.41208,
na.action
       = na.exclude)
Residuals:
    Min      1Q  Median     3Q   Max
 -1.394 -0.2919 0.05191 0.3164 1.192

Coefficients:
              Value Std. Error  t value Pr(>|t|)
(Intercept)   1.5613   0.1091    14.3149   0.0000
  Admit.DOA  -0.0980   0.1790    -0.5479   0.5875

Residual standard error: 0.5116 on 33 degrees of freedom
Multiple R-Squared: 0.009014
F-statistic: 0.3002 on 1 and 33 degrees of freedom, the p-value is 0.5875


*** Analysis of Variance Model ***

Short Output:
Call:
   aov(formula = Log.S.SAL.d.1 ~ SS + GEN + Admit.DOA + SS:Admit.DOA, data =
       COV.ANALYSIS.41208, na.action = na.exclude)

Terms:
                      SS      GEN Admit.DOA SS:Admit.DOA Residuals
 Sum of Squares 0.404638 0.357304  0.057277     0.334592  7.560858
Deg. of Freedom        2        1         1            2        28

Residual standard error: 0.5196447
Estimated effects may be unbalanced

             Df Sum of Sq   Mean Sq  F Value     Pr(F)
          SS  2  0.404638 0.2023190 0.749245 0.4819652
         GEN  1  0.357304 0.3573041 1.323198 0.2597487
   Admit.DOA  1  0.057277 0.0572768 0.212112 0.6486740
SS:Admit.DOA  2  0.334592 0.1672958 0.619544 0.5454048
   Residuals 28  7.560858 0.2700306
```

Table O-35: Regression and ANCOVA: Admit Drug of Abuse on Log R-SAL Day 1

```
*** Linear Model ***

Call: lm(formula = Log.R.SAL.d.1 ~ Admit.DOA, data = COV.ANALYSIS.41208,
na.action
       = na.exclude)
Residuals:
   Min     1Q  Median     3Q   Max
 -1.61 -0.377 0.06613 0.4493 1.394

Coefficients:
              Value Std. Error  t value Pr(>|t|)
(Intercept)   1.5404   0.1292    11.9223   0.0000
  Admit.DOA  -0.1569   0.2120    -0.7402   0.4644

Residual standard error: 0.606 on 33 degrees of freedom
Multiple R-Squared: 0.01633
F-statistic: 0.5478 on 1 and 33 degrees of freedom, the p-value is 0.4644



*** Analysis of Variance Model ***

Short Output:
Call:
   aov(formula = Log.R.SAL.d.1 ~ SS + GEN + Admit.DOA + SS:Admit.DOA, data =
       COV.ANALYSIS.41208, na.action = na.exclude)

Terms:
                      SS      GEN Admit.DOA SS:Admit.DOA Residuals
 Sum of Squares  0.13377  0.38933   0.17225      0.29998  11.32501
Deg. of Freedom        2        1         1            2        28

Residual standard error: 0.6359754
Estimated effects may be unbalanced

             Df Sum of Sq   Mean Sq   F Value     Pr(F)
          SS  2   0.13377 0.0668843 0.1653650 0.8484060
         GEN  1   0.38933 0.3893300 0.9625807 0.3349394
   Admit.DOA  1   0.17225 0.1722461 0.4258619 0.5193487
SS:Admit.DOA  2   0.29998 0.1499889 0.3708330 0.6934981
   Residuals 28  11.32501 0.4044647
```

Table O-36: Regression and ANCOVA: Admit Drug of Abuse on Log DA Day 1

```
       *** Linear Model ***

Call: lm(formula = Log.DA.d.1 ~ Admit.DOA, data = COV.ANALYSIS.41208,
na.action =
       na.exclude)
Residuals:
     Min      1Q   Median     3Q    Max
 -0.5689 -0.1256 -0.03078 0.1596 0.4603

Coefficients:
             Value Std. Error t value Pr(>|t|)
(Intercept)  0.6738  0.0515    13.0865  0.0000
  Admit.DOA  0.0264  0.0845     0.3119  0.7571

Residual standard error: 0.2415 on 33 degrees of freedom
Multiple R-Squared: 0.002939
F-statistic: 0.09729 on 1 and 33 degrees of freedom, the p-value is 0.7571



       *** Analysis of Variance Model ***

Short Output:
Call:
   aov(formula = Log.DA.d.1 ~ SS + GEN + Admit.DOA + SS:Admit.DOA, data =
       COV.ANALYSIS.41208, na.action = na.exclude)

Terms:
                      SS      GEN Admit.DOA SS:Admit.DOA Residuals
 Sum of Squares 0.114532 0.107844  0.007380     0.040886  1.659619
Deg. of Freedom        2        1         1            2        28

Residual standard error: 0.2434586
Estimated effects may be unbalanced

             Df Sum of Sq   Mean Sq  F Value     Pr(F)
          SS  2  0.114532 0.0572659 0.966153 0.3928709
         GEN  1  0.107844 0.1078437 1.819468 0.1881854
   Admit.DOA  1  0.007380 0.0073800 0.124510 0.7268365
SS:Admit.DOA  2  0.040886 0.0204431 0.344903 0.7112561
   Residuals 28  1.659619 0.0592721
```

Table O-37: Regression: AST on Log S- and R-SAL Day 1

```
*** Linear Model ***

Call: lm(formula = Log.S.SAL.d.1 ~ AST.U.L, data = COV.ANALYSIS.41208,
na.action =
      na.exclude)
Residuals:
    Min      1Q Median     3Q  Max
 -1.309 -0.1192 0.1088 0.2266 1.07

Coefficients:
              Value Std. Error  t value Pr(>|t|)
(Intercept)   1.7380   0.1205    14.4206   0.0000
    AST.U.L  -0.0037   0.0015    -2.4455   0.0202

Residual standard error: 0.4788 on 32 degrees of freedom
Multiple R-Squared: 0.1575
F-statistic: 5.98 on 1 and 32 degrees of freedom, the p-value is 0.02015
1 observations deleted due to missing values



      *** Linear Model ***

Call: lm(formula = Log.R.SAL.d.1 ~ AST.U.L, data = COV.ANALYSIS.41208,
na.action =
      na.exclude)
Residuals:
    Min      1Q Median     3Q   Max
 -1.524 -0.3227 0.1268 0.3586 1.241

Coefficients:
              Value Std. Error  t value Pr(>|t|)
(Intercept)   1.7650   0.1407    12.5456   0.0000
    AST.U.L  -0.0048   0.0017    -2.7232   0.0104

Residual standard error: 0.5589 on 32 degrees of freedom
Multiple R-Squared: 0.1881
F-statistic: 7.416 on 1 and 32 degrees of freedom, the p-value is 0.01038
1 observations deleted due to missing values
```

Table O-38: Regression model for main effects of time on CIWA-AR

```
*** Linear Model ***

Call: lm(formula = CIWA ~ DAY, data = CIWA.AR.ANAL.041208, na.action =
na.exclude)
Residuals:
    Min     1Q  Median    3Q  Max
 -6.295 -3.323 -0.8511 2.149 11.7

Coefficients:
              Value Std. Error t value Pr(>|t|)
(Intercept)  7.7389  1.1081     6.9839  0.0000
        DAY -1.4439  0.5178    -2.7886  0.0064

Residual standard error: 4.205 on 98 degrees of freedom
Multiple R-Squared: 0.07352
F-statistic: 7.776 on 1 and 98 degrees of freedom, the p-value is 0.00636
```

Table O-39: Regression model for main effects of SS and GEN on CIWA-AR

```
*** Linear Model ***

Call: lm(formula = CIWA ~ DAY + SS + GEN, data = CIWA.AR.ANAL.041208,
na.action =
      na.exclude)
Residuals:
    Min     1Q Median    3Q   Max
 -6.638 -2.721 -1.005 2.123 10.42

Coefficients:
              Value Std. Error t value Pr(>|t|)
(Intercept)  9.4308  1.2817     7.3581  0.0000
        DAY -1.3893  0.5083    -2.7330  0.0075
       SSLS -1.2979  1.0339    -1.2553  0.2124
       SSNS -1.4035  0.9872    -1.4217  0.1584
        GEN -1.7750  0.8257    -2.1497  0.0341


Multiple R-Squared: 0.1359
F-statistic: 3.737 on 4 and 95 degrees of freedom, the p-value is 0.007216
```

VITA

Satjit Singh Brar was born on May 17, 1974 in Suffern, New York, and is an American citizen. He graduated from Laguna Hills High School, Laguna Hills, CA, in 1992. He received his Bachelors of Science in Environmental Chemistry in 1998 from the University of California at Santa Barbara, California. He joined the Pharm.D. program in 2000 at Virginia Commonwealth University/Medical College of Virginia Campus and he subsequently joined the Ph.D. program in the Department of Pharmaceutics as a combined degree student in 2001. He conducted his research in the Pharmacokinetics-Pharmacodynamics Research Laboratory to study Clinical Pharmacology. Throughout his tenure, he served as a Graduate Teaching Assistant from 2002-2006 and was appointed a Teaching Fellow for the 2006 academic year in the Department of Pharmaceutics teaching pharmacokinetics to both professional pharmacy and graduate students. He is a member of the American Society for Clinical Pharmacology and Therapeutics, American College of Clinical Pharmacology and the American Association of Pharmaceutical Scientists. He has served as Vice-president (between 2001–2004) and President (2004-2005) of the Department of Pharmaceutics Graduate Student Association. He was treasurer for the Graduate Research Association for Students in Pharmacy conference held in 2003. He was Founder and Chair of the VCU Student Chapter of the American Association of Pharmaceutical Scientists as well as the VCU School of Pharmacy Combined Degree Student Committee. He was inducted into the Rho Chi Honor Society in 2003 and the Phi Kappa Phi Honor Society in 2006. He was honored with a Pre-doctoral Intramural Research Training Award from the National Institutes of Health to conduct his dissertation work. In 2007, Satjit was the recipient of the Department of Pharmaceutics John Wood Award, VCU Leadership and Service Award, VCU School of Graduate Studies Travel Award.