STUDIES INTO SULFUR AMINO ACID AND BILE SALT METABOLISM IN PANCREATIC AND LIVER DISEASES

Profiles of sulfur amino acids and glutathione in acute pancreatitis; method development for total and oxidized glutathione by liquid chromatography; bile salt profiles in liver disease by liquid chromatography-mass spectrometry.

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Keywords
Oxidative stress; acute pancreatitis; cysteine; homocysteine; cysteinyl-glycine; glutathione; liquid chromatography mass spectrometry; 1, methyl-2-vinyl-pyridinium trifluromethanesulfonate; liver disease; bile salts.

Abstract

Sulfur amino acids have critical function as intracellular redox buffers and maintain homeostasis in the external milieu by combating oxidative stress. Synthesis of glutathione (GSH) is regulated at a substrate level by cysteine, which is synthesized by homocysteine via the transsulfuration pathway. Oxidative stress and diminished glutathione pools play a sustained role in the pathogenesis of acute pancreatitis.

One of the aims of this study was to experimentally address the temporal relationship between plasma sulfur amino acid levels in patients suffering from acute pancreatitis. The data indicated low concentration of cysteine initially, at levels similar to those of healthy controls. Glutathione was found reduced whilst cysteinyl-glycine and γ-glutamyl transpeptidase activity were increased in both mild and severe attacks. As the disease progressed, glutathione and cysteinyl-glycine were further increased in mild attacks and cysteine levels correlated with homocysteine and γ-glutamyl transpeptidase activity. The progress of severe attacks was associated with glutathione depletion, reduced γ-glutamyl transpeptidase activity and increased cysteinyl-glycine, that correlated with glutathione depletion. The corollary that ample supply of cysteine and cysteinly-glycine does not contribute towards glutathione synthesis in acute pancreatitis poses an important issue that merits resolution. Heightened oxidative stress and depletion of glutathione rationalized the progression of disease in severe attacks.

An upsurge that reactive oxygen species can shift redox state of cells is determined by the ratio of the abundant redox couples reduced and oxidized glutathione (GSH: GSSG) in cell. The study reported a novel methodology for quantification of total oxidized glutathione (tGSSG) and total glutathione (tGSH) in whole blood using reverse phase high performance liquid chromatography. The novelty of the method is ascertained by the use of a mercaptan scavenger 1, methyl-2-vinyl-pyridinium trifluromethanesulfonate for the total oxidized glutathione determination. The results reported permit quantitation of tGSSG and tGSH and was applied to a control group.

Finally, the study was also focussed in developing a liquid chromatography-mass spectrometric method to evaluate free and conjugated bile acids in patients suffering from various degrees of cholestatic-hepatobiliary disorders. The study reported low levels of ursodeoxycholic acid (UDCA) and slightly high levels of lithocholic acid (LCA). All the primary bile acids seem to be conjugated with glycine and taurine amino acid.

Publications

a. Peer review articles

1. Rahman SH**,** **Srinivasan AR** and Nicolaou A **(2009).** *Digestive diseases and science.* Transsulfuration pathway defects and increased glutathione degradation in severe acute pancreatitis **(54),** 675-682.

b. Conference contributions

1. **Srinivasan AR,** Rahman SH, Nicolaou A **(2006).** Defects in the transsulfuration pathway and increased glutathione degradation contribute to the oxidative stress associated with severe acute pancreatitis. Iranian researchers' conference in Europe. Institute of Pharmaceutical innovation, University of Bradford, Bradford. UK (Poster presentation).

2. **Srinivasan AR,** Rahman SH, Nicolaou A **(2007).** Altered sulfur amino acid levels and glutathione depletion contribute to severe acute pancreatitis. Biochemical Society and British Pharmacological Society. Glasgow. UK (Poster presentation)

3. Rahman SH**,** **Srinivasan AR,** Nicolaou A **(2006)**. Defects in the transsulfuration pathway and increased glutathione degradation contribute to the oxidative stress associated with severe acute pancreatitis. International Hepato-Pancreato-Biliary association (IHPBA), Edinburgh. UK (Oral presentation).

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DEDICATION

Maa and Paa - Just saying thank you will not suffice the purpose for me being in Bradford miles away from you. Your selfless love has made me achieve this. Therefore, I dedicate this thesis to you.

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Abbreviations

γ-GT – Gamma glutamyl transpeptidase

2-VP – 2-Vinyl pyridine

AP – Acute pancreatitis

BrB – Monobromobiamine

BSO – Buthionine sulfoxide

CA – Cholic acid

CDCA – Chenodeoxycholic acid

CDER – Centre for drug evaluation and research

CP – Chronic pancreatitis.

CRP – C-reactive protein.

Cys – Cysteine

Cys-Gly – Cysteinyl-glycine

DCA – Deoxycholic acid

DTT – Dithiothreitol

EDTA – Ethylenediamineterta acetic acid

GCA – Glycocholic acid

GEE – Glutathione ethyl ester

GCL – Glutamate cystiene ligases

GCDCA – Glycochenodeoxycholic acid

GDCA – Glycodeoxycholic acid

GLCA – Glycolithocholic acid

GPx – Glutathione peroxidises

GR – Glutathione reductase

GSH – Reduced glutathione

GSSG – Oxidized glutathione

GST – Glutathione S transferease

GUDCA – Glycoursodeoxycholic acid

Hcy – Homocysteine

IAA – Iodoacetic acid

ICH – International conference on harmonisation

LCA – Lithocholic acid

M2VP – 1-methyl-2-vinyl pyridium trifluromethane sulfonate

MPA – Metaphosphoric acid

MPG – Mercaptopropionylglycine

NAC – N-acetylcysteine

NAT – N-acylamino transfereses

NEM – N- ethylmaleamide

ODFRS – Oxygen derived free radicals

OPA – *ortho*-phthalaldehyde

ROS – Reactive oxygen species

SAH – S-adenosylhomocysteine

SAM – S-adenosylmethionine

SBDf - 7-flurobenzofurazane-4-sulfonic ammonium salt

SOD – Superoxide dismutase

TCA – Taurocholic acid

TCDCA – Taurochenodeoxycholic acid

TDCA – Taurodeoxycholic acid

TLCA – Taurolithocholic acid

TUDCA – Tauroursodeoxycholic acid

tGSH – Total glutathione

tGSSG – Total oxidized glutathione

XO – Xanthine oxidase

XOD – Xanthine oxidase dismutase

γ-GCS – Gamma glutamyl-cysteine synthetase

SST – System suitability test

UDCA – Ursodeoxycholic acid

CHAPTER 1

INTRODUCTION

CHAPTER 1. Introduction

1.1. Oxidative stress

Evolution of organisms to adapt to oxygenated environment has heralded a permanent transformation in the development of effective cellular strategies to detect and detoxify metabolites of molecular oxygen widely known as reactive oxygen species (ROS). The term ROS encompasses a wide variety of chemical species that include oxygen centred radicals. Although, ROS have been known to actively participate in a diverse array of biological/ physiological process (such as regulation of vascular tone, normal cell growth, senescence, apoptosis) (Droge, 2002), they are also known to trigger a divergent response. The outcome of which reflects the subtle difference in the level and duration of the oxidant burst that accompanies oxidative stress (Frein et al., 2005).

The concept of oxidative stress has become interventional after the discovery of molecular oxygen by Joseph Priestley in 17th Century. Ever since its discovery, molecular oxygen has been a topic of intense debate and almost a paradox. Since then amassed wealth of scientific knowledge have suggested the formation and role of various free radicals produced during and after biochemical reactions in aerobic species. Further oxidation, describes an oxidative event that can or cannot be a reversible reaction and the term stress, delineates a deviation from the normal physiological process. Thus, the term “oxidative stress” is characterized as a persistent oxidative shift towards a pathophysiological event in the absence of a counterbalancing regulatory response called redox regulation. Redox, on the other hand, is a regulatory process, inducing a protective response against oxidative damage caused by accumulation of free radicals. Thus, the cells use redox process to reset to the original state of redox homeostasis after temporary exposure to ROS.

Nevertheless, the survival of the cell during and/ or after a stress insult is dependent upon the ability of the cell to adapt or resist the stress. Exposure of cells to high levels of ROS for prolonged time perturbs the normal redox regulation and shifts the cells into a state of oxidative stress. Free radicals thus formed during these processes create a stress environment in the cells resulting in a pattern of cumulative damage and/ or cell injury (Figure 1.1).

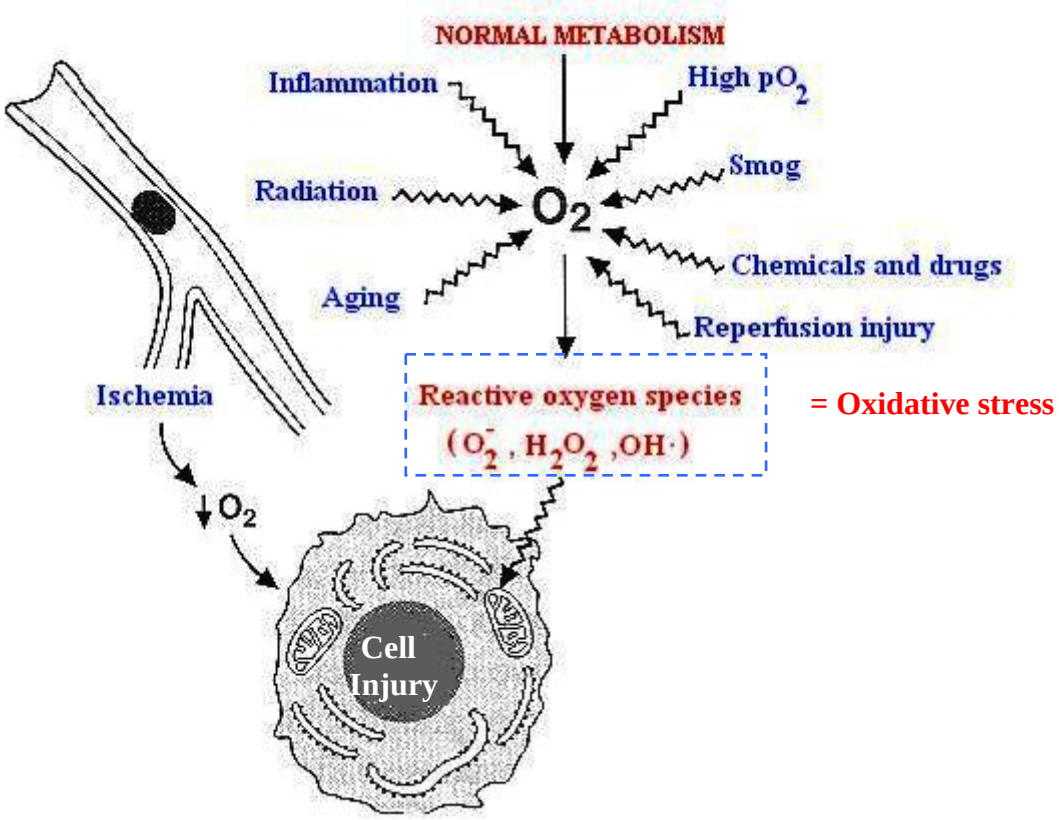


Figure 1.1. Schematic representation of production of free radicals as results various biochemical pathways and possible mechanism of cell injury. (Adapted from: (James et al., 2004)

1.1.1. Free radicals, their formation and types

Free radicals are electrically charged molecular species capable of independent existence that contains an unpaired electron in their atomic orbital (Halliwell and Gutteridge, 1988). These species are highly reactive and unstable due to the presence of

unpaired electron(s) thus, behaving as oxidants or reductants as they can either donate or extract an electron from other molecules.

Free radicals are formed when covalent bonds between entities are broken and one electron remains with each newly formed atom. Oxygen centred free radicals contain two unpaired electrons in the outer shell. When free radicals acquire an electron from a surrounding compound or molecule, a new free radical is formed in its place. The newly formed radical in an attempt to return to its ground state acquires or loses electron(s) with anti-parallel spins from cellular structures or molecules thus continuing the formation. A number of mechanisms within the body and external sources lead to formation of free radicals (Figure 1.2).

There are numerous types of free radicals formed within the body such as: superoxide anions (O_2^-), hydroxyl radical (•OH), and hydrogen peroxide (H_2O_2).

Superoxide (O_2^-) are abundant and 2% of the oxygen consumed by cells are converted into O_2^-(Ward, 1988). They are formed when oxygen acquires an additional electron, leaving the molecule with only one unpaired electron. This radical is not particularly reactive relative to other species. However, either by spontaneous action or through enzymatic action of superoxide dismutases, it is converted into H_2O_2 and O_2 (Fridovich, 1995). In biological systems, O_2^- can be converted (nonenzymatically) to hydrogen peroxide (H_2O_2) (Fridovich, 1978, Deby and Goutier, 1990). H_2O_2 can also be formed directly from O_2 through the two-electron transfer by a number of oxidases (*e.g.* urate oxidase, monoamine oxidase, and xanthine oxidase). Compared with oxygen radicals, H_2O_2 is relatively stable and can easily diffuse across cellular membranes. In the presence of reduced transition metal ions such as copper or iron, H_2O_2 can undergo reduction to the hydroxyl anion via the Fenton reaction, yielding the hydroxyl radical (•OH) (Halliwell and Gutteridge, 1988). •OH radical can also arise from the hemolytic

fission of H_2O_2 by ultraviolet light, the hydrolysis of water by ionizing radiation, or the metabolic reactions of certain chemicals (Halliwell, 1999). Thus, •OH is probably the most potent oxygen radical and can react non-specifically with any biological molecules.

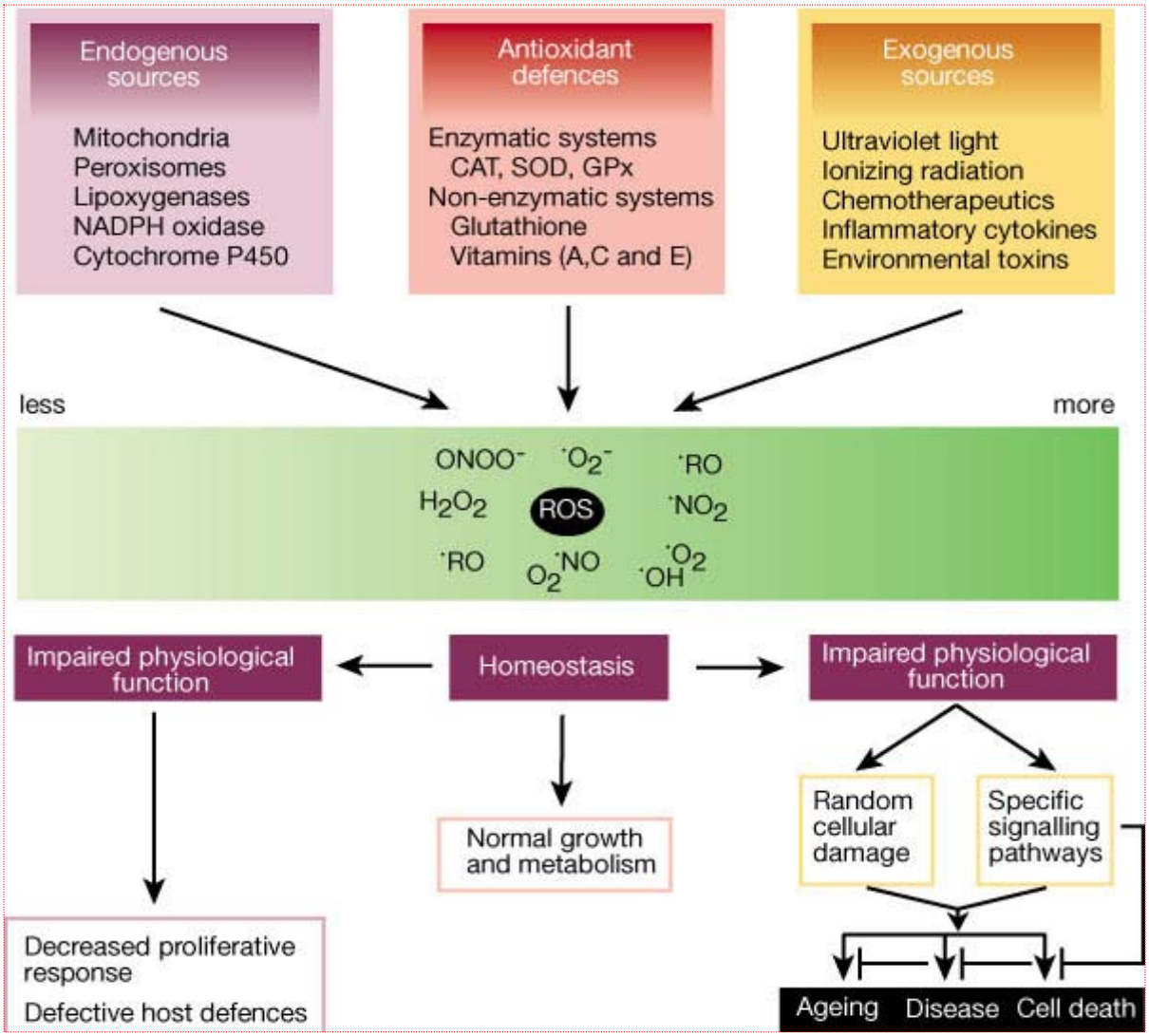


Figure 1.2. Possible endogenous and exogenous sources of free radicals and their effects on the cellular physiology. Possible cellular responses to counteract oxidative stress and regulate physiological homeostasis. (Adopted from (Finkel and Holbrook (2000).

1.1.2. Cellular sources of oxidants and oxidative stress response by organism

Four keys sources in aerobic organisms generate ROS, 1) the mitochondrial electron transpor, 2) peroxisomal metabolism 3) cytochrome P450 reactions and 4) phagocytotic cells. Mitochondrial respiratory chain produces the majority of ROS through NADH dehydrogenase and ubiquinone-cytochrome *c* reductase thus contributing towards the production of O_2^- radicals (Turrens, 1997). During respiratory process, the oxygen radicals capture electrons and leak out of electron transport cycle leading to the formation of O_2^- radical. Peroxisomes contain a number of H_2O_2 generating oxidases, including fatty acyl-CoA oxidase and urate oxidase. These enzymes remove two electrons from substrates and ultimately transfer the electrons to O_2 to form H_2O_2.

The cytochrome P450 enzymes also generate generous portions of ROS in the cell. These enzymes are primarily mixed-function oxidases found in the microsomes. The endogenous substrates of these enzymes include fatty acids, steroids, prostaglandins, leukotrienes, and thromboxanes (Puntarulo and Cederbaum, 1998). In addition, the P450s are able to act on a variety of xenobiotic compounds. The catalytic mechanism of cytochrome P450s involves several steps of one-electron transfers among NADPH, the substrates and O_2. Such steps, if uncoupled, may generate $O_2^{\bullet-}$ superoxide anion and H_2O_2 as by-products (Puntarulo and Cederbaum, 1998). Neutrophils and macrophages are able to enhance the uptake of oxygen and use it to generate a mixture of free radicals, including O_2^-, H_2O_2, and nitric oxide (Robinson and Badwey, 1994). These oxidants are part of the defence mechanism to allow immune cells to kill invading pathogens.

In order to counteract the oxidants, the cells in time with evolution have developed an impressive repertoire of strategies to detect and detoxify the metabolites of ROS. A number of stress response mechanisms in the cell, either in a cooperative or in an

antagonistic manner have evolved to adapt to the stress response. Many of these pathways and/ or response have led to the evolution of innate cellular protective mechanisms contrary to oxidation. These innate free radical defence mechanisms are maintained by the intracellular redox regulating molecules (Go and Jones, 2005, Rubartelli and Sitia, 2009, Nkabyo et al., 2005) and these molecules primarily function to create an intra and extracellular reducing environment. Thus, redox describes all the chemical reactions in which the atoms have their oxidation state changed. The redox systems, that include the glutathione (GSH) and thioredoxin (TRX) system, function primarily to contribute towards a reducing environment. In addition to these molecules, there are certain cellular antioxidant enzyme systems that contribute towards maintaining redox homeostasis by counteracting the ROS. Some of these antioxidant enzymes are superoxide dismutases (SOD), catalase, glutathione transferases (GST's), and glutathione peroxidise (GP). These protein antioxidant enzymes are combined with non-protein intracellular scavengers namely GSH, ascorbate and vitamin E (Table 1.1).

Table 1.1. Schematic representation of ROS produced, damage caused and antioxidant defence to counteract the oxidant production.

Oxidative stress	Damage caused by ROS	Antioxidant defences
Hydroxy radical	Adds bases to DNA, damage to lipid bilayer, mitochondrial damage	SOD, GPx, GR
Hydrogen peroxides	Diffuses nuclear membrane causing DNA damage, capable of penetrating plasma membrane	GPx, catalase
Superoxide's	Membrane diffusible	GR and SOD
$ONOO^-$	Homolysis to NO_2 and OH radicals	Vitamin E
4-Hyrdoxynonenal	Aldehyde product of lipid peroxidation	Lipoic acid
Xenobiotics	Endogenous origin	GST's and GPx

1.1.3. The emerging concept of redox regulation

A mechanism in the body subject the cells to follow redox regulation to achieve redox homeostasis, as redox balance is fundamental for a cell existence. Under normal physiological conditions, the human body is inevitably submitted to oxidative reactions through various biochemical pathways. However, to counteract this, large batteries of antioxidants swiftly repair and replace the likely harm. These antioxidant systems maintain the redox balance/ homeostasis by scavenging the oxidant species.

Under instances where the free radical activity suddenly increases as a consequence of either primary (e.g. excess radiation exposure) or secondary, (e.g. tissue damage by trauma) insults, the antioxidant defence will be weakened. Under these circumstances, the cells fail to tolerate these abruptive changes over long period leading to macroscopic shifts in redox potentials that divulge a failure in the stress responses (Jones, 2006). As a result, pathophysiological events such as accumulation of free radicals, lipid peroxidation products, DNA strand break following oxidation of proteins occurs, overwhelming cell leading to cell damage/ death. This damage can be apoptotic, necrotic or reversible, irreversible in nature.

Many current perspectives favour the evidence for the existence of a redox regulatory network intimately linked with cellular functions in both normal and pathological conditions (Finkel, 2003). These redox regulatory reactions describe a reversible phase during which oxidative reactions like zinc finger oxidation, methionine sulfoxidation, glutathiolation, S-nitrosation return to a resting state by reductive pathways including changes in oxidation states of metals (Frein et al., 2005).

Accumulated measurable evidence of such insults caused by free radical accretion instigating stress initiation, contribute to many consequences of aging and major disease process including cardiovascular (Stocker and Keaney, 2004), pulmonary diseases

(Cantin, 2004), diabetes (Bonnefont-Rosselot, 2004), neurodegenerative diseases (Vina et al., 2004) and cancer (Berger, 2005) are abundant. Also, increased oxidative stress presents an important mechanism in the genesis and progression of several diseases in humans (Halliwell, 1993). One of the classic examples of such a progressive inflammatory disease is acute pancreatitis.

1.1.4. Redox regulation in body systems

Redox regulation is exhibited in cells/ tissues that are subjected to oxidative/reductive stress and the response imparted reflects the state of the redox couples in the cells. As stated erstwhile by Sir Hans Krebs, "*Redox state describes the ratio of interconvertible oxidized and reduced form of specific redox couple emphasizing on* NAD^+*/ NADPH redox couple*". However the statement later was slightly modified with a contemporary definition – "Redox state not only describes the state of a particular redox pair but describes the redox environment of the cell and defines the half-cell reduction potential and the reducing capacity of the redox couples"– $NADP^+$/ NADPH, GSH/GSSG and reduced and oxidized thioredoxin (TRXss/TRX(SH_2). Therefore, the reducing capacity of the cell is important in maintaining the redox environment (Schafer and Buettner, 2001).

The three major redox systems in the body as mentioned above are interdependent with each other. GSH/GSSG and TrxSS/ Trx (SH_2) use NADPH as a source for their reducing equivalents. The GSH/GSSG system forms the representative principal redox buffer of the cell because of its concentration, which is far higher than the other two-redox buffers. Thus, the overall redox environment is the contribution of GSH/GSSG ratio.

1.1.5. Role of amino thiols in redox regulation

Amino thiols have a critical function as intracellular redox buffers and extend an important purpose as extracellular redox system. Low molecular weight thiol-containing amino acids have an essential role in maintaining the cells redox state. Oxidation of GSH, Cys and Met is one among cellular response to an increase ROS production (Schafer and Buettner, 2001).

1.2. Sulfur amino acid metabolism

Methionine (Met) and cysteine (Cys) are considered the principal sulfur-containing amino acids. Met is an essential amino acid initiating protein synthesis in eukaryotes. Cys plays a critical role as a precursor amino acid for proteins in maintaining protein structure by virtue of its ability to form disulfide bonds. Met is an essential amino acid obtained significantly from the diet while Cys is a semi-essential amino acid as it can be produced *de novo* by the site specific transsulfuration pathway (Finkelstein, 1990). Dietary amino acids are mainly absorbed in the apical membrane system and enter the plasma circulating as free amino acids. The metabolic cycling of Met is central to many important methylation reactions in the body. Met and Cys are metabolically linked via the transsulfuration pathway, allowing Met to serve as a source for Cys synthesis.

Much of the interest in sulfur amino acid metabolism in the past decade has been focussed on the possible role of homocysteine (Hcy) in various cardiovascular and neurological diseases (Janosikova et al., 2003, Graham et al., 1997). However, more recently Cys, has been implicated in cellular redox function and as a possible indicator of oxidative damage (El-Khairy et al., 2001, El-Khairy et al., 1999). Cys has pleotropic roles in the body. Most importantly, the sulfur atom in Cys *in vivo* has an ability to occur in ten different oxidation states. These different oxidation states perhaps helps in a range of cysteine modifications in peptides and proteins (Giles et al., 2003).

Transmethylation provides a pathway for methionine metabolism beginning with its activation of S-adenosylmethionine (SAM) (Figure 1.3). SAM plays a vital role in Met metabolism. Met is converted to SAM in presence of methionine adenosyl transferases (MAT) and is ATP dependent. In mammals, SAM serves as a source a primary methyl donor for various biological methylations in the body (Williams and Schalinske, 2007). The utility of SAM as a methyl donor lies in its active high-energy sulfonium ion and the electrophilic nature of its carbon atoms adjacent to sulfur atoms (Brosnan and Brosnan, 2006). SAM dependent methylations are essential in various biosynthetic cellular components like phospholipids, proteins, DNA, RNA, creatine, carnitine (Stipanuk, 2004). SAM is converted to S-adenosylhomocysteine (SAH) by methyltransferases. SAH is hydrolyzed to homocysteine upon losing adenosine by SAH hydrolase. These sequences of reactions constitute the transmethylation and are ubiquitously present in all the cells. Hcy either undergoes remethylation or is metabolised irreversibly to form Cys (Figure 1.3).

Thus, transmethylation claims Hcy back to methionine cycle, which occurs through two reactions, both of which are equally important in converting Hcy to Met (Finkelstein, 1990). In the first reaction, a methyl group is transferred from betaine (oxidation product of choline) to Hcy in the presence of BHMT independent of folate, to form Met and dimethylglycine (DMG). The second reaction uses folate and the methionine synthase to transfer the methyl group from 5-methyltetrahydrofolate (5-MTHF) to vitamin B12 to form methylcobalamin. The methylcobalamin in turn transfers the methyl group to Hcy to produce Met (Kharbanda, 2007). A deficiency in these vitamins (Vitamin B12, folic acid, riboflavin and pyridoxine) is associated with elevated plasma Hcy levels. Thus, Hcy functions as a co-substrate for two methyltransferase reactions.

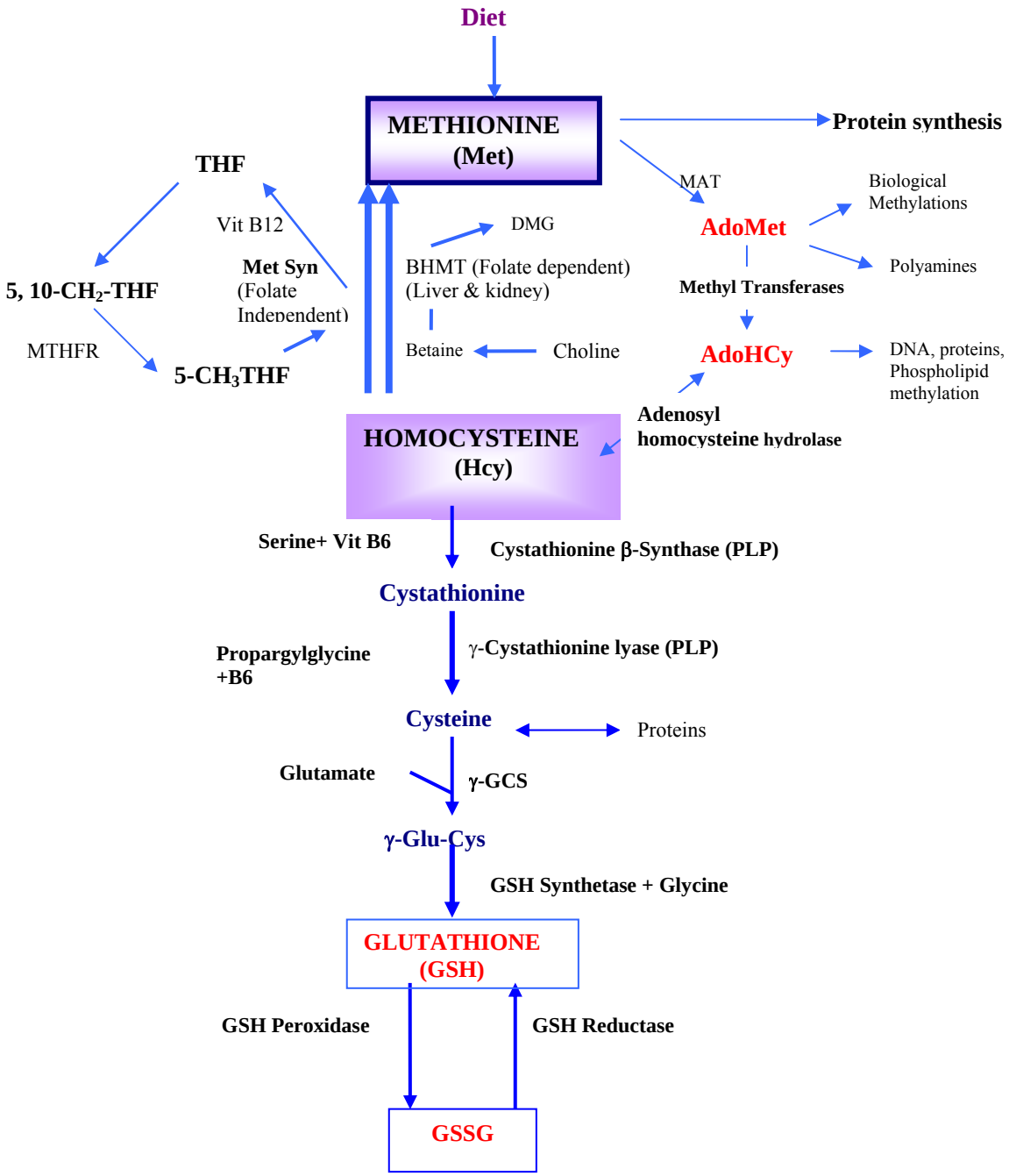


Figure 1.3. The transmethylation and transsulfuration pathways.

Met Syn - Methionine synthase, THF - Tetrahydrofolate, 5-CH_3-THF–5-Methyl tetrahydrofolate, CH_2-THF- Methylene tetrahydrofolate reductase, MTHFR-methylene tetrahydrofolate reductase, CBS-Cystathionine β-synthase, MAT- methionine adenosyl transferases, DMG-dimethyl glycine, BHMT- Beatine homocysteine methyltransfereases.

(Modified from Kenyon *et al.* (2002) and Mosharov *et al* (2000).

Hcy metabolism via remethylation versus transsulfuration seems to be highly coordinated in response to cellular SAM concentration or the need to generate methyl methionine groups (Selhub and Miller, 1992). For instance, excess dietary methionine intake in the liver, increases SAM concentration facilitating transsulfuration while limiting homocysteine remethylation. On the other hand, lower concentrations of SAM enhance methionine conservation. Thus, SAM facilitates a balance in allowing homocysteine to undergo either remethylation or transsulfuration. In order to perform this function, SAM is both an allosteric inhibitor of MTHFR and allosteric activator of cystathionine β-synthetase (CBS). Hence, low cellular concentrations of SAM, induces the synthesis of MTHF suppressing cystathionine synthesis resulting in the conservation of homocysteine for methionine synthesis. Conversely, higher SAM levels, diverts homocysteine through transsulfuration as cystathionine synthesis is stimulated following an inhibition of MTHF. This metabolic junction i.e. formation of Hcy, structures a delicate balance in the cells for providing SAM for methyl transfers and glutathione for redox buffering in the body (Martinov et al., 2000).

1.2.1. Transsulfuration Pathway

The transsulfuration pathway constitutes a major route for Cys biosynthesis and thus is central in controlling the intracellular redox milieu via GSH. A depletion in Cys not only alters GSH homeostasis but also inhibits protein synthesis (Jones et al., 2004). The major role of the transsulfuration pathway is to convert Met to Cys via Hcy (Figure 1.3). In other words, the sulfur group in Met is donated for GSH synthesis thus, essentially maintaining the sulfur balance. The body equilibrium favours SAH synthesis to keep the transmethylation pathway in motion, however, CBS competes with methyltransferases for Hcy to keep the transsulfuration in motion. Thus, the enzymes at this metabolic nexus compete to salvage Hcy and are tightly regulated to meet cellular needs. This

tightly regulated pathway is hence, subjected and is sensitive to redox changes (oxidative stress) in the body implying that transsulfuration is independent of methylation / remethylation status when the body has an increased need for Cys to promote glutathione synthesis (Zou and Banerjee, 2005). Thus, the transsulfuration pathway can be streamlined as an auto-corrective response of the cell that lead to an increased level of GSH synthesis challenged by oxidative stress.

Under oxidizing conditions, methionine synthase activity is diminished due to oxidative liability of its co-factor, cobalamin (Chen et al., 1995). Under these conditions SAM exerts a reciprocal regulation to activate CBS (Finkelstein, 1975, Taoka et al., 1998), decreasing the activity of methylenetetrahydrofolate reductase (MTHFR), thus depressing transmethylation. CBS, on the other hand, exerts a twofold increase in its activity under oxidizing conditions to flux Hcy through transsulfuration pathway to compromise with diminishing glutathione pool. Thus, CBS forms a highly regulated enzyme in humans. It was suggested that the heme-binding terminal in the mammalian CBS functions as redox sensor (Taoka et al., 2002, Meier et al., 2001). A reduction of the heme moiety was associated with decreased enzyme activity while an oxidation increased the flux of the enzyme (Zou and Banerjee, 2003). This juncture poses a critical indication in the cells, to preferentially increase transsulfuration and to generate more Cys for GSH synthesis during oxidative stress.

The hypothesis that CBS is activated under oxidizing conditions was confirmed from the studies conducted using cultured cells. Cells treated with H_2O_2 elicited a time and dose-dependent increase in transsulfuration flux (Masharov, 2000) followed by an increased consumption of radio-labelled Met into GSH. This posits that redox regulation is important in modulating the Hcy flux to autocorrect the function during

oxidative stress. Thus, Hcy forms a key junction metabolite in both the transmethylation and transsulfuration pathways.

Studies have shown that 50% of GSH is formed from Cys that is derived from Hcy in the human liver cells through the transsulfuration pathways. However, the transsulfuration pathway occurs completely only in the liver, kidney, pancreas and small intestine as they exhibit both CBS and cystathionine γ-lyase activity. Tissues that do not express the transsulfuration pathway depend on dietary Cys or on the transsulfuration pathway products of other tissues for GSH synthesis. Other catabolic products of the transsulfuration pathway include taurine and Coenzyme A (Vitvitsky et al., 2003, Mosharov et al., 2000)

During the transsulfuration cycle, Hcy undergoes a condensation reaction with serine in presence of heme-dependent CBS and vitamin B6 (pyridoxine) forming cystathionine. The formation of cystathionine irreversibly marks the end of transmethylation pathway. Cystathionine forms Cys and α-keto butyrate in presence of cystathionine-γ-lyase. Cys either formed from transsulfuration or supplied from diet serves as a precursor for protein synthesis and several other essential molecules like GSH, taurine, coenzyme A and inorganic sulfur. While high plasma concentrations of Cys can prove toxic to cells, it however remains a limiting amino acid for cellular glutathione pools (Vitvitsky et al., 2003).

1.3. Glutathione

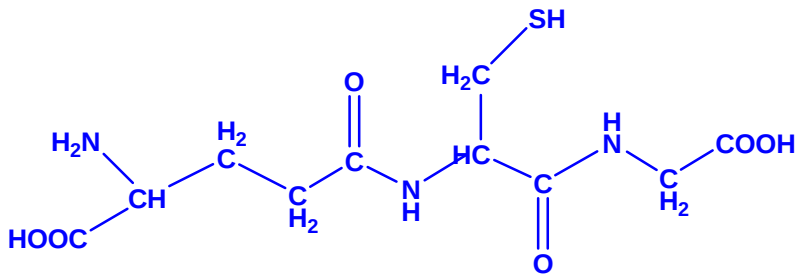
After its rediscovery in 1921, GSH was extensively studied only because of its ubiquitous distribution (Colowick et al., 1954, Hopkins, 1921). The reactivity of the sulfhydryl group was considered less important. However, the uniqueness of glutathione along with its sulfhydryl group was not known until 1969 (Kosower and Kosower,

1969). Since then GSH is considered an inevitable non-enzymatic scavenger (Sies, 1978).

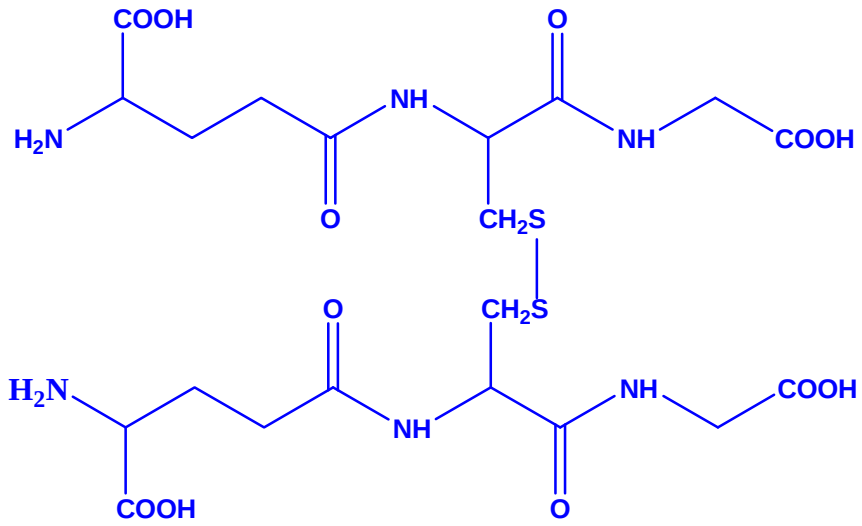
GSH is a tripeptide composed of Cys, glutamic acid and glycine (Figure 1.4). It exists in reduced (GSH) and oxidized form (GSSG). The two characteristic structural chemistry that make GSH potent are the γ-glutamyl linkage between glutamyl acid and Cys that prevents its degradation by proteases and the –SH group. The peptide bond between the amino group of Cys and the carboxyl group of glutamate renders GSH resistant to most of the peptidases except γ-glutamyl transpeptidase (γ-GT).

The reactive thiol group (-SH) of Cys is maintained in reduced state as it has a facile electron-donating capacity. The electronic structure of the sulfur atom in GSH permits an overlap during the formation of transition states and dissipation of electrons transferred from radicals, under lays the reactivity of thiol group towards nucleophilic addition and redox reactions. The ability of sulfur atom to donate electrons to other compounds makes GSH a well-known reductant (Josephy, 2006).

Further, this structural chemistry plays an important role in cellular stability and is intimately associated with glutathione functions. This major low molecular weight (307 kDalton) non-protein thiol is ubiquitously found in all living cells but abundantly found in cells/tissues vulnerable to oxidative stress (Meister, 1995b). GSH is found mainly in the cell cytoplasm (80-90%) and in the organelles that include lens of the eye, mitochondria, liver, peroxisomes, nuclear matrix.



(A)



(B)

Figure1.4. (A) Structure of glutathione (GSH) and (B) glutathione disulfide (GSSG).

1.3.1. Glutathione biosynthesis and γ-glutamyl cycle

GSH biosynthesis is closely associated with Met metabolism via the transsulfuration pathway (Figure 1.3). GSH synthesis from its constituent amino acids occurs intracellularly in two closely linked steps, which are ATP dependent and enzymatically controlled (Anderson, 1997, Lomaestro and Malone, 1995). The first step involves the formation of γ-glutamyl cysteine. The amino group in Cys and γ-carboxyl group in glutamate combine in the presence of the enzyme called glutamate cysteine ligase (GCL). This catalytic mechanism proceeds by phosphorylation of γ-carboxylate of

glutamate by ATP. The nucleophilic amino group in Cys attacks the γ-glutamyl phosphate intermediate forming γ-glutamyl cysteine (See reaction 1),

L-glutamate + *L*-Cys + ATP → γ-glutamyl cysteine + ADP + Pi (1)

The enzyme GCL is the rate limiting, inhibited by GSH following a regulation in response to oxidative stress suggesting a significant feedback mechanism physiologically (Richman and Meister, 1975). The enzyme is specific for the γ-glutamyl moiety and is regulated in two steps; a) feedback competitive inhibition of the γ-glutamate binding site by GSH (Richman and Meister, 1975) and b) availability of Cys (Vina et al., 1978).

The enzyme GCL is heterodimeric in mammals with catalytic (GCLC, 72.8 kDa) and modifier (GCLM, 30.8 kDa) subunits. It has been reported that the association of both of the subunits is necessary for GSH biosynthesis under normal physiological conditions of glutamate and GSH (Griffith, 1999). However, it was cited that over expression of GCLM increases cellular GSH by 2 fold, rendering cells resistant to oxidative stress (Tipnis et al., 1999). Contrarily, a down-regulation of this subunit decreased GSH levels in cultured pancreatic islet cells (Kijima et al., 1998).

Acute depletion of GSH can lead to a short-term increase in GSH synthesis. This is because of a transient increased activity in pre-existing GCL by decreasing the feedback inhibition by GSH which results in short-term increase in GSH synthesis (Richman and Meister, 1975). The enzyme is inhibited by S-alkyl moiety buthonine sulfoxamine (BSO) that binds to the active site of the enzyme that accepts Cys. The formation of glutathione conjugates and ROS have been shown to induce increased transcription of the GCL leading to increased glutathione biosynthesis (Wild and Mulcahy, 2000).

The second step in the GSH biosynthetic pathway involves the coupling of γ-glutamylcysteine with glycine by the catalytic action of glutathione synthetase. The transfer of the γ-phosphate of ATP to the carboxylate of γ-glutamylcysteine generates an acylphosphate intermediate. The α-amino group of the glycine then attacks the phosphorylated intermediate, displacing the inorganic phosphate to form GSH. Human glutathione synthetase is a homodimer. Glutathione synthetase rapidly converts γ-glutamyl cysteine to GSH (See reaction 2) *in vivo* and its activity is several fold higher than GCL in liver. However, in other tissues, like the skeletal muscles, the activity is comparable to GCL, suggesting that glutathione synthetase activity may affect the intracellular GSH levels. There is a notion that the over expression of GSH synthetase fails to increase GSH levels, while an induction of GCL increases GSH levels (Mulcahy et al., 1995).

γ-glutamylcysteine + *L*-glycine + ATP → GSH + ADP + Pi (2)

GSH undergoes metabolism via the γ-glutamyl cycle (Figure 1.5). This cycle was proposed by Alton Meister and co-workers, describes six major reactions that constitute the γ-glutamyl cycle accounting for functional metabolism and reclaim of GSH. The breakdown of GSH is catalysed by an ectoenzyme, γ-GT, an abundant enzyme found in major fractions in the external surface of the cell membrane. The enzyme distribution in the organs and tissues is similar in several species, highest in kidney and low in adult liver (Orlowski and Meister, 1965). This is the first enzyme in the salvage pathway of GSH. γ-GT is synthesized in liver as an inactive 60-kDa polypeptide. Cleavage of this proenzyme yields an active heterodimer comprising a 40-kDa and a 20-kDa subunit.

The concentration of GSH is highest in liver followed by kidney, small intestine and pancreas (Githens, 1991). Regulation of GSH levels within organs is looked in terms of

entire organism, since some organs are net synthesizers of GSH while others are net exporters. The liver is both a synthesizer and exporter of GSH. The liver exports large quantities of GSH into the blood. The blood plasma contains 20-30 μM GSH with high turnover rate (Meister and Anderson, 1983). The kidneys either take up GSH from plasma via the metabolism of GSH by γ-GT reaction or direct up take of intact GSH into kidney cells (Anderson et al., 1980, Griffith and Meister, 1979).

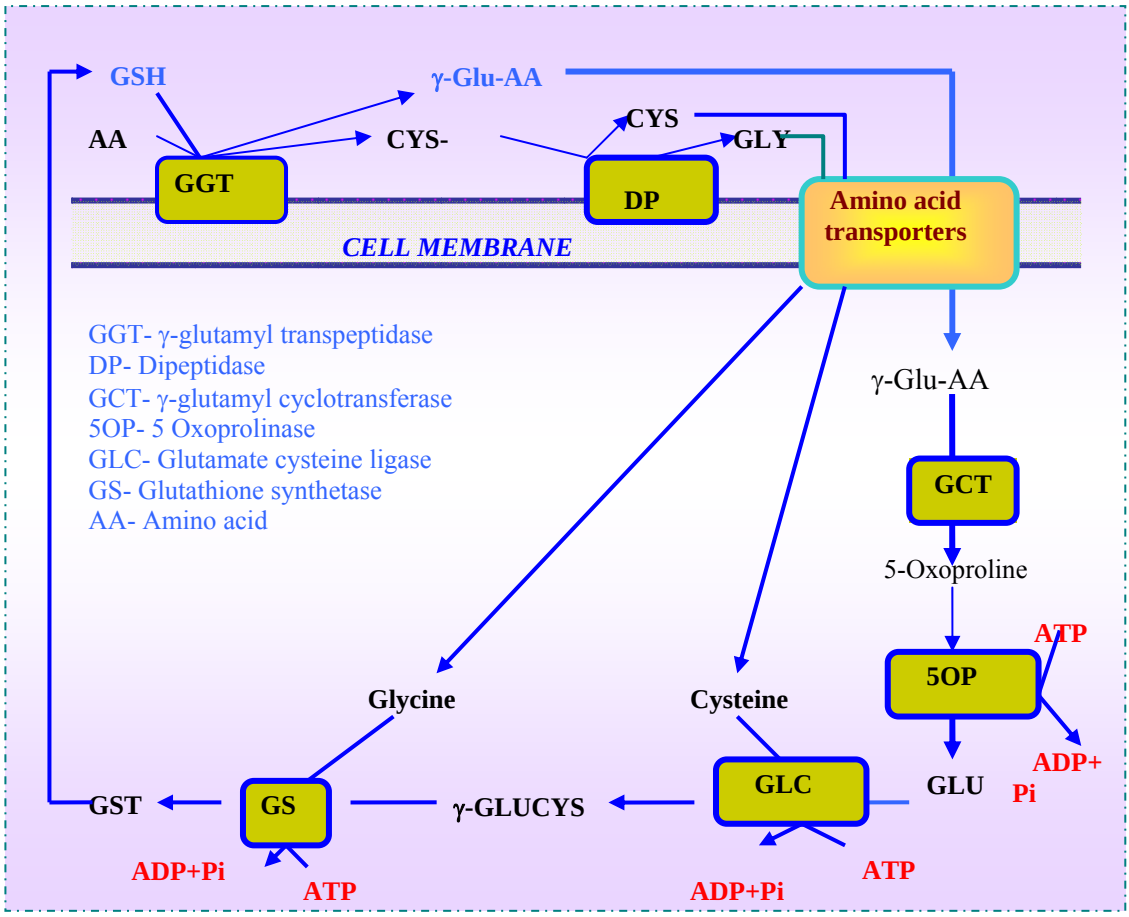


Figure 1.5. The γ-glutamyl cycle.

(Adapted from: Redox Biochemistry, Banerjee R, 2007, p18)

Organs with high γ-GT activity can utilize plasma GSH. On the other hand, liver and other organs with low γ-GT activity are net exporters of GSH into the plasma (Meister and Anderson, 1983). Hence, liver GSH depletion is followed by GSH depletion in

other tissues. The plasma and erythrocyte concentration are thought to reflect the synthetic capacity of the liver. Intracellular GSH levels in cells that do not export GSH directly, regulates GSH by controlling the levels of γ-GCS. A study *in vitro* has indicated that a high concentration of GSH itself inhibits the enzyme. Although, high levels of GSH inhibit γ-GCS, glutamic acid however, competes with GSH for binding at the regulatory site of the enzyme (Richman and Meister, 1975). Thus, GSH regulates its own synthesis (Meister, 1995a).

γ-GT cleaves the γ-glutamyl amide bond in GSH. The ectoenzyme γ-GT in ATP dependent manner cleaves extracellularly the peptide linkage between glutamate and Cys transferring the glutamyl residue to cystine, which is a disulphide form of Cys generating cysteinyl-glycine (Cys-Gly). Extracellular dipeptidase cleaves Cys-Gly to yield Cys and glycine. Dipeptidase is involved in both γ-glutamyl cycle and in detoxification process in normal physiology. These products Cys and glycine re-enter the cells through specific amino acid transporters. This transport of Cys is crucial as Cys is a limiting amino acid in the *de novo* GSH synthesis (Dickinson and Forman, 2002). The carrier amino acid is often cystine facilitating the re-entry of γ-glutamyl-amino acid into the cell, thereby recycling Cys. 5-oxoproline is formed as a residue of γ-glutamyl-amino acid by 5-oxoprolinases, which yields glutamate. Thus entire cycle assist in the synthesis and recycle of GSH (Griffith et al., 1978).

The γ-glutamyl cycle thus, provides all the properties that fulfil the requirements for the amino acid transport system. The fact that γ- glutamyl amino acids are formed in or on the cell membrane by the interaction of γ-GT, forms the corner stone for the hypothesis that γ-GT acts as a carrier for amino acids transport where they are subsequently released into the intracellular milieu by the action of γ-glutamyl cyclotransferases (Orlowski and Meister, 1970).

1.3.2. Hepatic glutathione transport

Liver has the greatest content of GSH and maintenance of hepato-cellular GSH is a dynamic process. A steady state hepatic cellular GSH concentration is achieved by a balance between: 1. The rate of synthesis catalysed by γ-GCS and GSH synthetase and its utilization through GSH peroxidises and alkylating reactions by GST's. 2. The rate at which it is exported from the hepatocytes (Figure 1.6). Hepatic GSH synthesis is largely dependent or limited by the availability of Cys (Betty and Reed, 1980). A complete transsulfuration cycle taking place in liver contributes towards Cys formation, which is otherwise derived from diet or protein breakdown. Thus, Cys is rapidly incorporated into GSH. Hepatic GSH is thought to play a central role in inter-organ GSH homeostasis by providing Cys to extra-hepatic tissues. Following sinusoidal efflux from liver into the blood stream, GSH is hydrolyzed at tissue sites by two membrane-bound enzymes: γ-GT and dipeptidase. Hydrolysis of GSH results in the liberation of Cys, which is then taken up into the cells to synthesize GSH and/or proteins. Liver releases GSH in both reduced and oxidized forms. The hepatocytes have distinct plasma membrane domains for sinusoidal and canlicular transport. Sinusoidal GSH release to the plasma is by carrier-mediated transport. This was supported by studies on isolated hepatocytes, which have demonstrated that Met selectively inhibits GSH efflux, suggesting that Met shares the same carrier (Aw et al., 1984). Thus, Met maintains hepatic GSH by inhibiting its efflux and serving as a Cys precursor.

On the other hand, canalicular GSH transport occurs by specific carrier mediated transport (Ballatori and Truong, 1992). This efflux, studied in the perfused liver model, was exclusively GSSG (Kaplowitz et al., 1985). Studies on rat bile have indicated high GSSG levels. In order resolve this apparent contradiction, Abbot *et al*, detected 1-2 mM GSH and low transpeptidase activity in pure bile (Abbott, 1983). This canalicular GSH

efflux across the canalicular plasma membrane into bile significantly contributes to bile formation (Ballatori and Truong, 1992, Grattagliano et al., 2005). A significant proportion of this GSH in bile is degraded by γ-GT in the biliary epithelial brush border. Hepatic GSH also plays a central role in inter-organ GSH homeostasis by supplying plasma GSH through sinusoidal efflux (Ookhtens and Kaplowitz, 1998). A decrease in GSH has been found in association with a variety of oxidative stress-mediated liver diseases such in various cholestatic hepato-biliary diseases.

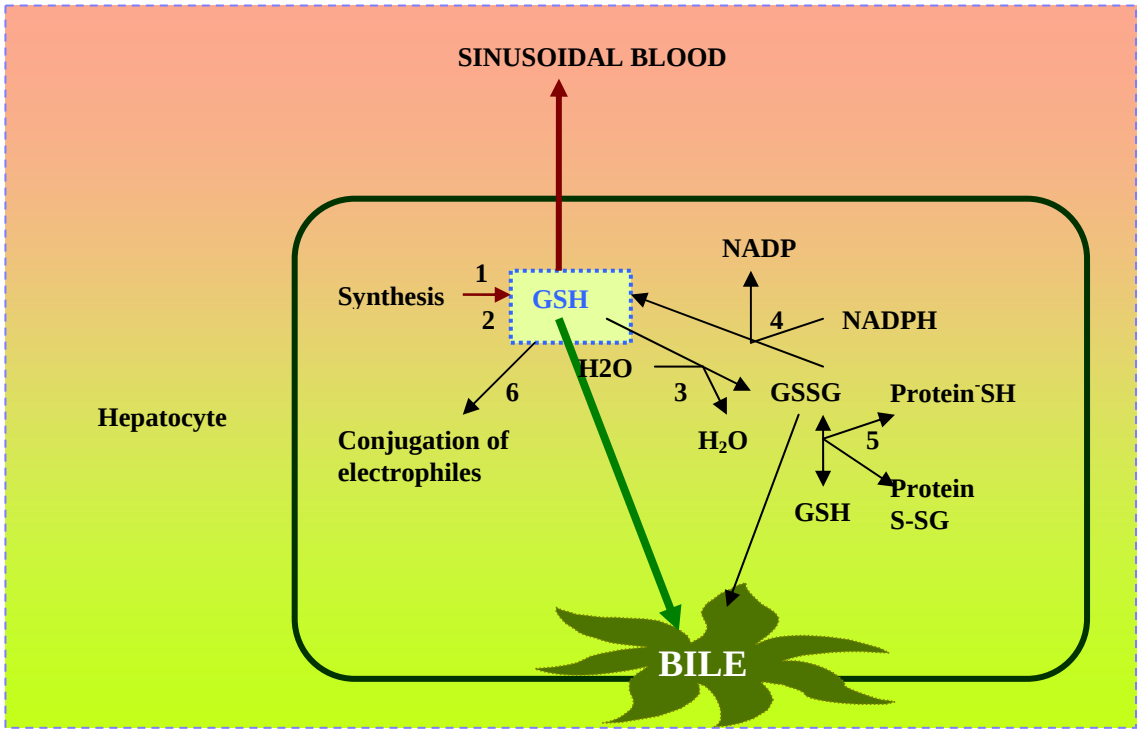


Figure 1.6. Regulation of hepatic glutathione.

Reactions: 1. γ-glutamyl-Cys synthetase, 2. GSH synthetase, 3. GSH peroxidises, 4. GSSG reductase, 5. Thioltransferases, 6. Glutathione -S-transferases. (Adopted from (Kaplowitz et al., 1985).

1.3.3. Role of glutathione in health and disease

GSH has a critical role in the maintenance of reduced environment by maintaining a balanced cellular redox potential (Townsend et al., 2003). It has numerous roles in the both physiological and biochemical pathways in the body (Figure 1.7). It participates in many biological reactions related to cellular function, protection against oxidative damage, detoxification of endogenous and exogenous compounds, storage and transport of Cys, regulation of membrane transporters, as well as cell growth and DNA synthesis (Manciu et al., 2003, Meister, 1995a, Sies, 1999).

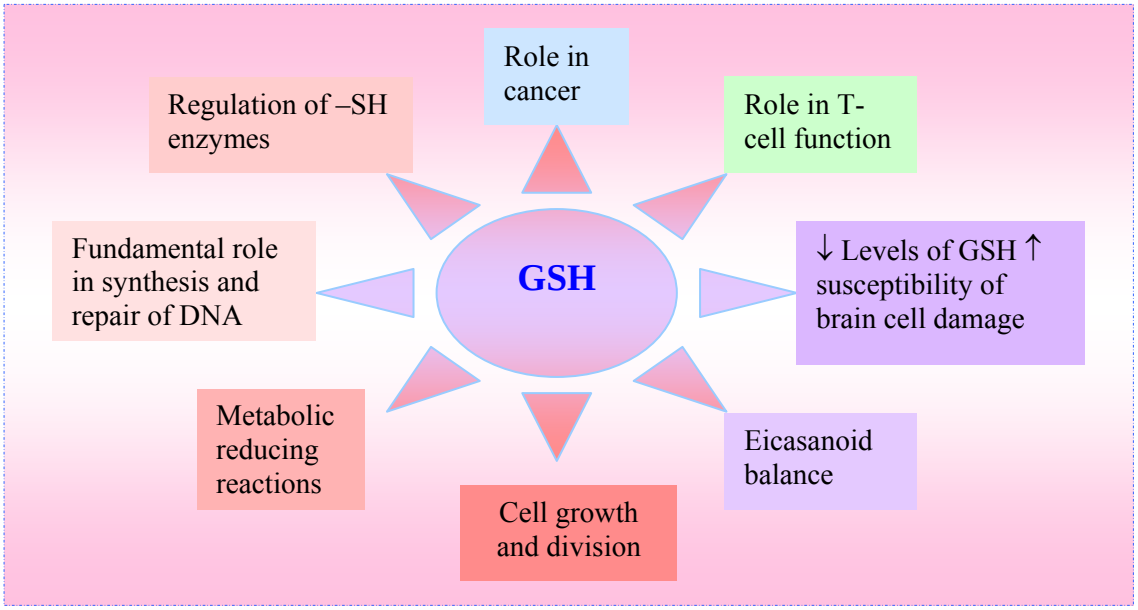


Figure 1.7. Role of glutathione in the body and in health and disease.

1.3.4. Redox regulation by glutathione

Redox phenomena have a profound role in life processess. GSH is one of the major pro-homeostatic modulator of intracellular –SH groups on proteins (Hidalgo et al., 1990, Ondarza, 1989). Enzymes involved in glucose metabolism are regulated by redox balance largely defined by the –SH groups (Ondarza, 1989). The –SH group in GSH

sets the redox stage for metallothioneins which are able to bind to heavy metals and subsequently are removed from the body (Hidalgo et al., 1990). Thus, the homeostasis of various cellular environments is finely tuned by the reducing power of GSH.

A redox status of a cell describes the ratio of inter-convertable reduced/oxidized forms of a molecule. The thiol group of GSH is oxidized to form a disulfide;

$$2GSH \longrightarrow GSSG + 2H^{+} + 2e^{-}$$

The 2GSH/GSSG is considered to be the major thiol-disulphide redox couple of the cell (Gilbert, 1990). The 2GSH/GSSG ratio is tightly regulated and strongly is the favour of GSH. In the endoplasmic reticulum, where, GSH is implicated in protein disulphide bond formation, the GSH/GSSG ratio is 3:1 (Hwang et al., 1992). Over 99% of cellular GSH in the mitochondria and cytosol however, is maintained in the reduced state (GSH), essentially though GSSG reductase (GR) (Meredith and Reed, 1982). These enzymes belong to a group of a flavoprotein disulfide oxidoreductase family. This reaction is fast and irreversible providing cells with high GSH/GSSG ratio. GR is found to have high affinity for their substrates (GSSG) and other mixed disulfides. This enzyme uses NADPH to convert GSSG to GSH to replenish the GSH levels under cellular stress conditions.

$$GSSG + H^{+} + NADPH \longrightarrow 2GSH + NADP^{+}$$

As GSH potently modulates cell signalling in an oxidation-dependent fashion, it is crucial for the cells to have efficient ways to replenish reduced levels. Impaired GR activity and/ or excessive GSSG accumulation may react with protein sulfhydryl via a mixed disulfide reaction, thereby impairing protein function (DeLucia et al., 1975). Alternatively, if GSSG is not reduced to GSH, the former is removed from the cell as it can readily permeate through the cell lipid membrane (Bannai, 1984).

The reaction mechanism of GR involves, i) receiving two electrons from NADPH and is tightly bound to FAD, ii) transfer the reducing equivalents to the disulfide formed by the two Cys residues in the active site of the same subunit, iii) the electrons are transferred to GSSG and interact with the reduced Cys residues, iv) finally the enzyme releases two GSH molecules (Argyrou and Blanchard, 2004). A steady state production of NADPH depends on the supply of glucose and its flux through the pentose phosphate pathway, mitochondrial production and shuttle within the two pathways. Changes in the pentose phosphate activity can affect NADPH supply and consequently GSH/GSSG status. Thus, the ratio of GSH/GSSG is tied with NADPH levels, which is in turn determined by the energy status of the cell (Figure 1.8).

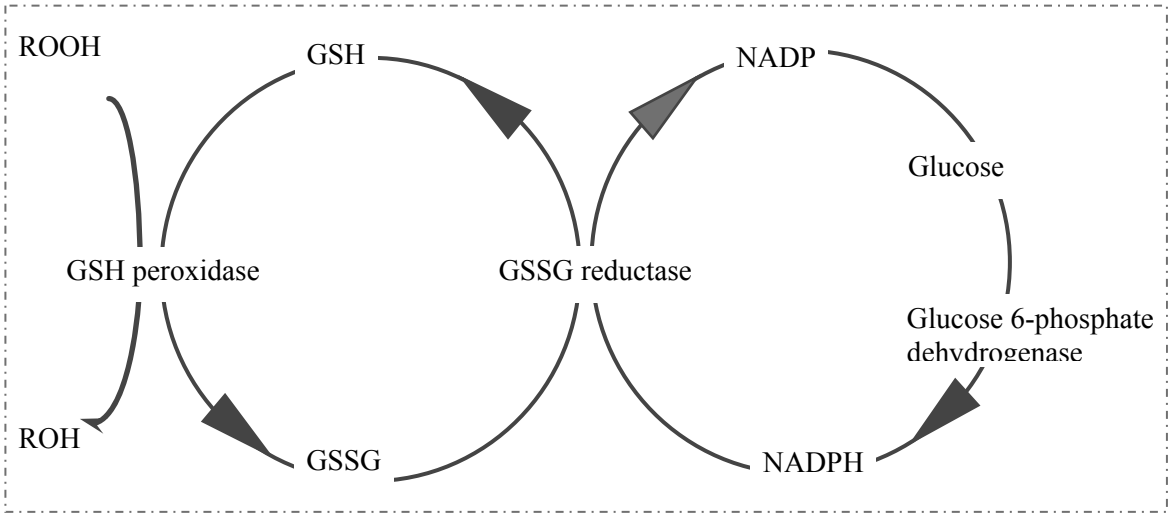


Figure 1.8. Association GSH: GSSG ratio with NADPH levels in pentose phosphate pathway.

During an oxidative insult, the GSH/GSSG ratio shifts the redox state resulting in a positive potential (Filomeni et al., 2003, Schafer and Buettner, 2001). The export of GSSG under these circumstances prevents positive potential increase thereby protecting the sensitivity of the cells towards disturbed equilibrium. This exhibits a protective mechanism for the cells and tissues from oxidative stress. Mechanisms that alter GSH levels and the GSH/GSSG ratio also alter the cellular redox state. Cellular redox state

can be altered by an increase in ROS (RNS) generation as seen during inflammation/ metabolism of drugs/ xenobiotics that decreases the GSH/GSSG ratio. Higher threshold levels of drugs and xenobiotic compounds can lead to extreme depletion in the GSH levels thus, drastically shifting the cellular redox status. Therefore, it is critical for the cells to have efficient ways to replenish this loss in GSH levels and maintain the GSH/GSSG ratio.

1.3.5. Antioxidant function of GSH

One of the major functions of GSH involves its antioxidant properties. Reduced GSH is capable of directly scavenging radicals and peroxides by being oxidized to either GSSG or to a mixed disulfide (Sies, 1999). GSH can also reduce H_2O_2 and lipid peroxide in the presence of GSH peroxidise (GPx) (Zhang et al., 1989). GSSG is then converted back to GSH in presence of GR and NADPH to complete the cycle.

$$2GSH + H_2O_2 \xrightarrow{GPx} GSSG + 2H_2O$$

$$GSSG + NADPH + H^+ \xrightarrow{GR} 2GSH + NADP^+$$

The GPx are a class of selenocysteine containing proteins. In addition to scavenging free radicals, GSH is essential in maintaining the intracellular redox balance and the essential thiol status of proteins. To achieve this, GSH (or GSSG) undergoes thiol-disulfide exchange in a reversible reaction catalyzed by thiol-transferases. Meanwhile, GSH reduces (or GSSG oxidizes) the sulfhydryl groups of the proteins. Whereas many proteins are active when the key sulfhydryl are in the thiol form, others require them to be in the oxidized disulfide form (Cotgreave and Gerdes, 1998). The equilibrium is determined by the redox state of the cell, which depends on the concentrations of GSH and GSSG.

GSH is also considered as the storage and transfer form of Cys. Circulating GSH is stable in that it reacts very slowly with oxygen. Cys, on the other hand, is unstable extracellularly and rapidly auto-oxidized to cystine, producing potentially toxic oxygen free radicals (Olney et al., 1990). To avoid the toxicity of auto-oxidation, most of the nonprotein Cys is stored as GSH. After GSH is exported from the cell, it can be quickly converted to Cys through the γ-glutamyl cycle. The resulting Cys is then used elsewhere for protein synthesis and for the biosynthesis of taurine and other sulfur metabolites. The liver, which provides more than 80% of plasma GSH, plays a major role in the homeostasis of GSH and Cys .

1.3.6. Role of glutathione in detoxification and mercapturic acid pathway

GSH also plays a major role in detoxifying many reactive metabolites by either spontaneous conjugation or by a reaction catalyzed by the glutathione S-transferases (GST). Compounds with electrophilic centres readily conjugate with GSH, catalyzed by GST (Ouwerkerk-Mahadevan et al., 1997). GST's are a group of antioxidant enzymes that play a critical role in providing protection against the electrophiles and metabolities of oxidative stress. In mammals, GST's play a vital role in the detoxification of harmful electrophilic compounds of both endogenous and exogenous origin. Liver receives toxins absorbed by the intestine through the portal vein. Many of these toxins undergo oxidation by adding a functional group that is attacked by GST's. This is followed by a sequential cleavage of residues of GSH (glutamic acid and glycine moieties) and acetylate the amino group of the remaining Cys moiety prior its export.

Kidney forms the primary site for the conversion of GSH conjugates to Cys conjugates. In the kidneys, degradation by peptidase to Cys conjugates occurs and is eliminated in the urine. This conversion of GSH-conjugates to Cys conjugates constitutes the

mercapturic acid pathway. GSH conjugates form Cys-Gly S-conjugates catalyzed by γ-GT and Cys-S-conjugates catalyzed by dipeptidase (Commandeur et al., 1995). The resulting Cys-S-conjugates are acetylated at the amino position of Cys in kidney or transported to the liver via Cys-S-conjugate N-acetyltransferases to form N-acetylcysteine or mercapturic acid metabolites (Figure 1.9).

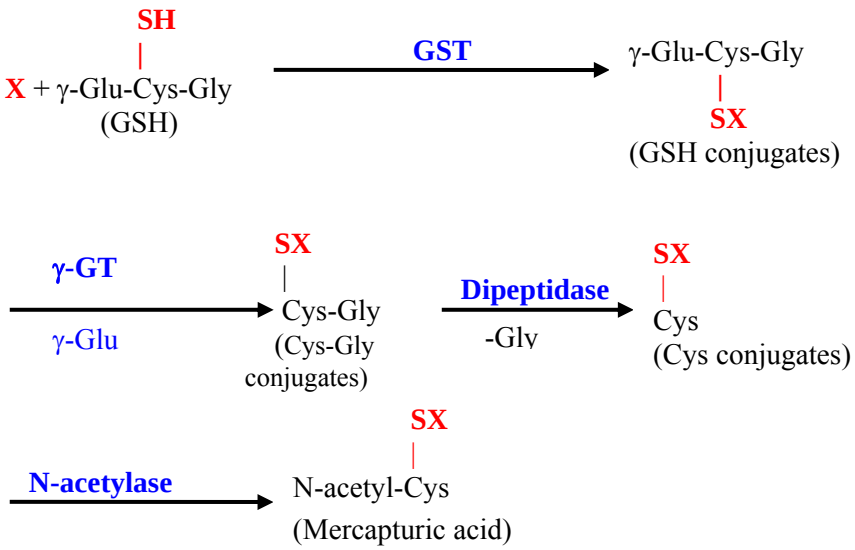


Figure 1.9. Mercapturic acid pathway. (**X**=Electrophilic compounds react with GSH to yield conjugates and it can be xenobiotics, leukotrienes, steroids, prostaglandins). (Adopted from (Lu, 1999).

1.3.7. Genetic variation in human glutathione –S-transferases

There are four main groups of human cytosolic GST's: GSTAlpha (A), GSTMu (M), GSTPi (P) and GSTTheta (T) and these groups have also been described in human pancreatic tissues (Rahman et al., 2004) and are up-regulated during oxidant stress. A substantial depletion in pancreatic GSH is elucidated in clinical and experimental models of pancreatic diseases (Deneke, 1989). GSH represents a critical substrate for

GST and GSH peroxidises. Depletion of GSH due to oxidative stress is more profound in the cells with higher GST activity (Rahman et al., 2005a, Kraus and Kloft, 1980) compared with those with low or no GST activity. It is reported that, GSTT-1 genotype may be a significant factor in the pathogenesis of AP and the possession of the functional GSTT-1*A genotype appears to be associated with a severe attack of AP (Rahman et al., 2005).

1.4. Pancreatitis

Pancreas is a retroperitoneal gland located on the posterior of the abdominal cavity functioning both as an exocrine and endocrine gland. The exocrine portion of the pancreas is mainly concerned with the secretion of digestive enzymes that are required for the digestion of lipids and proteins. A highly coordinated timed release (in volume and content) of the pancreatic juice from the acinar cells, to suit the body's digestive requirements, reflects a complex synergy of actions of both hormonal and neural pathways. The endocrine pancreas is composed of small clusters of cells embedded in the exocrine tissue-the highly vascularised islets of Langerhans cells. These islets of Langerhans cells are of two types – the alpha cells those that secret glucagon and the beta cells that produce insulin. Glucagon and insulin function simultaneously.

Diseases of the pancreas can irreversibly affect both the exocrine and endocrine secretions thereby leading to insufficient production of digestive enzymes thus impairing fat and protein absorption and bring an imbalance in the blood sugar level by affecting the production of insulin secreted from the endocrine pancreas. Some of the common pancreatic disorders are diabetes mellitus, malabsorption of lipid or proteins, pancreatitis and pancreatic tumours.

Pancreatitis is an inflammatory process of the pancreas associated with severe upper abdominal pain with nausea and vomiting. The initial stage of pancreatitis does not

affect digestion or the normal blood sugar levels. However, a single severe attack of pancreatitis can trigger series of events which can destroy the pancreatic acinar cells causing bleeding in the gland thereby leading to tissue damage, impaired enzyme secretion that could auto-digest the organ (Mergener and Baillie, 1998). Clinical judgement upon admitting patients with pancreatitis has often proved unreliable in predicting the severity of pancreatitis. In an attempt to predict morbidity and mortality, a number of prognostic scoring systems have been developed. This was first coined in 1974 by John HC Ranson, who introduced the concept of a prognostic scoring system for early identification of patients with severe pancreatitis (Ranson et al., 1974). Multifarious scoring systems have evolved since then, attempting to identify an "at risk" group that may potentially benefit from aggressive medical or surgical management (Karimgani et al., 1992).

According to UK recommendations, severity stratification should be made in all patients within 48 hours. It is recommended that all patients should be assessed by the Glasgow score and CRP (Anonymous, 1998). The APACHE-II score is equally accurate and may be used for initial assessment. A dynamic CT scan is recommended to be performed on all severe cases between three and ten days after admission.

1.4.1. Acute pancreatitis- a model of oxidative stress

Acute pancreatitis (AP) is defined as an acute inflammatory process of the pancreas, with variable involvement of other regional tissues or remote organs. AP initially starts with an unrecognised aetiology. A localized inflammatory process in the pancreas is capable of spreading or involving remote organ systems. The typical severe abdominal pain appearing suddenly and radiating back is associated with non-specific symptoms like nausea, vomiting and pyrexia. AP remains a disease with considerable morbidity and mortality, with up to a quarter of patients developing life-threatening complications.

In humans, AP leads to severe acinar damage, extensive interstital edema, hemorrhage and migration of neutrophils into the damaged pancreatic gland (Aho et al., 1982). There is increasing evidence to suggest that the local and systemic manifestations of AP are related to the activation of cellular oxidant generating pathways and oxidative stress (Altomare et al., 1996, Kikuchi et al., 1997, Schulz et al., 1999).

AP develops as an inflammatory disorder with a complex cascade of immunological events associated with it. The disease progression is viewed in three stages; with a local inflammation of the pancreas, following a generalized inflammatory response and eventually multiorgan dysfunction (Bhatia et al., 2001a, Bhatia, 2000, Bhatia, 2002). Despite this, the exact mechanism of development and pathogenesis of AP remains poorly understood, partly because the process is fully established when patients are present with AP.

AP can be either mild or severe:

Mild AP is defined as that associated with minimal organ dysfunction and an uneventful recovery, and it lacks the features of severe AP. Severe AP is defined as that associated with organ failure and/or local complications, such as abscess, pseudocyst or necrosis. However, in certain conditions of severe AP, a single attack may be followed by a complete recovery or a life threatening illness.

AP accounts for 3% of all cases of abdominal pain admitted to hospital in the UK (de, 1991). Epidemiological evidence suggest an increase in the incidence of AP reflecting increased consumption of alcohol among the younger generation (Wilson and Imrie, 1990). A majority of patients fall under mild attacks while 25% of patients of AP suffer severe attacks. At least about 30-50% of patients with severe attacks are known to die due to secondary infections and/ or organ failure (Bhatia et al., 2001a, Bhatia, 2002).

1.4.2. Current biochemical methods of diagnosis

Commonly employed methods of diagnosis include biochemical tests (serum amylase, lipase, and trypsinogen). AP is usually confirmed by assay of circulating higher than control (or healthy) levels of digestive enzymes (amylase, lipase, trypsinogen and C-reactive protein). The causes for AP can be major like a block in the biliary tract by gallstones leading to a mechanical obstruction or alcoholism, which accounts for ≥ 80% upon admission to the hospital. Minor causes like cystic fibrosis, pancreatic trauma, peptic ulcers, cardiopulmonary bypass producing an insult to the gland, carcinoma of pancreas and viral infections like mumps, hepatitis and certain bacterial infections like mycoplasma attribute for 20% of AP.

1.4.3. Oxidative stress in acute pancreatitis

Mounting evidence has accumulated that oxidative stress plays pivotal role in the pathophysiology of AP. Oxygen radicals mediate an important step in the initiation of AP (Schoenberg et al., 1995). However, there are no direct evidences so far that free radicals are the prime culprits for the onset of human AP as there has always been an inevitable delay in the patients arriving at the hospital. This delay has excluded the investigation of preliminary mechanisms of the initiation of acinar cell injuries during the onset of AP (Tsai et al., 2005).

Much of the evidence supporting the role of ROS in AP has largely been experimental, and supported by three lines of evidence: (1) heightened free radical production; (2) depletion of antioxidant systems; and (3) amelioration of pancreatic injury by free radical scavengers (Sanfey et al., 1985). These three evidences are germane to the present clinical setting. However, the difficulty in relating this work to the clinical condition is that much of the observed responses vary from one experimental model to

the next, although the caerulin model has been the most widely used (Dabrowski et al., 1999).

1.4.4. Evidence of heightened free radical production

The first line of evidence was adduced by measuring the products of lipid peroxidation in various animal models (Braganza, 1990, Schoenberg et al., 1995). Excessive accumulation of lipid peroxidation products was seen in caerulin induced-AP (Dabrowski et al., 1988). In the caerulin model, Dabrowski (1988) also showed that severe oxidative stress is associated with a depletion of SOD in the pancreas to 16% of normal activity. A number of reports suggest that lipid peroxidation parallels a reduction in pancreatic sulfhydryl compounds that include key antioxidant enzymes such as GPx (Dabrowski and Chwiecko, 1990), SOD and catalase (Nonaka et al., 1990). Direct evidence of oxygen free radical activity by chemiluminescence was demonstrated in caeruline and taurocholate induced AP model (Gough, 1990). Most of these studies provided unequivocal evidence that oxidant stress was involved at a very early stage in the evolution of experimental AP.

1.4.5. Depletion of antioxidant systems

Depletion of antioxidant system in pancreatitis was investigated in the cearulein model (Dabrowski et al., 1991, Luthen and Grendell, 1994). The study concluded that depletion of non-protein sulfhydryl (GSH) correlates with an increase in lipid peroxidation products and depletion of protein thiols. Although the depletion of total GSH was a consistent observation in caerulin and taurocholate models (Luthen and Niederau, 1995, Schoenberg et al., 1994), its relationship with the pathogenesis of AP has not been fully explored. Furthermore, the idea that GSH depletion is the initial step in the development of AP has not gone undisputed, because Fu *et al* (1997), could not induce AP in a caerulin rodent model through GSH depletion with diethylmalonate (Fu

et al., 1997). These observations suggested that oxidative stress was not solely responsible for initiating pancreatitis.

1.4.6. Amelioration of pancreatic injury by free radical scavengers

The third line of evidence of ameliorating pancreatic injury by free radical scavengers was demonstrated a decade ago in an *ex vivo* canine pancreas (Sanfey et al., 1985). The study involved initiation of AP by free radical infusion or by partial duct occlusion with secretin stimulation. Addition of allupurinol resulted in a decrease in interstitial oedema and hyperamylaseaemia by inhibiting the production of superoxide anion by inhibiting xanthine oxidase. In general, various results upon pre-treatment with antioxidant enzymes were reviewed many times (Schoenberg et al., 1995, Braganza, 1990, Sweiry and Mann, 1996). Treatment with glutathione monoethylester partially abrogates GSH depletion, acinar cell lesions and hyperamylaseaemia in a rodent model of caerulin AP, while Cys pro-drug l-2-oxothiazolidine-4-carboxylate produced variable results (Luthen et al., 1997). The pro-drug was cleaved by 5-oxoprolinase in an ATP-dependent reaction to form L-Cys and subsequently increases GSH levels.

Table 1.2 suggests ways in which GSH may be depleted in pancreas.

Table 1.2. List of possible etiologies of GSH depletion in the pancreas.

Source of depletion	Etiology	Mechanism
Decreased synthesis		
SAA deficiency	Poor diet	Lack of substrate (Cys)
Protein deficiency	Poor diet	Lack of substrate (Cys)
GGT inhibition	AT-125	↓ transport of precursor
Enhanced oxidation		
Oxidant stress (↑$O^{\bullet}{}_2^-$, ↑H_2O_2)	Hypoxia	Mitochondrial dysfunction, XO
↓ GSH peroxidise activity	Selenium deficiency	Activation producing (↑$O^{\bullet-}{}_2$, ↑H_2O_2)
Vitamin C deficiency	Poor diet	Generation of free radicals via ↑H_2O_2
Vitamin E deficiency	Poor diet	↑ cytosolic free radicals
	↑ unsaturated fat high-fat diets	↑ membrane free radicals
	Hypoxia	↑ lipid peroxides, CYP induction
		↑H_2O_2 generating membrane free radicals
↑ lipid peroxidation	Dietary and environmental, xenobiotic, drugs	Direct injury to membrane by toxins or metabolites
	Biliary reflux	Lipid peroxidation products of bile, surfactants
	Inflammation	Generation of $O^{\bullet}{}_2^-$, H_2O_2 by leucocytes
Increased consumption		
Oxidant stress	Hypoxia	↑ efflux of excess GSSG from cell
GSH conjugation	↑ xenobiotic exposure	Binding of GSH to metabolite and efflux of conjugate from cell

Adopted from (Wallig, 1998).

1.5. Bile acids

Bile, an organic fluid, is largely a heterogeneous group of water-soluble compounds characterized by steroid scaffolding with carboxylic group located in the side chains. Bile is essentially a mixture of catabolic products of cholesterol. Newly synthesized bile acids from the liver are secreted into the lumen of the small intestine where they act as emulsifiers of cholesterol, dietary lipids and fat-soluble vitamins. From the intestine, the bile acids are transported back to the liver via the portal circulation and resecreted into the bile . About 95% of bile acids are recovered in the gut during the enterohepatic circulation and about 5% loss of bile acids is replaced by the synthesis in the liver. Bile acid synthesis is tightly regulated ensuring that there is catabolism of sufficient amounts of cholesterol to maintain the homeostasis and provide adequate emulsification in the intestine.

1.5.1. Bile acid biosynthesis

An approximate of 500 mg of cholesterol is converted to bile acid each day in an adult liver. Cholesterol is an important biological molecule and an precursor of bile acid formation. It has roles in membrane structure as well as being a precursor for the synthesis of the various steroid hormones and bile acids. Both dietary cholesterol and that synthesized *de novo* are transported through the circulation in lipoprotein particles such as LDL, HDL. The synthesis and utilization of cholesterol is tightly regulated in order to prevent over-accumulation and abnormal deposition within the body. Bile acid synthesis is the major pathway for cholesterol catabolism in mammals. The pathway for bile acid biosynthesis involves 17 different enzymes (Table 1.3) most of which are expressed in the liver. The four main steps involved in the synthesis of bile acids are: 1. Initiation, 2. Modification in the ring structures, 3. Oxidation and shortening of the side chain and 4. Conjugation of bile acids with an amino acid. Figures 1.12 a, b, c and d illustrate those steps.

Table 1.3. Enzymes involved in bile acid biosynthesis.

Reaction	Enzyme	Subcellular location	Family
1	Cholesterol 7α-hydroxylase	ER	P450 (CYP7A1)
2	Cholesterol 24-hydroxylase	ER	P450 (CYP46A1)
3	Cholesterol 25-hydroxylase	ER	Diiorn co-factor
4	Sterol 27-hydroxylase	Mitochondria	P450 (CYP27A1)
5	Oxysterol 7α-hydroxylase	ER	P450 (CYP39A1)
6	Oxysterol 7α-hydroxylase	ER	P450 (CYP7B1)
7	3β-Hydroxy-Δ^5-C27steroid oxidoreductase	ER	Specific for C_{27} substrates
8	Sterol 12α-hydroxylase	ER	P450 (CYP8B1)
9	Δ^4-3-oxosteroid5-β-reductase	Cytoplasm	Aldo-Keto reductase (AKR1D1)
10	3α-hydroxysteroid dehrogenase	Cytoplasm	Aldo-Keto reductase (AKR1C4)
11	Sterol 27-hydroxylase	Mitochondria	P450 (CYP27A1)
12	Bile acid CoA ligase	ER	Uses log-chain FA's
13	2-Methylacyl-CoA racemase	ER, Peroxisome	Acts on 2-methyl FA's
14	Branched chain acyl-CoA oxidase	Peroxisome	ACOX2
15	D-bifunctional protein	Peroxisome	Multiple enzyme activity
16	Peroxisomal thiolase 2	Peroxisome	Multiple isoforms
17	Bile acid CoA: amino acid N-acyltransferase	Peroxisome	Liver selective

*****ER – Endoplasmic reticulum

1.5.2. Initiation (Step1)

Figure 1.10a shows the enzymatic steps involved in step 1. Bile acid synthesis begins by one of the several pathways. Cholesterol is converted to 7α-hydroxycholesterol by cholesterol 7α-hydroxylase (Figure 1.10a, reaction 1), a microsomal enzyme expressed only in liver. 7α-hydroxycholesterol is totally committed for bile acid production (Einarsson et al., 1973). This constitutes the classical pathway in the bile acid

biosynthesis. However, mice deficient in cholesterol 7α-hydroxylase produce bile acids from an alternative pathway, which led to the identification of three enzymes required to synthesize bile acids. Hydroxylation of cholesterol at three different positions on the side chain produce oxysterols at C24, C25 and C27–hydroxycholesterol in the presence of cholesterol 24-hydroxylase, cholesterol 25-hydroxylase and sterol 27-hydroxylase.

Cholesterol 24-hydroxylase synthesizes oxysterol 24(S) hydorxycholesterol (Figure 1.10a, reaction 2). Humans produce 6-7 mg of C24-hydroxycholesterol per day (Bjorkhem et al., 1998), of which ~3.5 mg is catabolized to bile acids (Bjorkhem et al., 2001). Hence, the enzyme cholesterol 24-hydroxylase, contributes little towards bile acid synthesis. Cholesterol 25-hydroxylase (Figure 1.10a, Reaction 3) is expressed in low levels in endoplasmic reticulum and Golgi apparatus in most tissues (Fuchs, 2003). This enzyme forms the substrate for oxysterol 7α-hydroxylases, which produces 7α-hydroxylated oxysterols that are funneled into steps for bile acid biosynthesis. 27-hydroxy cholesterol is the most abundant oxysterol in human plasma synthesized by the sterol-27 hydroxylase (Figure 1.10a, Reaction 4). About 25% of the bile acid pool originates from sterol-27 hydroxylase.

Oxysterols undergo 7α-hydroxylation in order to be converted to bile acids. Two members of the microsomal P450 enzymes (CYP39A1 and CYP7B1) catalyze this sequence of steps. The CYP39A1 acts on oxysterol 24(S) hydorxycholesterol (Figure 1.10a, Reaction 5). The 25 and 27-hydroxycholesterol are converted to bile acid intermediates and catalyzed by CYP7B1 oxysterol 7α-hydroxylase (Figure 1.10a, Reaction 6).

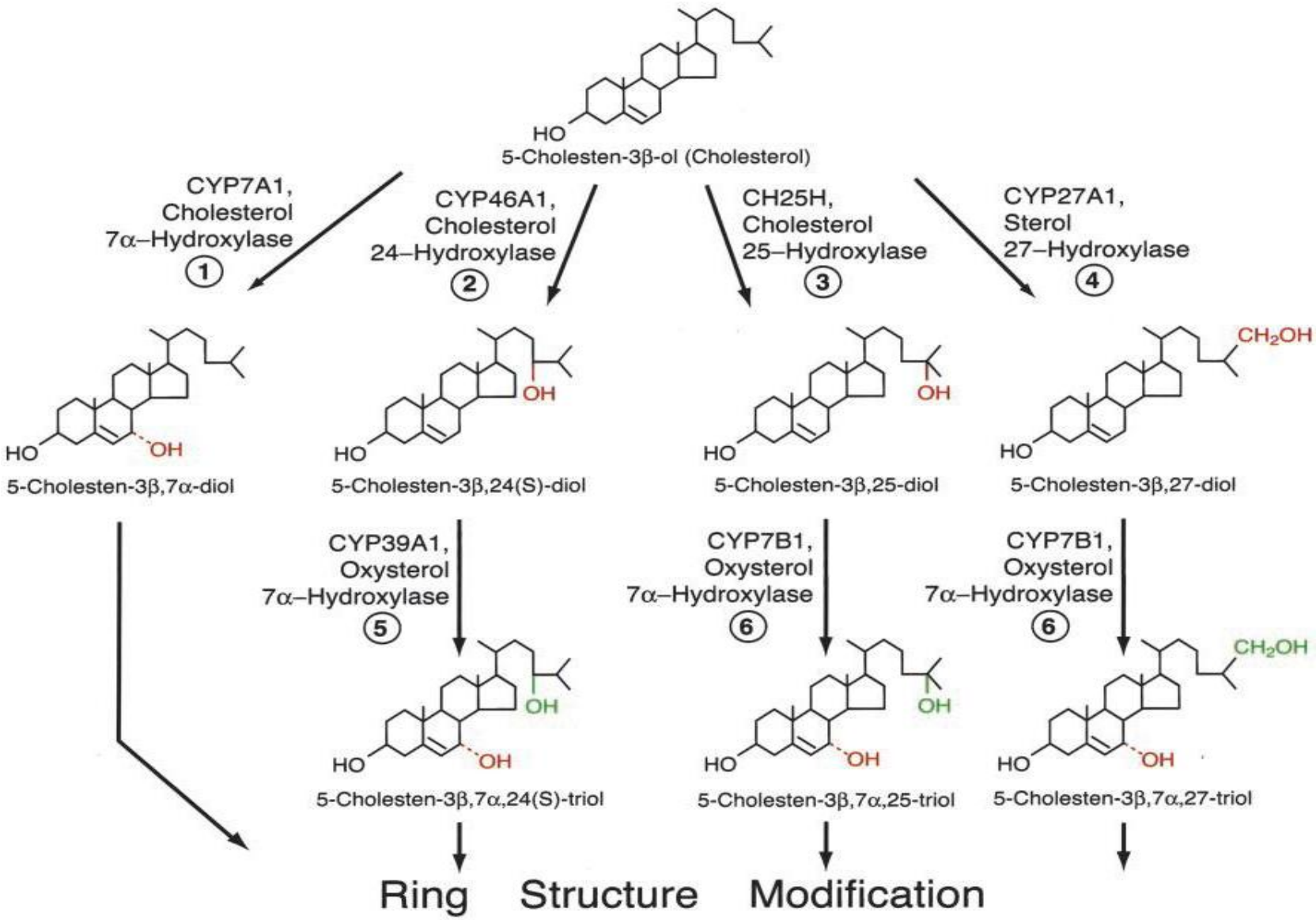


Figure 1.10a. Enzymatic steps involved in the initiation of the bile acid biosynthesis (Step 1).

1.5.3. Ring structure modification (Step 2)

The steps involved in this pathway are shown in Figure 1.10.b. In this step, the 7α-hydroxylated intermediates those derived from cholesterol and oxysterols are converted to their 3-oxo Δ^4 forms in the presence of a microsomal 3β-hydroxy-Δ^5-C_{27} steroid oxidoreductase or C_{27}-3β-HSD. The complex reaction catalyzed by this enzyme involves isomerization of the double bonds from the 5^{th} to the 4^{th} position and oxidation of the 3β-hydroxyl to a 3-oxo group (Figure 1.10a, Reaction 7). The enzyme acts only on sterols with 7α-hydroxyl group. Therefore, loss of this enzyme blocks all the bile acid synthesis, as there is only one C_{27}-3β-HSD (Schwarz et al., 2000, Wikvall, 1981).

The products of C_{27}-3β-HSD-steroid oxidoreductase forms 7α-4-hydroxy-4-cholesten-3-one (4-cholestene -7α-ol-3-one) which is a common precursor for both cholic acid (CA) and chenodeoxycholic acid (CDCA). However, if the intermediate acted upon by sterol 12α-hydroxylase (Figure 1.10b, Reaction 8), is converted ultimately to CA (right arm of the reaction pathway, Figure 1.10b). In the absence of 12α hydroxylation, CDCA is formed (left arm of the reaction pathway, Figure 1.10b). CA and CDCA are the two primary bile acids.

The 12α-hydroxylated intermediates, produced from C_{27}-3β-HSD and those that escape 12α-hydroxylation, undergo reduction of the double bond by the enzyme Δ^4-3-oxosteroid 5β-reductase (Figure 1.10b, Reaction 9). The last step of the ring structure modification involves reduction of the 3-oxo group, which is catalyzed by 3α-hydoxysteroid dehydrogenase (Figure 1.10b, Reaction 10).

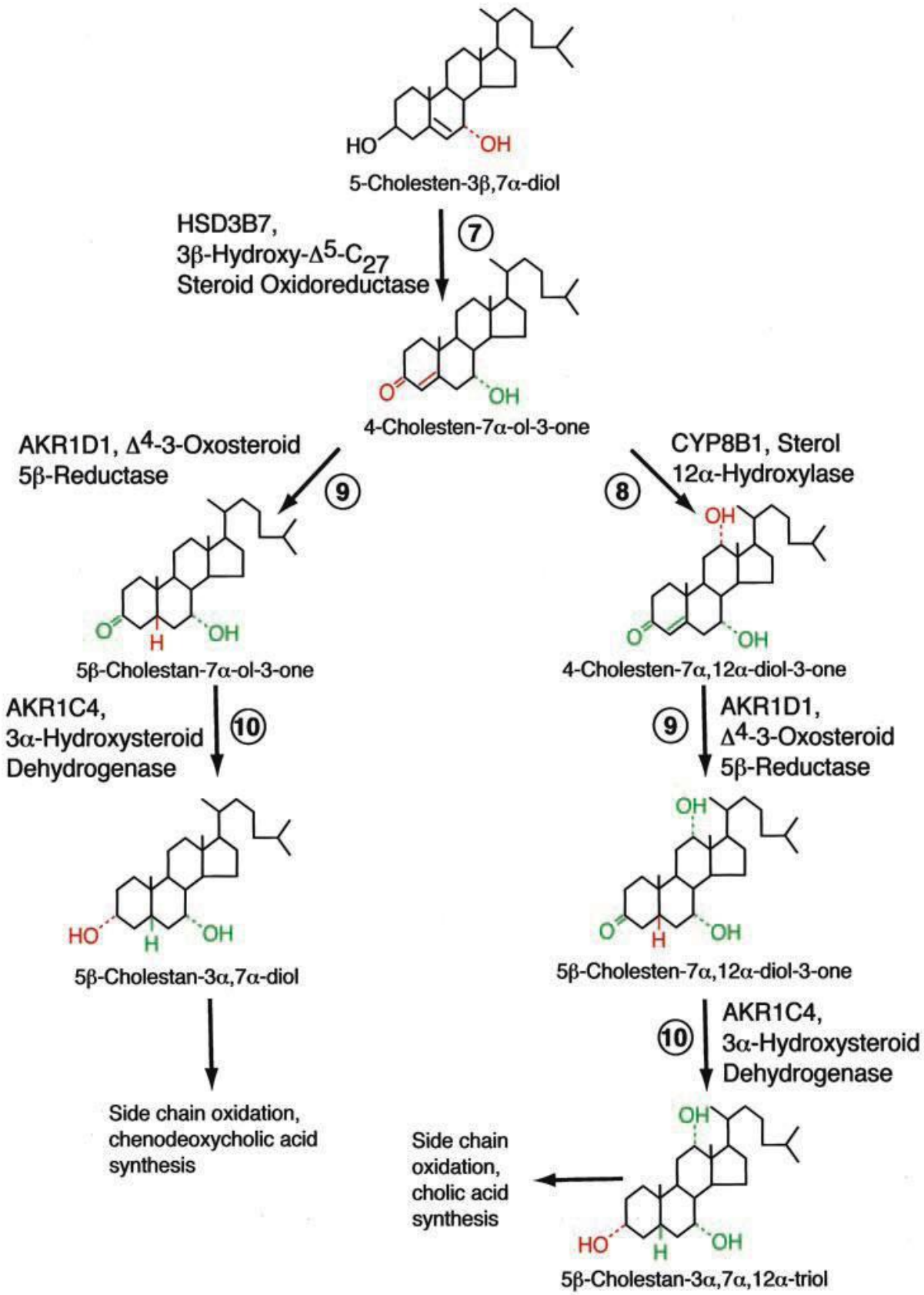


Figure 1.10b. Enzymatic steps leading to changes in sterol ring structure in the bile acid biosynthesis (Step 2).

1.5.4. Side chain oxidation (Step 3)

The steps involved in side chain oxidation reactions are shown in Figure 1.10c. The products of ring structure modification undergo oxidation and shortening of the sterol side chain and this is performed by sterol 27-hydorxylase (Figure 1.10c, Reaction 11). This is the same enzyme that initiates bile acid synthesis (See Figure 1.10a, Reaction 4). The enzyme introduces a hydroxyl group (at C27), oxidizes the hydroxyl group to an aldehyde and finally to carboxylic acid. The products from the above reaction exit mitochondria and are subjected to shortening of the side chain. This shortening of the side chain takes place in the peroxisomes catalyzed by bile acid coenzyme A ligase (Figure 1.10c, Reaction 12). The intermediates of bile acid coenzyme A ligase undergo isomerization i.e. the C25(R) position is converted to C25 (S) by 2-methylacylcoenzyme A racemase (Figure 1.10c, Reaction 13).

The products from the above reaction are subjected to dehydrogenation by a branched chain enzyme acyl-coenzyme A oxidase to yield 24, 25-trans unsaturated derivatives (Figure 1.10c, Reaction 14). They then undergo hydration and oxidation by a D-bifunctional protein (Figure 1.10c, Reaction 15). A peroxisomal thiolase cleaves the C24 – C25 bond to form propionyl – coenzyme A and a C24-coenzyme A bile acid intermediate (Figure 1.10c, Reaction 16).

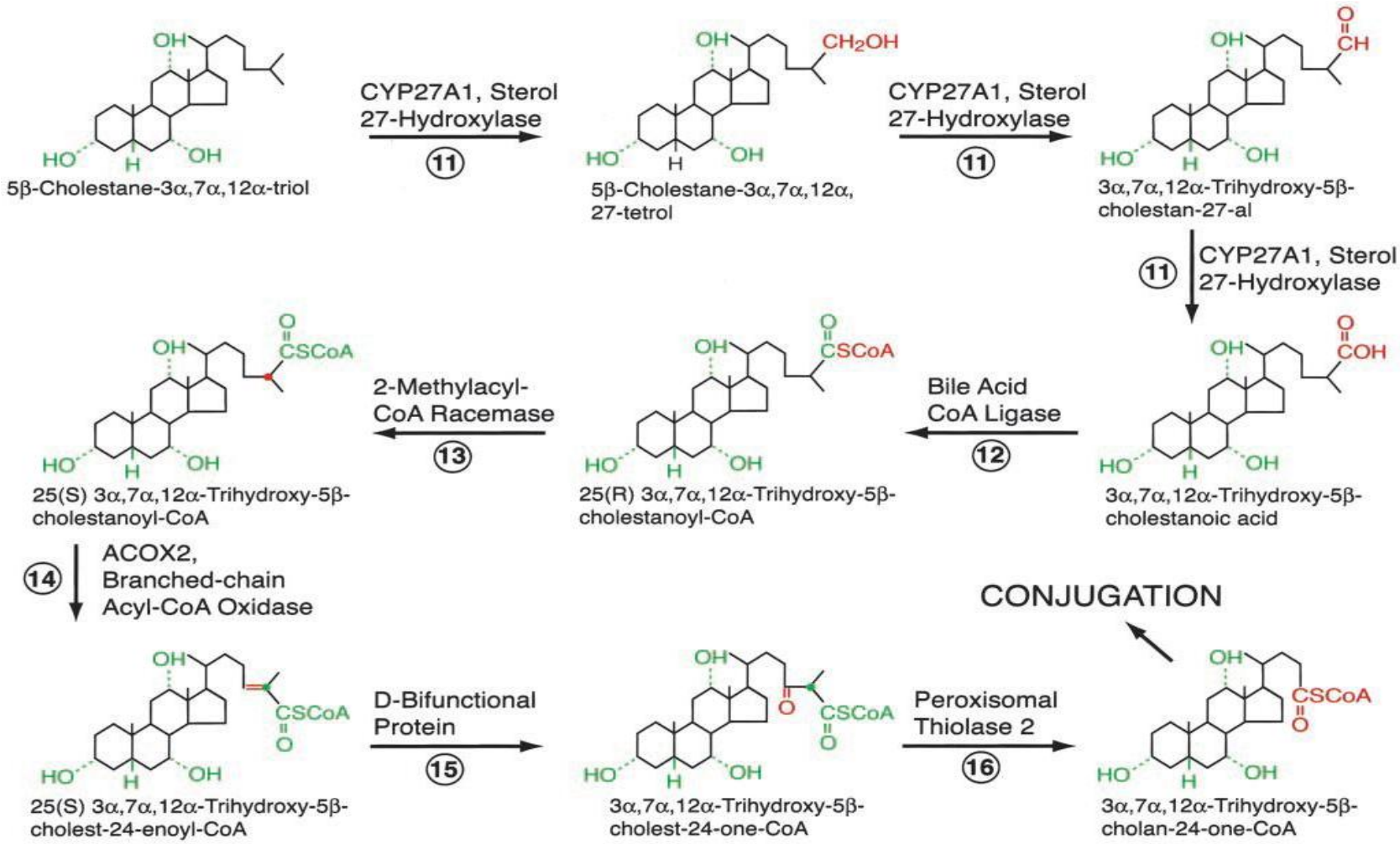


Figure 1.10c. Side chain oxidation in bile acid biosynthesis (Step 3).

1.5.5. Conjugation with amino acids (Step 4)

The terminal step before the exit of bile acids into the bile involves conjugation with amino acids in the liver, usually with glycine or taurine in an amide linkage to C24 catalyzed by the bile acid coenzyme A and amino acid-N-acyltransferase (Figure 1.10d, Reaction 17). N-acyltransferase is located in the peroxisomes and the amino acids glycine and taurine (in humans) taurine (mice) and bile acid coenzyme A thioester are the substrates (Falany et al., 1997). The ratio of conjugation of the amino acids with bile acids in the humans is dependent on the relative abundance of the two amino acids. The conjugated bile acids are then stored in the gall bladder and subsequently secreted into the duodenum via the bile duct.

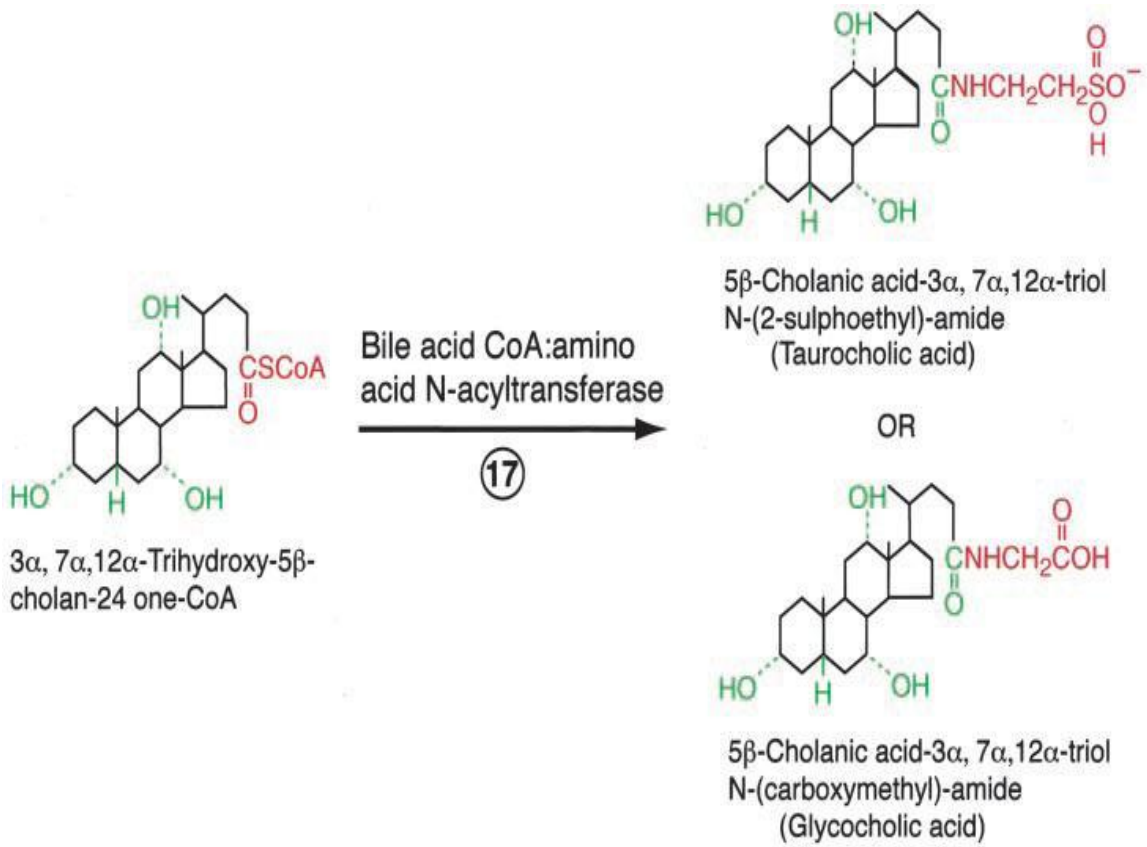


Figure 1.10d. Conjugation of bile acids (Step 4).

Figures 1.10a, b, c and d adopted from (Russell, 2003).

This conjugation increases the amphipathicity and enhances the water solubility of the molecules thus making them impermeable to the cell membranes. Conjugated and free bile acids do not cross cell membranes thus, specific transport systems are essential for moving the bile acids in and out of the cells (Love and Dawson, 1998). The classical pathway preferentially forms CA while the alternative pathway produces CDCA.

During the enterohepatic circulation, the primary bile acids, CA and CDCA synthesized in the liver are converted to secondary bile acids deoxycholic acid (DCA), lithocholic acid (LCA) and ursodeoxycholic acid (UDCA) by the action of intestinal microflora. The primary bile acids are metabolized and follow deconjugation by the intestinal bacteria that remove the hydroxyl group in the 7^{th} position from the steroid molecule. The principally formed secondary bile acids are DCA, LCA and UDCA. LCA is highly insoluble and largely excreted in the stools adhered to dietary fiber (Dowling, 1973). DCA is partially reabsorbed from the colon as it passes to liver where it is conjugated with glycine or taurine before joining the primary bile acids that are already in the enterohepatic circulation.

A defect on bile acid synthesis can be related to deficiencies of the enzymes CYP7B1, 3β-hydroxy-Δ^5-C_{27} steroid oxidoreductase and 3-oxo-Δ^4-steroid 5β-reducatse leading to liver diseases (Fuchs and Stange, 1999). Clinical manifestations of these deficiencies include progressive cholestasis, fat malabsorption and various neurological symptoms. Deficiency in CYP7A1 on the contrary, is not associated with liver diseases in adults but lead to hyperlipidemia, premature atherosclerosis and gall stone formation (Pullinger et al., 2002).

1.5.6. Physiology of bile formation

Bile formation is a vital process driven by the osmotic gradient generated by the active secretion of the non-permeant solutes into the canalicular lumen (Ballatori and Truong, 1992, Sperber, 1959). Both endogenous organic solutes like the bile salts and inorganic ions and their secretion from the blood to the bile has been identified as a major osmotic driving force in the bile formation. Bile salts and their conjugates have been noted to account for 40-50% of bile flow (Boyer, 1986). Bicarbonate ions, electrolytes, GSH and its conjugates constitute the rest (Ballatori and Truong, 1992, Graf, 1983, Hardison and Wood, 1978). The concentration of bile acids in the bile is up to 1000-fold necessitating active transport by the hepatocytes against the gradient (Tomer and Shneider, 2003).

Hepatocytes are polarized cells with two plasma membrane domains; a basolateral (sinusoidal) and an apical (canalicular) domain (Suchy et al., 1997). Uptake of biliary constituents into the hepatocytes occurs at the basolateral membrane, which is in contact with sinusoidal blood. The uptake is mainly sodium dependent driven by Na^+ gradient generated by Na^+/K^+ ATPase. The Na^+ taurocholate co-transporting polypeptide (NTCP) accounts for the major hepatocellular uptake for the conjugated bile salts (Hagenbuch et al., 1991, Jacquemin et al., 1994). Sodium independent transport is mediated by organic anion transporting polypeptides (OATP). ATP-dependent multi drug resistant proteins mediate the basolateral efflux of bile salts across the canalicular membrane.

1.5.7. Determination of bile levels in hepatobiliary diseases

Liver as an organ is involved in variety of synthetic, degradative and excretory functions. Although, the liver is made up of three types of cells (hepatocytes, biliary epithelial cells and the Kupffer cells) the hepatocytes are mainly involved in most of the metabolic activities. Hence, to meet the diverse demand by these metabolic pathways, a

number of enzymes are engineered for assisting these pathways. Hepatobiliary and intestinal diseases disturb these metabolic pathways giving rise to altered enzyme or bile acid synthesis and / or secretion, which are reflected in the serum, plasma or in the blood stream. The majority of bile acids are contained in the enterohepatic circulation, very little exist outside this circulation under normal healthy conditions (Hofmann, 1999).

In an abnormal condition as in hepatic and obstructive biliary diseases, bile metabolic rates are altered thereby increased levels of bile in serum, blood, plasma and urine can occur. Determination of primary and secondary bile acids in the serum and other biological matrices is a good indicator for the diagnosis of hepatobiliary diseases, disorders in lipid metabolism and other gastro-intestinal dysfunction (Street et al., 1983, Staggers et al., 1990). Bile acid analysis is becoming essential in many clinical biliary abnormalities.

1.6. HPLC in bioanalysis

1.6.1. Principles of HPLC

Optimising a HPLC method is required in order to ensure data quality in pharmacological assays. A HPLC system validation under good laboratory practices (GLP) is routinely followed to obtain highest quality data. The main aspects of GLP are:

System suitability tests (SST) - verifies the functionality of the chromatographic system on a day-to-day basis. According to International Conference on Harmonisation (ICH) and Center for Drug Research and Evaluation (CDER), SST forms an integral part of any analytical procedure. The parameters that include in any SST are resolution, tailing factor (T), theoretical plate number (N) and plate height (H).

Resolution (R_s): Separation in chromatographic systems occur when the rate of migration of components differs. This is defined by resolution which calculates the

distance between two adjacent solute zones centre divided by average peak width (Lindsay, 1992).

Theoretical plates/ Plate number (N) or plate height (H): This describes the column efficiency and quality of a chromatographic system. The efficiency of a chromatographic column is assessed by the time spent by the solute in the column. The longer the time spent, more dispersed it becomes. This efficiency is calculated in quantities called plate number or plate height (H). N is used to compare the efficiencies of different systems as it indicates column performance.

Other aspects of SST include are describes as follows:

Initial system qualification- verifies the functional performance of each component of the HPLC instrument (Dong et al., 2001, Dowling, 1973) which includes:

The pumping systems: the pumping module has an ability to maintain reproducible consistent and accurate flow of the mobile phase, which is necessary for high resolution and high-speed analyses. A good pumping system should be able to generate stable flow of the mobile phase, which minimizes detector noise. Anomalies in flow rate can affect retention time and resolution.

Gradient accuracy: Gradient accuracy defines the ability of the pumps to deliver accurately the required percentage of mobile phase at various time points within the run. This is a critical to achieving proper chromatographic resolution and reproducibility.

Pressure Test: The pressure test elucidates the proper functioning of the check valve and the tubing system with the pumps. An appropriate pressure is a pre-requisite for stable mobile phase flow.

Injector Module/ Auto-sampler: The injector module includes the injector and the needle seat. Injector describes the ability of the needle to draw prescribed volume of sample at each replicate injection made. This reflects on the peak area and peak height gained at each injection. Anomalies in the injection volume can lead to inaccurate

quantitation. Needle seat contamination/ carryover, on the other hand, can lead to contamination from the previous injection to the next injection. This could affect quantitation. Contamination can be avoided by injecting blanks in between the samples. This can be effective after injecting samples containing high concentration of analyte.

Detector Module: A detector constantly monitors the mobile phase emerging from the column. Detectors function based on the principle of wavelengths. Each component emerging from the column is detected at a particular wavelength followed by its emission at different wavelength. Wavelength deviations/ wavelength accuracy problems can lead to less sensitive response. Further, it is necessary to programme the detector output to obtain specific response.

Method validation- verifies the performance of entire analytical procedure.

1.6.2. Detection techniques in HPLC analysis

The main detection mode systems used in liquid chromatography are visible wavelength, electrochemical, UV, photodiode array and fluorescent detectors. Visible wavelength detectors are those used for measuring colour of sample component obtained by colorimetric reactions. Electrochemical detector measures the current associated with the oxidation or reduction of solutes. UV detectors detect solutes that absorb UV or visible light. Photodiode array detectors use diodes to measure narrow bands of wavelength. Fluorescence detection involves introducing a fluorophore conjugated to the analyte of interest.

1.7. Mass spectrometry in bioanalysis

1.7.1. Principles of mass spectrometry

Mass spectrometry is used to identify ions based on their mass-to-charge ratio *(m/z)* and their relative abundances. A mass spectrometer can be coupled to a separation method. The separation method can be either GC (GC-MS) or liquid chromatography (LC-MS).

The use of LC-MS has become one of the choicest analytical techniques. Its main features are high sensitivity, high resolution, wide dynamic range, cover wide chemical diversity, robustness and feasibility to elucidate the molecular weight and structure of unknown compounds. A mass spectrometer has includes three fundamental parts- an ion source, the analyser and the detector.

1.7.2. Electrospray ionization

Several ionization techniques can be used at the LC-MS interface. Commonly used sources are atmospheric-pressure chemical ionisation (APCI) and electrospray ionisation (ESI). Both of these require high voltage and heat which can generate ions from solutions of the analyte. The introduction of ESI has proven to be a great tool in biochemistry, allowing the mass spectrometric characterization and sequencing of peptides, proteins and other biopolymers of great importance to human life and medicine.

ESI ionises a liquid sample at atmospheric pressure. A high voltage (3- 4kV) produces nebulisation of the solution producing charged droplets. These charged droplets get smaller as the heated drying gas causes the solvent to evaporate. Individual ions are formed in the process that are then, separated by the mass analyzer (Figure 1.14). ESI is described as a soft ionisation technique because it does not fragment biomolecules . The main drawback of ESI is its liability for ion suppression. This is due to matrix effects that occur through competition between undetected matrix components co-eluting with the analyte of interest that can interfere during the ionisation process.

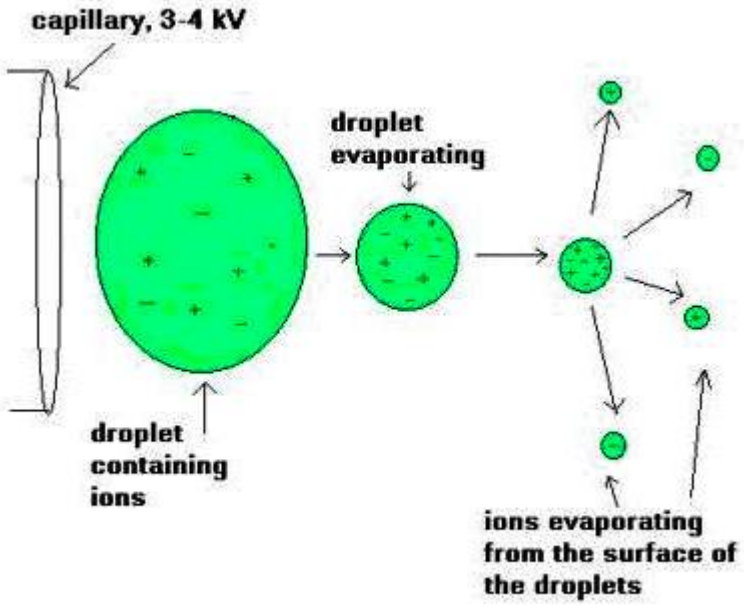


Figure 1.11. Illustration of the electrospray ionisation process. (Adapted from .

1.8. Aims and objectives

AP is an inflammatory disease. Extensive research on various animal models and human studies have shown and proved the role of oxygen-derived free radicals in the pathogenesis of AP. Depletion of non-protein thiols and antioxidant GSH in the pancreas is an early feature in both the cases. The hypothesis is that "oxidant stress" caused by free radicals and depletion of antioxidant systems (including GSH) in the pancreatic acinar cell, sets into motion a series of changes leading to AP. In order to investigate the role of GSH, it is important to understand the kinetics of the transsulfuration pathway in normal and disease conditions. The precursor amino acids for GSH synthesis are transported via the γ-glutamyl cycle, therefore any derangement in transsulfuration pathway invariably affects the availability of GSH.

Determination of GSH and GSSG is challenging because GSH is prone to auto-oxidation, giving erroneous GSSG values. The ratio GSH: GSSG are used to evaluate the oxidative stress status in biological systems. Oxidative stress can be assessed by measurement of the GSH: GSSG ratio in blood. Alterations in this ratio have been

demonstrated in various disease states including AP. Therefore, it is important to determine the GSH: GSSG ratio accurately.

Bile acids are major components of the bile. Synthesis and subsequent excretion of bile salts in the feces represent a significant mechanism for elimination of excess dietary cholesterol. The concentrations of bile salts are important prognostic and diagnostic indicators of any hepato-biliary/intestinal dysfunction.

The main aims of this thesis were to:

- To investigate the temporal relationship between the plasma amino thiols Cys, Hcy, Cys-Gly and kinetics of GSH during the attack of AP.
- To develop an accurate method for the determination of GSSG and tGSH levels in whole blood.
- Study the profile of bile acids from patients suffering from various hepato-biliary diseases.

The specific objectives were to:

1. Collaborate with clinicians at hospital, who recruited patient criteria for both AP and bile acid studies.

2. Set up an HPLC method with fluorescence detection to profile the sulfur amino acids and analyse their profiles in AP.

3. Identify a reliable scavenger for GSH and develop an HPLC method for the analysis of GSSG and tGSH. 4. Set up an LC/MS method for the analysis of bile salts in clinical samples and use it to analyse their profiles in patients with cholestatic heapto-biliary diseases, an a pilot study.

CHAPTER 2

Materials and Methods

CHAPTER 2. Materials and Methods

2.1. Materials

2.1.1. Sulfur amino acid analysis

(D, L)-homocyseteine (Hcy), L-cysteine (Cys), cysteinyl-glycine (Cys-Gly), and mercaptopropionylglycine (MPG) were obtained from Sigma Aldrich (Dorset, UK). Tri *n*-butylphosphine (TBP) and 7-flurobenzofurazane-4-sulfonic acid ammonium salt (SBD-f) and sodium acetate-3-hydrate ($CH_3COONa.H_2O$) were purchased from Fluka, (Dorset UK). Dimethylformamide (DMF) was purchased from Riedl-deHaen GmbH & CO. KG (Zeelze, Germany). Tris (hydroxylmethyl) amino methane (TRIS), sodium di-hydrogen phosphate monohydrate, sodium hydroxide (NaOH) and ethylenediaminetertraacetic acid (EDTA). HPLC-grade acetonitrile was obtained from Merck (Nogent-sur-Marne, France). HPLC-grade acetic acid and HPLC grade methanol were obtained from Fischer (Loughborough, UK).

2.1.2. Glutathione analysis

Reduced Glutathione (GSH), oxidized glutathione disulphide (GSSG), Glutathione ethyl ester (GEE), metaphosphoric acid (MPA), *o*-phthalaldehyde powder (OPA), OPA readymade reagent, dithiothreitol (DTT), 1-methyl-2-vinylpyridium trifluromethanesulfonate (M2VP), N-ethylmaleimide (NEM), potassium tetraborate and sodium acetate-3-hydrate ($CH_3COONa.\ 3H_2O$) were obtained from Sigma-Aldrich (Dorset, UK). Tris (hydroxylmethyl) amino methane (TRIS) and sodium di-hydrogen phosphate monohydrate were obtained from BDH (Poole, England). HPLC-grade acetonitrile was obtained from Merck (Nogent-sur-Marne, France). HPLC grade acetic acid and HPLC grade methanol were obtained from Fischer (Loughborough, UK).

2.1.3. Bile salts analysis

Sodium salts of taurocholic acid (TCA), taurolithocholic acid (TLCA), taurodeoxycholic acid (TDCA), taurochenodeoxycholic acid (TCDCA), tauroursodeoxycholic acid (TUDCA), glycocholic acid (GCA), glycodeoxycholic acid (GDCA), glycochenodeoxycholic acid (GCDCA), glycoursodeoxycholic acid (GUDCA), cholic acid (CA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA), ursodeoxycholic acid (UDCA), lithocholic acid (LCA) and ammonium acetate were obtained from Sigma-Aldrich (Dorset, UK). Glycolithocholic acid (GLCA) was procured from Calbiochem. HPLC grade methanol was obtained from Fischer (Loughborough, UK). Formic acid was obtained BDH, (Poole England). The internal standard cholic acid *d*-4 was obtained from QMX laboratories, Canada. Solid phase extraction (SPE) cartridges (C18-E, 500 mg, 6 mL) were purchased from Phenomenex (Macclesfield, UK).

2.1.4. Other consumables

All HPLC buffers for the present study were filtered through 0.45 μM, 47mm Millipore Nylaflo® nylon membrane filters (Pall Gelman). HPLC vials, vial inserts (250 μL, colourless flat bottom), septas (8mm x 0.010 PTFT), screw-cap lids and. Eppendroff s and disposable pipette tips were supplied by Sarstedt, Germany. Glass pasture pipettes (6'') and extraction glass tubes were purchased from Western Laboratories Services Ltd (Aldershot, Hampshire, UK). Broad range (4.5-10.0) pH indicator paper strips were purchased from Merck, UK. 4 mL vacutainer® plastic tubes were provided from Becton Dickson, Rutherford, NJ. Nitrogen gas was supplied by BOC gases, UK. Water was filtered using a resistance of 18Ω to provide Milli Q grade quality.

2.2. Equipment

2.2.1. General laboratory apparatus

An analytical balance and micro-balance (Sartorious Basic, BP110) to 4 decimal places was used for preparation of all standards. For HPLC and ESI/LC-MS analysis, all buffer mobile phases were prepared using a calibrated pH meter (Basic Denver Instruments). The pH meter were calibrated with standard pH 4 and pH 7 solutions prepared by dissolving standard pH 4 and pH7 tablets in Milli Q grade water. Vortex mixtures (Jensons Scientific, UK). Desktop Centrifuge (Micro Centaur, Quality laboratory equipments London, UK), oven and an SPE vacuum manifold and SPE drying attachment for vacuum manifold were purchased from Phenomenex, Macclesfield, UK.

2.2.2. HPLC for sulfur amino acid and glutathione study

A 1050 series Hewlett Packard equipped with a Hewlett Packard 1050 pumping systems and Prime Multi Channel Data Station for Windows ™, HPLC Technology, Herts, UK was used for analysis for both studies. A Millipore Waters 470 programmable Scanning Fluorescence Detector was used for detecting fluorescent derivatives. A Hypersil C-18 reversed-phase ODS-2 silica column (250x4.6 mm: 5 µm particle size) (Chrompack, Les Ulis, France) and Alletech Allsphere ™ ODS-2 guard cartridges (7.5x4.6mm, 5µm) were used for both assays. The HP 3396A integrator was operated in parallel (with Prime Multi Channel Data station) under following conditions: Attenuation 3, chart speed =0.5mm^{-1}, area of rejection=5000, threshold=2.0 and peak width 0.04 mm.

2.2.3. LC/MS for bile salt analysis

The LC-MS analysis for bile salts was performed using a Waters Alliance 2695 HPLC pump coupled to an electrospary (ESI), single quadrupole mass spectrometer (Waters,

Elstree, Hertsfordshire, UK) operated in negative mode. Full scan spectra were taken in the mass range of *m/z* 200-600. Instrument control and data acquisition were performed using the Masslynx ™ V4.0 software. The chromatographic analysis on a C_{18} column (Gemini, 5μ, 150 x 2mm; Phenomenex). Sample injections were performed with a Waters 2690 auto sampler and the sample chamber temperature was set at 8°C. The column was maintained at ambient temperature.

2.3. Clinical samples

2.3.1. Acute pancreatitis study

Human plasma samples from patients, who satisfied the criteria for acute pancreatitis, were supplied from The Leeds Teaching Hospitals, NHS Trust, Leeds UK. The samples were collected according to the protocol approved by the local ethics committee. The plasma samples were collected in 1 mL eppendroffs and kept in -80ºC.

2.3.1.1. Clinical Criteria for acute pancreatitis classification

All the clinical criteria for the AP patients were assessed by Dr Z Rahman at The Leeds Teaching Hospitals NHS Trust, Leeds, U.K in accordance with the Atlanta criteria of 1992 and The Acute Physiology and Chronic Health Evaluation – II score (APACHE-II) and Acute Physiology Scores (APS) at 24-hrs after the onset of abdominal pain and at 48 and 72 hours (Knaus et al., 1981). A control cohort of healthy, age matched subjects, with no history of AP, chronic disease, or malignancy was recruited from among the in-hospital medical and nursing staff. Samples for biochemical analyses collected at 24 and 72 h after the onset of abdominal pain were analysed immediately for erythrocyte GSH, γ-GT activity and disease severity determination at LGI, Leeds, UK, by Dr Z Rahman.

2.3.2. Glutathione study

Human whole blood samples were supplied from St. Luke's General Hospital, Leeds, UK and Leeds General Infirmary (LGI), Leeds, UK, by Dr Rahman SH. The samples were collected according to the protocol approved by the local ethics committee.

2.3.3. Bile salts

Human bile samples from patients suffering from cholestatic hepato-biliary diseases were collected at the Leeds General Infirmary (LGI), Leeds, UK, by Dr Rahman SH. The samples were collected according to the protocol approved by the local ethics committee. The samples were collected in 5 mL plastic screw top specimen containers and stored in -80ºC.

2.4. Biochemical analyses

2.4.1. Total erythrocyte glutathione determination

Total reduced erythrocyte glutathione (GSH) concentration was determined colorimetrically using the GSH-400 (OXIS, BIOMED; Nycomed Amersham plc, Buckinghamshire, UK). Assay using 1 ml of erythrocyte pellet separated from an EDTA treated 5ml sample of peripheral venous blood. The assay was performed according to the manufacturer's instructions by Dr Rahman SH.

2.4.2. γ-Glutamyl transpeptidase activity

Venus blood after an overnight fast was used for the determination of serum activity of γ-glutamyl transpeptidase (γ-GT). Enzyme activity was determined through a colorimetric method based on the recommendation of European Committee for Clinical Laboratory Standards (Klauke et al., 1993). Serum γ-GT greater than 50 IU/L was defined as an elevated γ-GT. The assay was performed by Dr Rahman SH.

2.4.3. C-reactive protein assay

A further 5 ml sample of heparin treated venous blood was obtained from all patients every 24 hours for at least 4 days from the onset of pain, from which C-reactive protein (CRP) levels were measured using an enzyme-linked immunosorbent assay (ELISA) (DAKO, High Wycombe, UK). The assay was performed by Dr Rahman SH.

2.5. Sulfur amino acid analysis

2.5.1. Reagents

2.5.1.1. Reducing solution (TBP)

1 mL of Tri n-butylphosphine (TBP) was thoroughly mixed with 9 mL of dimethylformamide in a fume cupboard. This solution was made fresh each time and discarded after use.

2.5.1.2. Protein precipitating solution (TCA)

1 g of trichloroacetic acid (TCA) and 3.72 mg EDTA were dissolved in 10 mL water using 10 mL volumetric flask. The final concentration of TCA was 100mg/ mL and that of EDTA was 37.2 mg/ mL. The solution was stored at 4˚C for one week.

2.5.1.3. Borate buffer

3.82 g of potassium tetraborate and 0.15 g of EDTA were dissolved in water. The solution was made up to 100 mL in volumetric flask. The solution was stored at 4˚C for one week.

2.5.1.4. Mercaptopropinyl glycine (MPG)

19.6 mg of MPG was weighed and dissolved in a 100 mL volumetric flask with water. The concentration of this solution was 1.2 mM.

2.5.1.5. Sodium hydroxide solution

This solution was made by dissolving 6.2 g NaOH in 100 mL water using a volumetric flask. The solution was stored at 4˚C for one month.

2.5.1.6. SPD-f derivatizing solution

A solution of 1 mg/mL SPD-f was prepared fresh every time in borate buffer. The volume of this solution was adapted to the number of samples to be derivatized. The solution is light sensitive and was kept at 4˚C in a covered container for up to 7 days.

2.5.2. HPLC mobile phase solutions

Buffer A: (0.1 M acetate buffer, pH 4)

13.6 g of sodium acetate 3- hydrate was dissolved in 900 mL Milli Q Water to make a 0.1M acetate buffer. The pH of the buffer was adjusted to pH 4 by the addition of HPLC grade glacial acetic acid, drop wise. The solution was then transferred to 1000 mL volumetric flask and the volume was made up with Milli Q water. The buffer was filtered through 0.45 μM Millipore filter and degassed for 10 min.

Buffer B (0.1 M acetate buffer, pH 4, 5% MeOH)

13.6 g of sodium acetate 3- hydrate was dissolved in 800 mL Milli Q water. The pH of the buffer was adjusted to pH 4 by the addition of glacial acetic acid, drop wise. 50 mL HPLC grade MeOH was added. The solution was then transferred to a 1000 ml volumetric flask and made up with Milli Q water. The buffer was filtered through 0.45 μM Millipore filter and degassed for 10 min.

2.5.3. Sulfur amino acid solutions

2.5.3.1. Stock solutions

1.2 mM stock solution of each sulfur amino acid standard and the internal standard (MPG) were made. HCY (4.2 mg), CYS (3.62 mg), CYS-GLY (5.35 mg), GSH (9.2 mg) and MPG (4.9 mg) were dissolved in 25 mL water using volumetric flasks. These stock solutions were aliquoted in 1 mL eppendroff tubes and stored at -20˚C for one month.

2.5.3.2. Preparation of sulfur amino acid combined standard

1 mL of each one of the 1.2 mM standard stock solutions was taken in a 10 mL volumetric flask. The final volume was made up to 10 mL with water. This solution is was the combined standard of all sulfur amino acid at a concentration of 0.12 mM each and was made fresh every time.

2.5.3.3. Standard solutions of sulfur amino acids for the calibration line

The combined standard (0.12 mM) was further diluted as shown in the table below to obtain a linear range of concentrations. These were used as calibration points.

Dilution	7	6	5	4	3	2	1
Volume of 0.12 mM combined standard (µL)	1000	750	500	250	125	63	31
Milli Q water (µL)	0	250	500	750	875	938	969
Final concentration (µM)	120	90	60	30	15	7.5	3.75

2.5.3.4. Preparation of sulfur amino acid single standards

Single standards of all the amino acids were made from the 1.2 mM stock standards (Section 2.5.3.1) and this was used as to determine the retention time for compound. Each standard was diluted to a final concentration at 60 μM (50 μL of 1.2 mM stock solution added and mixed with 950 μL water.

2.5.3.5. Preparation of the internal standard (I/S)

20 μL of the 1.2 mM stock solution of MPG (I/S) was mixed with 980 μL water to make a working solution of the internal standard at a concentration of 24 μM.

2.5.3.6. Derivatization of sulfur amino acids

60 μL of biological sample (or standard) were mixed with 40 μL of MPG (24 μM) and 10 μL of TBP solution. This step was performed in the fume cupboard. The samples were mixed and then incubated at 4˚C in the dark for 30 min. 100 μL of TCA solution was then added to each sample solution and the solutions were centrifuged at 2817 x g (6000 rpm) for 10 min.

50 μL of the resulting supernatant was transferred into an eppendroff containing 125 μL of borate buffer and 10 μL of NaOH. The samples were then derivatized by the addition of 50 μL of SBD-f solution and incubated in the oven at 60˚C for 60min. The derivatized samples were left to cool to room temperature for 10 min and analyzed immediately by HPLC.

2.5.3.7. HPLC analysis of sulfur amino acids

HPLC analysis was based on a gradient system with two solvents: Buffer A and Buffer B. the gradient was programmed as shown in the table below. The injection volume was 20 μL, the flow rate was 1 mL/min with a overall runtime of 29 min.

Time (min)	% Buffer A	% Buffer B
0	100	0
2	50	50
12	50	50
14	0	100
22	0	100
23	100	0
29	100	0

The fluorescence detector was operated at attenuation 2.0 gain x 100 and at excitation wavelength of 385 nm and emission wavelength of 515 nm. The integrator was operated under the following conditions: Attenuation 2, chart speed= 0.5 mm/min, Area of rejection= 5000, Threshold= 2.0 and Peak width= 0.04.

2.6. Glutathione Analysis by HPLC

2.6.1. Reagents

2.6.1.1. 1-Methyl-2-vinyl-pyridium trifluromethane sulfonate (M2VP)

A 15 mM M2VP was made (2 mg/ 500 μL) in 0.01M HCl solution. This was prepared fresh every time before use.

2.6.1.2. N-ethylmaleimide (NEM)

A 15 mM NEM solution was prepared fresh (9 mg/ 500 μL) in 0.01M HCl solution. This was prepared fresh every time before use.

2.6.1.3. Dithiothreitol solution

A 25 mM DTT (19 mg/5 mL) solution was made in water, fresh every time before use. The solution can also be stored for 3-5 days at 4ºC.

2.6.1.4. TRIS buffer

A 0.1 M TRIS buffer (121 mg/100 mL) was prepared in water and kept at 4ºC for up to 10 days.

2.6.1.5. Sodium phosphate buffer

A 500 mM of sodium dihydrogen phosphate (6.89 g/100 mL) was prepared in water. This solution was stored at 4ºC for one week.

2.6.1.6. Metaphosphoric acid solution

A 6% (w/v) solution of MPA acid solution was prepared by dissolving 6 gm of MPA in 100 mL water. The MPA solution was stored at 4ºC for one week.

2.6.1.7. Potassium tetraborate solution

A 0.4 M potassium tetraborate solution was prepared by dissolving 1.2 in 10 mL of water. This solution has a pH of 9.90 unadjusted.

2.6.1.8. OPA reagent

The OPA reagent was purchased as a complete ready-made reagent. It contained Brij 35, methanol, *o*-phthaldialdehyde, 2-mercaptoethanol, potassium hydroxide and boric acid with a of pH 10.4. The solution is light sensitive and can be oxidized when exposed to air. Hence, the reagent bottle was flushed with nitrogen every time after use, sealed in parafilm and store at 4ºC as advised in material data sheet.

2.6.1.9. Preparation of OPA solution from powder

A 5 mg/mL OPA solution was made by dissolving 50 mg of OPA powder in 0.5 mL HPLC grade methanol. The volume was made up to 10 mL with freshly prepared 0.4 M potassium tetraborate solution. This OPA solution can be stored for a 1week at 4ºC or for four weeks at -20ºC.

2.6.2. Preparation of GSH, GSSG and GEE standards

Stock solution of GSH (10 mM; 37 mg/ 10mL), GSSG (1 mM; 32 mg/ 5 mL) and GEE (1 mM; 1 mg/ 3 mL) were prepared in 0.01M HCl. These solutions were aliquoted (100 µL) in eppendroff tubes and stored at -20ºC up to 1 week. Working standard solutions of GSH (5 mM, 2 mM, 1 mM, 500-10 µM) and GSSG (500-10 µM) were prepared daily from these stock solutions by diluting the solutions with 0.01M HCl. All the standard and working standard solutions for GSH or GSSG were prepared in 0.01M HCl to protonate the reactive thiolate anions.

2.6.3. Preparation HPLC mobile phase solutions

Buffer A (50 mM acetate buffer, 10 % acetonitrile, pH 6.2)

6.80 g of sodium acetate-3-hydrate was dissolved in 900 mL water. The pH of the solution was adjusted to 6.2 using glacial acetic acid. 100 mL of HPLC grade acetonitrile was added. The aqueous solution and the organic solvent were mixed thoroughly on a magnetic stirrer. The buffer was filtered through 0.45 µM Millipore filter and degassed for 10 min.

Buffer B (50 mM acetate buffer, 80% acetonitrile, pH 6.2)

6.80 g of sodium acetate-3-hydrate was dissolved in 200 mL water. The pH of the solution was adjusted to 6.2 using glacial acetic acid. This solution was mixed with 800

mL HPLC grade acetonitrile and mixed thoroughly on a magnetic stirrer. The buffer was filtered through 0.45 μM Millipore filter and degassed for 10 min.

2.6.4. Collection of blood samples

Blood samples were collected in 4 mL vacutainer tubes coated with K_2EDTA as anticoagulant by vein puncturing healthy volunteers. Immediately, 100 μL of the blood sample were placed in 1 mL eppendroff containing 10 μL of M2VP (15 mM) solution and mixed thoroughly. These samples were used for tGSSG analysis. The concentration of M2VP in the blood samples was 1.3 mM. Another aliquote of blood (100 μL) was collected 1 mL eppendroff. These samples were used for tGSH analysis. All samples were frozen immediately in an upright position and kept at -80ºC.

2.6.5. Total GSH (tGSH) assay

100 μL of whole blood collected for tGSH assay was thawed on ice. 50 μL of 100 μM GEE (internal standard) was then added followed by 100 μL of 25 mM DTT. The samples were mixed gently and then incubated at 4ºC for 30 min. 800 μL of ice-cold MPA solution was then added to each sample. The resulting solution was mixed and left on ice for 10-15 min to complete the precipitation of proteins. The solutions were then centrifuged at 7826 x g (10,000 rpm) for 10 min. 100 μL of the clear supernatant was transferred to a clean eppendroff and mixed with 200 μL of 0.1M TRIS, pH 8.5. 100 μL of this neutralized solution was then mixed with 100 μL of freshly made OPA solution (from OPA powder). The samples were mixed and left in dark for 10 min at room temperature to derivatize. 800 μL of 500 mM phosphate buffer, pH 7.00 was then added to the sample to adjust the pH to 9.0. 20 μL of this derivatized sample were taken for HPLC analysis immediately.

2.6.6. Oxidized GSH (GSSG assay)

100 µL of whole blood collected with M2VP (1.3 mM), was thawed on ice. 50 µL of 100 µM GEE (internal standard) was added followed by 100 µL of 25 mM DTT. The samples were mixed gently and incubated at 4ºC for 30 min. 800 µL of ice-cold MPA solution was then added. The solution was mixed and left on ice for 10-15 min to complete precipitate the proteins. The resulting solution was centrifuged at 7826 x g (10,000 rpm) for 10 min. 100 µL of the clear supernatant was transferred to a clean eppendroff and mixed with 200 µL of 0.1M TRIS, pH 8.5. 100 µL of this neutralized sample was then mixed with 100 µL of freshly made OPA solution (from OPA powder). The samples were mixed and left in dark for 10 min at room temperature to derivatize. 800 µL of 500 mM phosphate buffer, pH 7.00 was then added to the sample to adjust the pH to 9.0. 20 µL of this derivatized sample were taken for HPLC analysis immediately.

2.6.7. HPLC analysis for tGSH and tGSSG

The HPLC analysis was based on a gradient system with two buffers, Buffer A and Buffer B. The gradient was programmed as shown below. The flow rate was 1 mL/min with an overall runtime of 29 min.

Time (min)	% Buffer A 10% acetonitrile	% Buffer B 80% acetonitrile
0	90	10
10	80	20
15	50	50
16	0	100
20	0	100
21	100	0
29	100	0

The fluorescence detector was operated at attenuation 2.0 gain x 100 and at excitation wavelength of 340 nm and emission wavelength of 420 nm. An integrator was operated under the following conditions: Attenuation 2, chart speed= 0.5 mm/min, Area of rejection= 5000, Threshold= 2.0 and Peak width= 0.04. Prime Multi Channel Data Station for Windows ™ was also operated in parallel.

2.6.8. Calibration lines

The peak to area ratio of each analyte to the relevant internal standard was calculated and plotted against the concentration of the calibration standards. Calibration lines were calculated by the least square linear regression method. Peak integrations and S/N calculations were performed using the Prime Multi Channel data station software for Windows The method development followed for tGSH and tGSSG and relevant calculations are described in detail in chapter 3.

2.7. Bile acid analysis using ESI/LC-MS

2.7.1. Preparation of bile acid stock solutions

All the primary (CA and CDCA) and secondary bile acid standards (DCA, UDCA and LCA), conjugates of taurocholic (TCA, TCDCA, TDCA, TUDCA, TLCA) and glycocholic acids (GCA, GCDCA, GDCA, GUDCA, GLCA) and the internal standard cholic-2, 2, 4, 4-d_4 acid were prepared in methanol at a concentration of 1 mg/mL (stock solution) individually, and stored in -80ºC. 10 µL of each individual bile acid and internal standard solutions were diluted with 990 µL MeOH to prepare working standard solutions of 10 µg/ mL separately. These working standard solutions were stored in -20ºC. A cocktail of bile acid standard solutions were prepared by mixing 15 working bile acid standard solutions (10 µg/mL): 10 µL of each bile acid standard (total

volume 150 µL) were mixed and the volume was made up to 300 µL by adding 140 µL MeOH and 10 µL of 1 µg/mL internal standard. The cocktail was then stored in -20 ºC. The final concentration of this composite standard was 0.33 µg/mL/ bile salt.

2.7.2. Preparation of HPLC solvents

The chromatographic separation of the 15 bile salts were achieved by gradient system using two buffers Buffer A (5 mM ammonium acetate in 0.012% formic acid) and Buffer B (5 mM ammonium acetate in MeOH and 0.012%formic acid). The flow rate was 0.2 µL/min with a total run time of 40 min.

2.7.3. Bile standards and calibration lines

The peak to area ratio of each bile acid standard to the relevant internal standard was calculated and plotted against the concentration of the calibration standards (25 µg- 2.5 µg/ mL). Calibration lines were calculated by the least square linear regression method.

2.7.4. Preparation and extraction of bile salts from clinical samples

Approximately, of 1 mL of frozen bile sample was scooped out from each frozen bile sample and transferred into a 1.5 mL eppendroff vial. These samples were defrosted on ice. 50 µL of each bile sample was then transferred into a clean eppendroff vial, and mixed with 940 µL of water, by gentle vortexing. 10 µL of the cholic 2, 2, 4, 4-*d4* acid (internal standard) (1 µg/mL) was then added to this sample. The samples were kept on ice.

2.7.5. Solid phase extraction

The SPE cartridges were attached to the vacuum manifold and a pressure of approximately 5mm Hg was applied. The SPE cartridges were pre-conditioned using 5 mL methanol adjusting the vacuum to attain a steady drop-wise flow through the

cartridge. The flow was stopped just as the meniscus touched the filter taking care not to allow any air through. This was judged by constant visual inspection. Then the cartridges were washed with 5 mL water adjusting the vacuum to attain a steady drop wise flow through the cartridge. The flow was stopped similarly, when the meniscus just touched the filter taking care not allow any air through.

The bile samples prepared (as described in section 2.7.4) were transferred to the SPE cartridge and allowed to pass through in a drop-wise manner taking care not allow air through the filter at any point. The cartridge was then washed with 10 mL water. The bile salts were eluted from each cartridge with 5 mL methanol. Care was taken to maintain a slow drop-wise flow (under gravity) through the cartridge. The eluent was collected in clean, dry extraction glass tubes. At this stage, air was allowed to pass though the cartridge for 2 min to ensure the entire methanol extract had been collected.

2.7.6. Drying and reconstitution of bile salt extract

The drying apparatus were set up as follows:

Drying apparatus were set with the desired number of needles; the sample extracts collected in clean glass vials were placed so that the needle was directly above the sample making sure that the needle was not in contact with the solvent. The stream of nitrogen gas was to ensure a gentle flow of nitrogen over the surface of the methanol causing it to slightly ripple. The flow was periodically readjusted as the methanol evaporated.

Once the tube containing the extracted sample was completely dry, the glass tubes were removed from the drying apparatus and the residue was reconstituted in 500 µL methanol. This reconstituted sample was then transferred to clean glass vial sealed with open screw caps with Teflon septa and stored at -20ºC until analysis.

2.7.7. LC-MS analysis

The chromatographic separation of bile salts were achieved by gradient system using two buffers buffer A (5 mM ammonium acetate in 0.012% formic acid) and buffer B (5 mM ammonium acetate in MeOH and 0.012%formic acid). The flow rate was 0.2 µl/min with a total run time of 40 min. The gradient was programmed as shown below:

Time (min)	% Buffer A	% Buffer B
0	30	70
20	5	95
24	5	95
40	30	70

Ionization was achieved in a negative electrospary ionisation mode (ESI) and data acquired using single ion recording (SIR). The optimum conditions for ionization of the individual bile acids are shown in the following summary table.

Sodium salts of bile acid standards	Cone voltage (V)	Indicative retention time (min)
TCA	82	8.08
TUDCA	60	5.81
TDCA	90	11.60
TCDCA	60	12.57
TLCA	90	15.82
GCA	60	8.62
GUDCA	60	6.17
GDCA	60	12.08
GCDCA	60	13.20
GLCA	60	16.40
CA	60	12.31
CDCA	60	10.08
DCA	60	17.07
UDCA	60	10.08
LCA	80	20.72
CA-*d*4 internal standard	92	12.26

2.7.8. Limits of detection and quantitation

The limit of detection (LoD) was estimated using signal-to-noise (S/N) ratio of 3. The limit of quantitation (LoQ) was determined using a S/N ratio of 10. Peak integrations and S/N calculations were performed using the MassLynx ^{TM} V4.0 software.

2.7.9. Extraction recovery

In order to assess the recovery of the extraction methodology, bile samples were spiked with 10 μL cocktail of standards (330 ng/ mL/ standard), without internal standard. The samples were cleaned up by SPE and analysed as described in section 2.7.5. These spiked

bile samples were analysed in parallel with unspiked bile samples and the difference in the peak-area values was related to the amount of the spike.

At the same time, standards at the same amount, as used to spike the biological samples, were analysed directly by ESI-LC/MS directly. The peak areas of the analyte from the spiked, unspiked and directly analysed standards were used to calculate the recovery of the spiked standards using the equation:

$$\% \text{ Recovery} = \frac{\text{Peak area of the spiked} - \text{Peak area of the unspiked}}{\text{Peak area of directly analysed standards}} \times 100$$

2.8. Statistical Analysis

All statistical analyses were performed using SPSS v16.00 statistical analysis software (SPSS Inc, Cary, NC, USA). Statistical analyses included both parametric and non-parametric tests. Data were assessed for normality using the Kolmigornov-Smirnov (KS) test together with visual inspection of the p-p plots and histograms. Parametric data's was compared using paired sample T-test. Non-parametric data was compared data using Mann-Whitney U-test. Pearson's correlation and Spearman's rank test was used to correlate continuous and non-continuous data respectively. P values <0.05 were considered stastically significant.

CHAPTER 3

Method development for oxidized and reduced glutathione

CHAPTER 3: Method development for oxidized and reduced glutathione

3.1. Introduction

Oxidative stress has long been implicated as one of the morbid factors leading to various premature cellular complications. This is particularly evident in erythrocytes as they are important mobile detoxifiers and carriers of GSH (Dumaswala et al., 2001). Given the importance of GSH in many biochemical reactions, there has always been an active interest in measuring whole blood GSH concentrations. As whole blood GSH concentrations reflect GSH status in other less accessible tissues, measuring both GSH and GSSG in whole blood is considered essential as an index of the whole subject oxidative status and as a useful indicator of disease risk in humans.

Under normal physiological conditions, GSH is present in its reduced form and in millimolar (mM) levels, while GSSG is present in micromolar (μM) levels. Variations in these levels, caused by pathophysiological conditions can make them mediators of important functions or parameters of clinical significance. Thus, methods that can accurately and specifically measure tGSH and/ or reduced or oxidized GSH levels in biological matrix are crucial for understanding the redox status of the cells or the tissues.

The wide spread heterogeneous distribution of GSH and its role against oxidative stress and various other vital functions have aroused continuous analytical interest. Accurate and precise measurement of GSH or GSSG is not a straightforward procedure for a number of reasons. Auto-oxidation is one of the major drawbacks in GSH estimation

and GSH levels in blood is liable to this. Possible errors that can occur during sample collection and preparation can be as follows:

1. Haemolysis of blood- results in an over estimation GSH, as the GSH levels in red blood cells is more than plasma.

2. Storage at room temperature- if it were stored at room temperature, there would be under estimation of GSH, as GSH would undergo auto-oxidation.

3. Oxidation during acid protein precipitation - oxidation during acid precipitation can happen in blood depending on the concentration of heme groups. In blood, oxidants formed from oxy-hemoglobin can oxidize GSH (Gallemann and Eyer, 1990) This means whatever GSH values acquired, may or may not be reliable. Thus, accurate determinations of GSH/GSSG largely depend on proper sample preparation (Mills et al., 1994).

Numerous methods are available for measuring reduced and oxidized GSH in biological samples. Among spectrophotometeric determination, the most popular assay is enzymatic recycling, first discovered in 1965 (Owens and Belcher, 1965) and was later developed by Tietze (1969). GSH reacts with 5, 5- dithiobis-(nitrobenzoic acid) (DTNB) or Ellman reagent. Ellman reagent oxidizes GSH yielding 2-nitro-5-thiobenzoic acid (TNB) which was spectrophotometrically measured at 412 nm. The method also measures GSSG by reducing it with NADPH in the presence of GR. The disadvantage was the lack of automation (Tietze, 1969).

In the intervening periods, these techniques have been further advanced and these advancements have been briefly considered. Significant developments were made in fluorimetric methods. Pioneering work using OPA, a fluorophore for the determinations of reduced GSH were first reported in 1966 (Cohn and Lyle, 1966). The method was modified to allow GSH and GSSG determination using OPA as a derivatizing agent to

produce highly fluorescent derivatives, which were measured using spectrofluorometer (Hissin and Hilf, 1976).

Methods involving capillary electrophoresis have also been described (Serru et al., 2001). However, considerable oxidation of GSH during analysis was noticed leading to loss of GSH. Some of the detection modes coupled with capillary electrophoresis are UV-Visible detection, diode array detector, electrochemical detector and mass spectrometer (Desiderio et al., 1999, Dankova et al., 1999). Although, some mass spectrometric methods for GSH determination have been described (Camera et al., 2001, Norris et al., 2001), LC/MS is currently not an equipment in many hospital laboratories (Steghens et al., 2003).

A majority of assays involved in separation and/ or determination of GSH/ GSSG/ tGSH from a given biological matrix are by HPLC systems. However, selecting a suitable detection technique for HPLC analysis is one of the most important factors in developing an analytical HPLC method. Relatively few HPLC methods for the GSH and its congeners with UV-Vis absorbance or diode array detector (DAD) have been described (Reed et al., 1980). The authors estimated high amounts of GSSG due to oxidation of GSH. Due to low sensitivity and specificity, methods involving UV/ DAD are currently out of use. HPLC-ECD (electrochemical detector) analyses of GSH and GSSG (Rabenstein and Saetre, 1977) have shown a high oxidation potential and have proved to reduce the performance of ECD (Rose and Bode, 1995).

The most common and popular mode of detection employed in the determination of GSH in biological matrices is fluorescence. Fluorescence detection provides variable excitation and detection wavelengths favouring trace analysis. Further, it is considered

more selective and sensitive as only a small number of compounds fluoresce. The method of fluorescence detection involves introducing a fluorophore that changes the chemical structure of the compound to be studied. The choice of a fluorophore comes from the chemical structure that selectively combines and forms a fluorescent product. Hence, a fluorophore increases the sensitivity of the assay. The most frequently used flurophores in the determination GSH are monobromobiamine (BrB), benzofluron derivatives namely 7-flurobenzofurazane-4-sulfonic ammonium salt (SBD-f) and *ortho*-phthalaldehyde (OPA).

BrB combines with the functional thiol group in GSH forming GSH-Bimane adducts. The adducts formed with high fluorescence allows emission followed by detection even at low concentrations (2x 10^{-8} M). Although, bimane adducts are widely used, studies have shown that BrB is not selective for GSH determination (Ivanov et al., 2000). BrB being fluorescent by itself undergoes photo-degradation yielding fluorescent products that appear as unknown peaks in the chromatograms. SPDf was considered a low-reactive reagent that requires drastic conditions such as 60º C for approximately 60 min to derivatize thiols (Toyo'oka and Imai, 1983).

Unlike BrB, OPA is non-florescent reagent (Neuschwander-Tetri and Roll, 1989, Senft et al., 2000, Yan and Huxtable, 1995), capable of reacting with primary amines in the presence of thiol, cyanide or sulfite forming a highly fluorescent tricyclic isoindole (Yan and Huxtable, 1995, Molnar-Perl, 2001). The reaction between the –SH group and OPA is pH dependent. It is widely reported that the reaction between amines and OPA takes place in strongly alkaline pH (Svedas et al., 1980). The free thiol group in GSH reacts with OPA forming a GSH-OPA adduct (Figure 3.1).

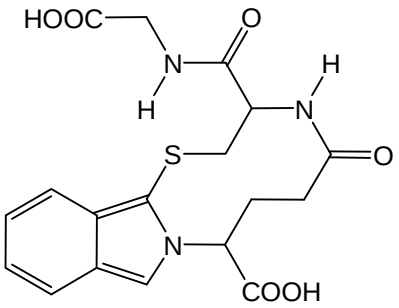


Figure 3.1. Structure of GSH-OPA adduct.

3.1.1. Disulfide reduction

Introducing fluorophore does not allow GSSG detection due to the presence of disulfide bond. Determination of tGSH or GSSG requires the reduction of all the disulphide species in the biological fluid. This reduction can either be enzymatic (Ercal et al., 2001) by electrolysis (Ivanov et al., 2000) or by chemical reaction (Lenton et al., 1999, Yang et al., 1995, Cereser et al., 2001). A number of reducing agents have been identified such as 2-mercaptoethanol, TBP, dithiothreitol (DTT) and sodium borohydride. Choosing an appropriate reagent is critical for assay performance.

3.1.2. Thiol scavengers in GSH determinations

One of the innate difficulties is the metal ions (heme in blood) can possibly react with sulfhydryl in GSH to yield GSSG during sample preparation. Although, the amount of tGSH remains the same, GSSG would be expected to increase under such conditions. In order to quantitate intracellular GSSG concentrations accurately, the reduced GSH pool has to remain unchanged (without oxidation). A strategy to block changes in GSH in a biological sample before protein precipitation, has led to the invention of thiol blockers or scavengers.

Griffith reported 2-vinyl pyridine (2-VP) as an efficient thiol blocker (Griffith, 1980). However, the masking reaction with 2-VP required 20-60 min incubation at room temperature (Shaik and Mehvar, 2006). This time interval can possibly cause GSH auto-

oxidation to GSSG at room temperature. There have been two known thiol scavengers N-ethylmaleimide (NEM) and indole-3-acetic acid (IAA) since 1969. NEM can effectively react with free thiol groups facilitating a GSSG determination (Camera et al., 2001, Piccoli et al., 1994). Nevertheless, there have always been certain disadvantages when the NEM or IAA were used.

NEM is a potent inhibitor of GR (Griffith, 1980) which is required for GSSG formation while IAA acid forms S-carboxymethyl derivatives by nucleophilic substitution (Camera et al., 2001, Loughlin AF, 2001, Piccoli et al., 1994). The principle of action behind these two thiol scavengers is the same i.e., both of these react with the thiol group in GSH. NEM blocks the reduced sulfhydryl group adding a thiol group across the double bond of maleimide.

Lately a more reliable scavenger, a derivative of 2-VP namely 1-methyl-2 vinylpyridinium trifluoromethane sulfonate (M2VP), a mercaptan scavenger, that completely scavenges GSH in less than 1 min has been identified (Fu and Murray, 2001). The chemical design of the mercaptan scavenger is to bind with the free thiol group as shown in the reaction below (Figure 3.2).

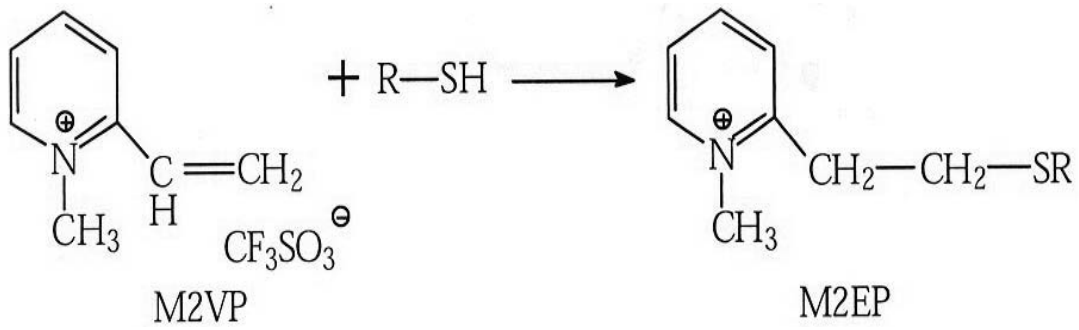


Figure 3.2. Reaction of M2VP with thiol forming 1-methy l-2-(2-thioethyl)-pyidinium salt.

The aim of the current study therefore, was to develop a reverse phase HPLC method for determination of tGSH and GSSG with fluorescence detection using OPA. In order to avoid GSH auto-oxidation, GSSG was measured using a mercaptan scavenger

M2VP. The concentration of GSH was determined by subtracting tGSH and GSSG levels and calculating the ratio GSH/GSSG under normal physiological conditions.

3.2. Material and methods

The materials used for this study are fully listed in section 2.1.2. The equipment conditions are described in section 2.2.2. The sampling procedure has been described in method section 2.6.4, whilst the optimised method is described in detail sections 2.6.5 and 2.6.6.

3.3. Results

3.3.1. Preliminary tests for glutathione

The initial analysis for the assay involved a simple step of derivatizing GSH with OPA. Sodium phosphate solution was added to neutralise the derivatized solution and directly taken for HPLC analysis. An isocratic elution with 50 mM sodium acetate with 5% acetonitrile was used to analyse the GSH-OPA derivative. The GSH-OPA peak was detected at 11 min (Figure 3.3). GSH solution were made in 0.01M HCl. Mild acidic condition for the preparation of standards can possibly minimize auto-oxidation of GSH and aids to protonate the reactive thiolate anions.

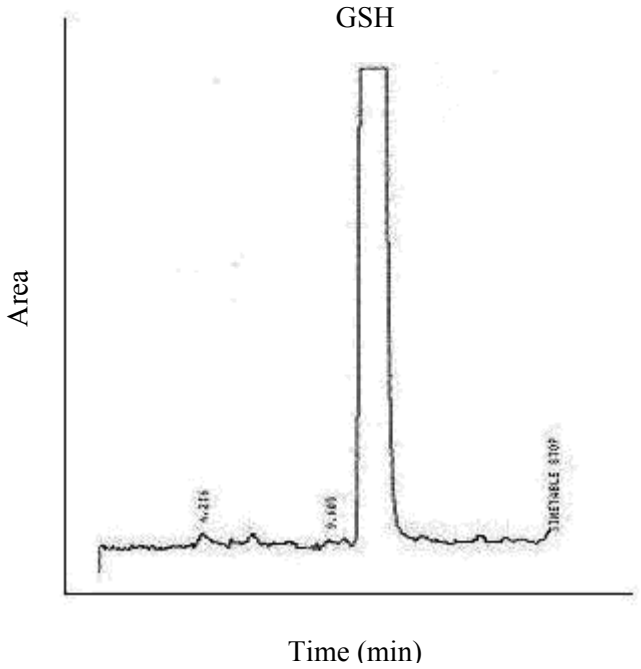


Figure 3.3. Sample chromatogram showing GSH peak (10 mM).

The concentration of GSH present in blood is approximately 10 mM. Hence, a calibration line was prepared to cover the entire range of expected concentrations (10 mM- 1mM) in whole blood (Figure 3.4) (See appendix Table 3.1).

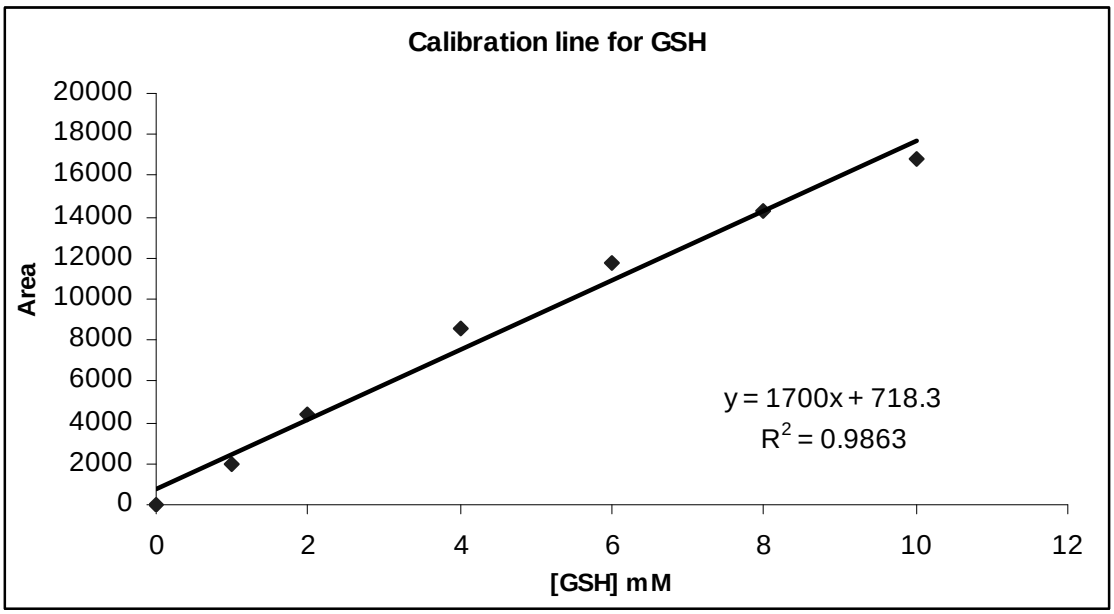


Figure 3.4. Representative calibration line for GSH to show the linearity of the assay over a range of concentrations (10 mM-1 mM).

3.3.2. Internal standard selection

An internal standard is a known amount of standard substance that is added in the assay, which can negate some of the problems with changes brought about, by the detector response, non-eluting sample constituents and variable injection volume. A number of criteria were considered prior selecting an appropriate internal standard.

An internal standard should, completely be resolved from other sample compounds, should not be already present in the test sample, should not react/ interfere with other components, should not co-elute with compound of interest, should be highly pure and readily available and should have similar physiochemical properties as the analyte(s).

As the method involved derivatization with OPA, an internal standard that can also derivatized with OPA was chosen. Two compounds were considered as potential internal standards and their chromatographic behaviour were studied. These were; mercaptopropionylglycine (MPG) and glutathione ethyl ester (GEE).

However, MPG (α-ketothiol) gave very low fluorescence intensity with OPA (Figure 3.5) due to low quantum yields of derivatives (Sano and Nakamura, 2001). GEE, on the other hand, was considered as a potential internal standard due to its structural proximity with GSH. However, GEE-OPA eluted at the same time as GSH-OPA peak under isocratic conditions (Figure 3.6).

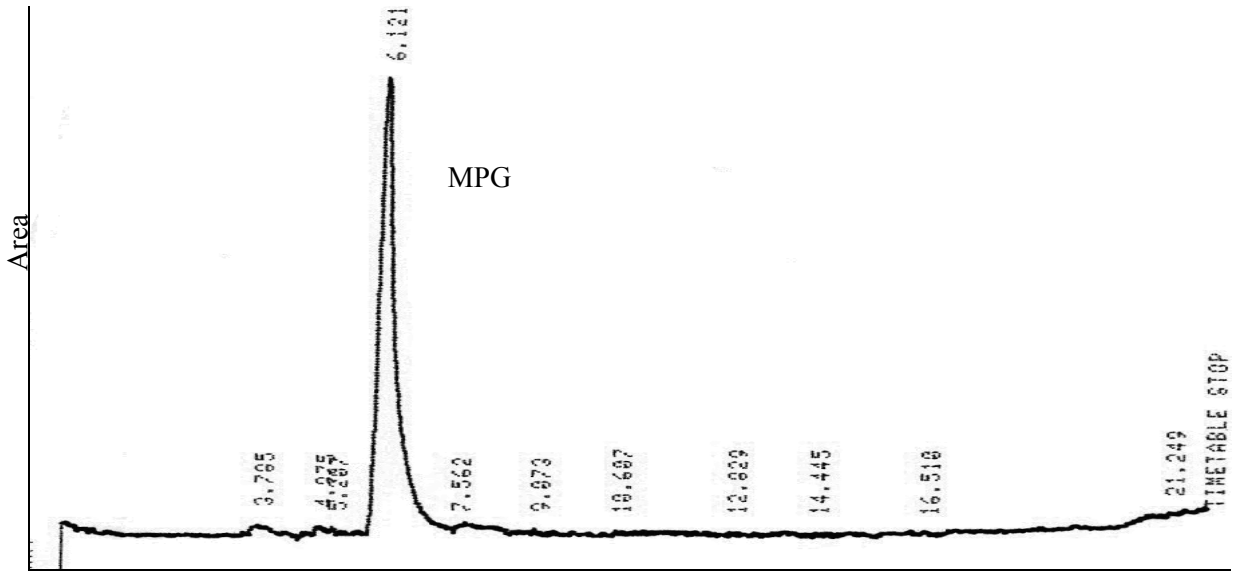


Figure 3.5. Sample chromatogram of MPG (100 μM).

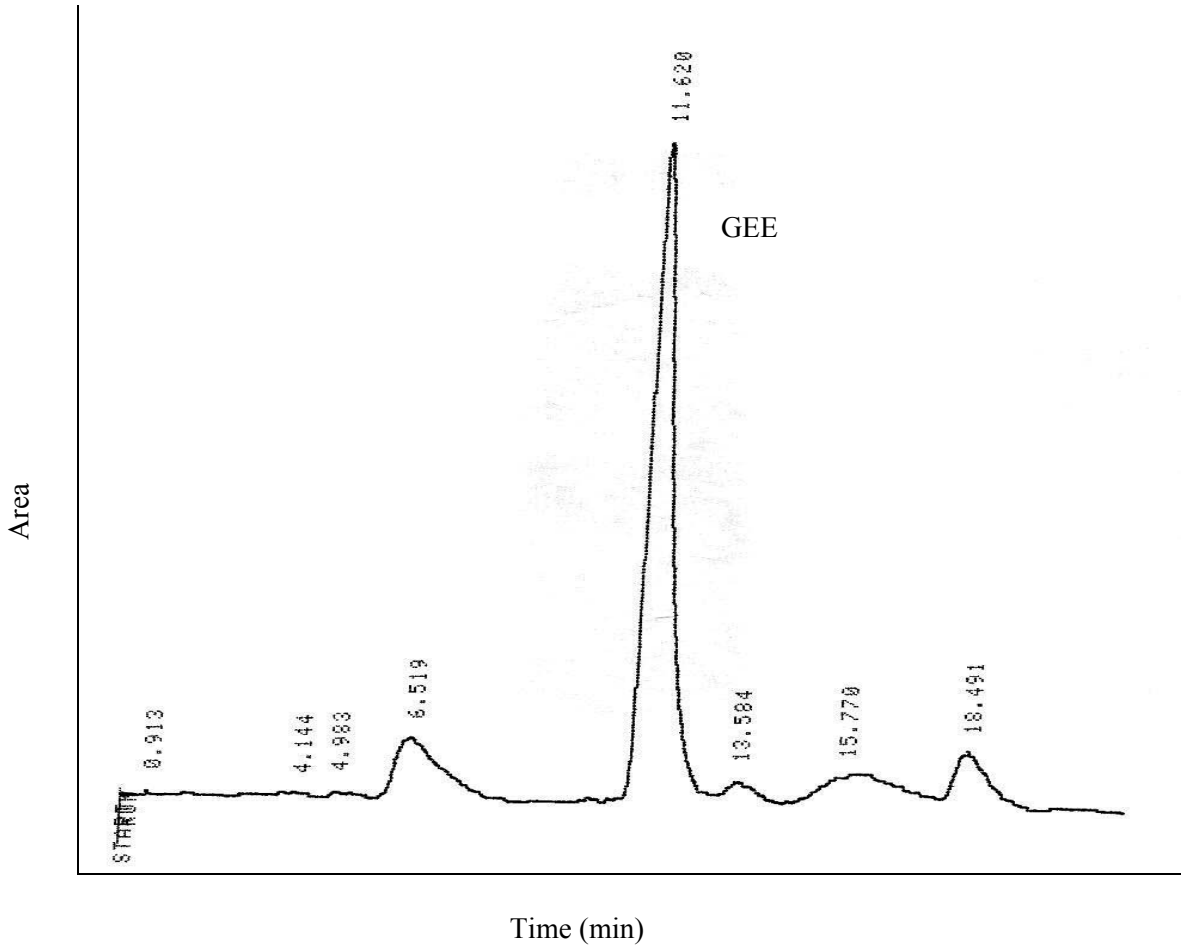


Figure 3.6. Sample chromatogram of GEE (100 μM)**.**

3.3.3. Effect of mobile phase pH on the separation of GSH and GEE

To overcome the problem of co-elution and to obtain adequate separation between GSH and GEE, a study of buffers with varying pH were undertaken. A very low pH (less than pH 5) was avoided as it could possibly lead to alterations in the fluorescent properties of the analyte i.e. shift the excitation and emission wavelengths of the analyte which, can occur due to stabilization or destabilization of electronic structure (Sadek, 1996).

In order to test different pH during the experiments, Buffer A, was 50 mM sodium acetate buffer adjusted to pH 5.8 containing and 5% acetonitrile (95: 5) and Buffer B was 20 mM sodium acetate buffer containing 50% acetonitrile (50: 50) corrected to pH 5.8 were made. A gradient was programmed as follows:

Time (min)	Buffer A 50 mM sodium acetate 5% acetonitrile pH 5.8	Buffer B 20 mM sodium acetate 50% acetonitrile pH 5.8
0	90	10
30	60	40
31	0	100
36	0	100
37	100	0
45	100	0

GSH eluted at 6.2 min and GEE appeared as a shouldering peak with non-derivatized OPA at 12.6 min. (Figure 3.7).

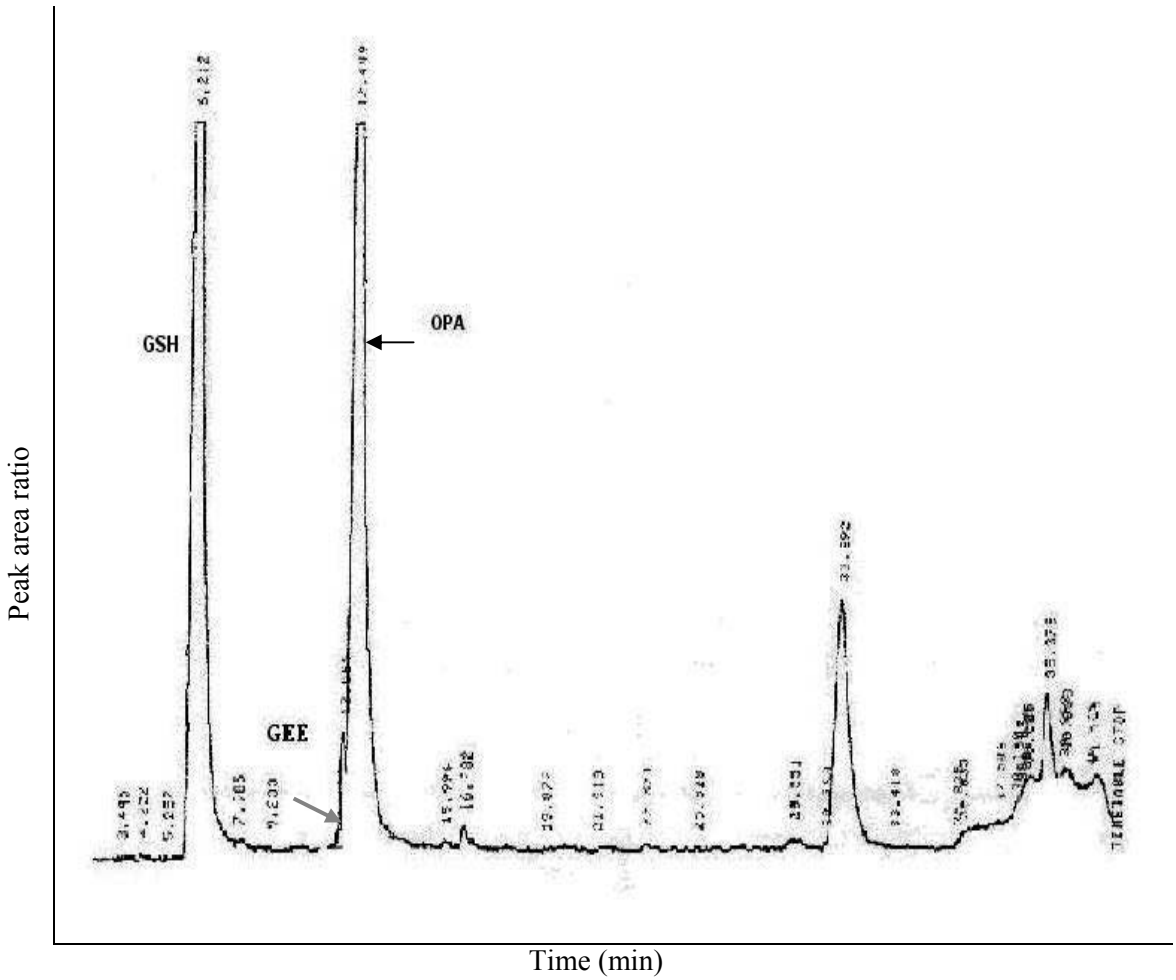


Figure 3.7. Sample chromatogram showing the separation of GSH from GEE at pH 5.8. GEE appearing as tail peak with OPA (Red arrow).

This experiment indicated that the acidity or the pH of the mobile phase was an important factor that could possible effect OPA derivatization and GEE separation. As a result, buffers with pH 5.8 were discarded. As a solution to this problem, low pH (<5.0) and very high pH (> 8.0) were avoided as the pH could possibly cause cleavage of OPA and also break down of C18 chains in the stationary phase and damage the stationary phase support (Walshe et al., 1995). Studies undertaken by changing the pH of buffers beginning from pH 5.0 to pH 5.8 did not produced any conclusive results in terms of separating GSH from GEE.

Cereser et al., (2001) used a pH of 6.20 with acetonitrile as the organic modifier for the analysis of GSH. Therefore, method development was continued using pH 6.20. However, the separation between GSH and GEE was still not achieved even with pH 6.20. Therefore, further analysis was continued addressing the role of the organic modifier with regard to GSH and GEE separation.

3.3.4. Separation of GSH and GEE using a gradient system

Two buffers were made (Buffer A and Buffer B) of sodium acetate 3- hydrate (50 mM) with pH 6.2. A number of gradient systems with various percentages of acetonitrile (v/v) were tested aiming to separate GSH and GEE. The effect of each of the gradients along with comments are discussed below;

Gradient table 1- Buffer A contained acetate buffer with 5 % acetonitrile (950:50), while Buffer B contained acetate buffer with 10 % acetonitrile (900: 100) (Figure 3.8).

Time (min)	% Buffer A 5% acetonitrile	% Buffer B 10% acetonitrile	RT* of GSH (min)	RT* of GEE (min)
0	100	0	8	17
20	80	20		
25	0	100		
30	0	100		
32	100	0		
40	100	0		

*RT- Retention time

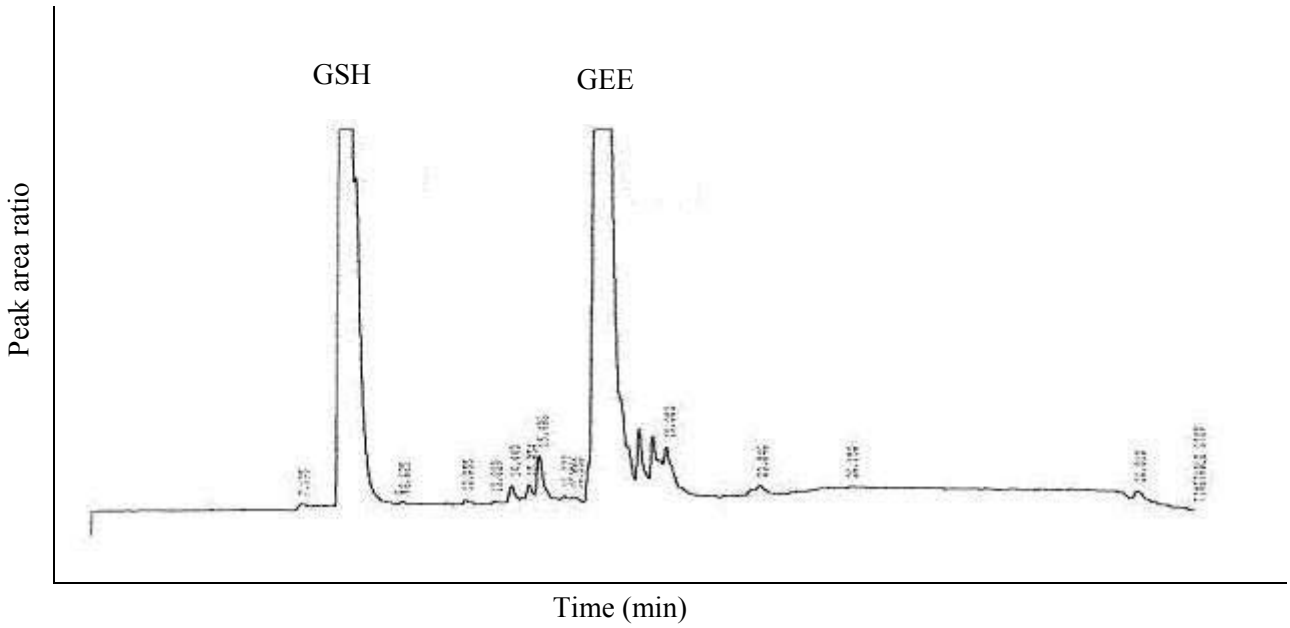


Figure 3.8. Sample chromatogram for gradient 1.

Although, GSH and GEE were separated with gradient 1, the time difference between GSH and GEE appeared to be vey long and there were unwanted tailing as GEE eluted. Further, the run time (40 min) was too long for to be consider for routine analysis. To seek a solution, the volume of acetonitrile in both the buffer A and buffer B was increased and gradient 2 was programmed as shown below.

Gradient table 2

Time (min)	% Buffer A 10% acetonitrile	% Buffer B 50% acetonitrile	RT of GSH (min)	RT of GEE (min)
0	100	0	8	13
12	80	50		
20	0	100		
30	0	100		
33	100	0		
40	100	0		

Increasing the % acetonitrile in both the buffers resulted in the separation of GSH and GEE and the time difference between the GSH (8 min) and GEE (13 min) was considerably smaller (Figure 3.9). However, the runtime still considered long.

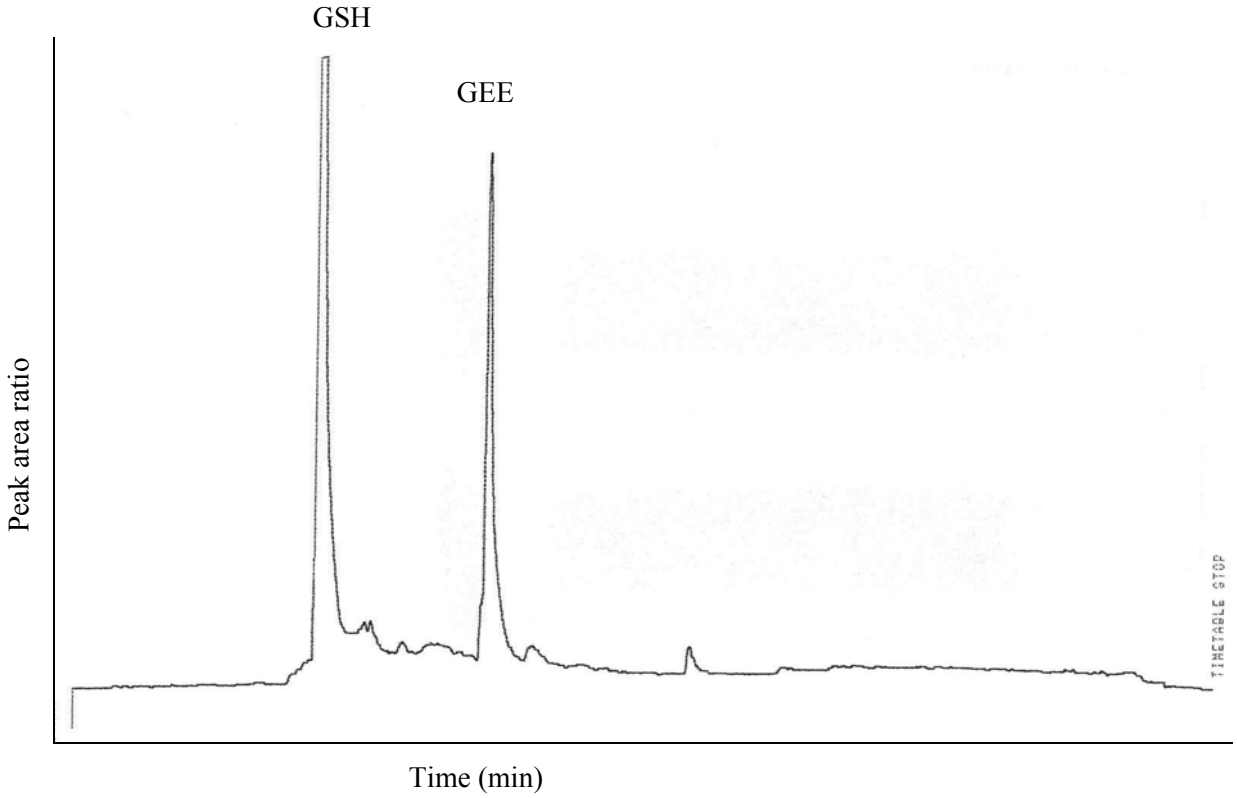


Figure 3.9. Sample chromatogram for gradient 2.

Gradient table 3

Further amendments were made in the % acetonitrile used. Buffer A containing 50 mM sodium acetate, pH 6.2 with 10% acetonitrile and Buffer B containing 50 mM sodium acetate, pH 6.2 with 80% acetonitrile.

Time (min)	% Buffer A 10% acetonitrile	% Buffer B 80% acetonitrile	RT of GSH (min)	RT of GEE (min)
0	90	10	4.2 min	11.8 min
10	80	20		
15	50	50		
16	0	100		
20	0	100		
21	100	0		
29	100	0		

Gradient system 3 gave the best results with shorter run time and good separation of GSH and GEE (Figure 3.10). Hence, this gradient was used for the estimation of blood tGSH and GSSG.

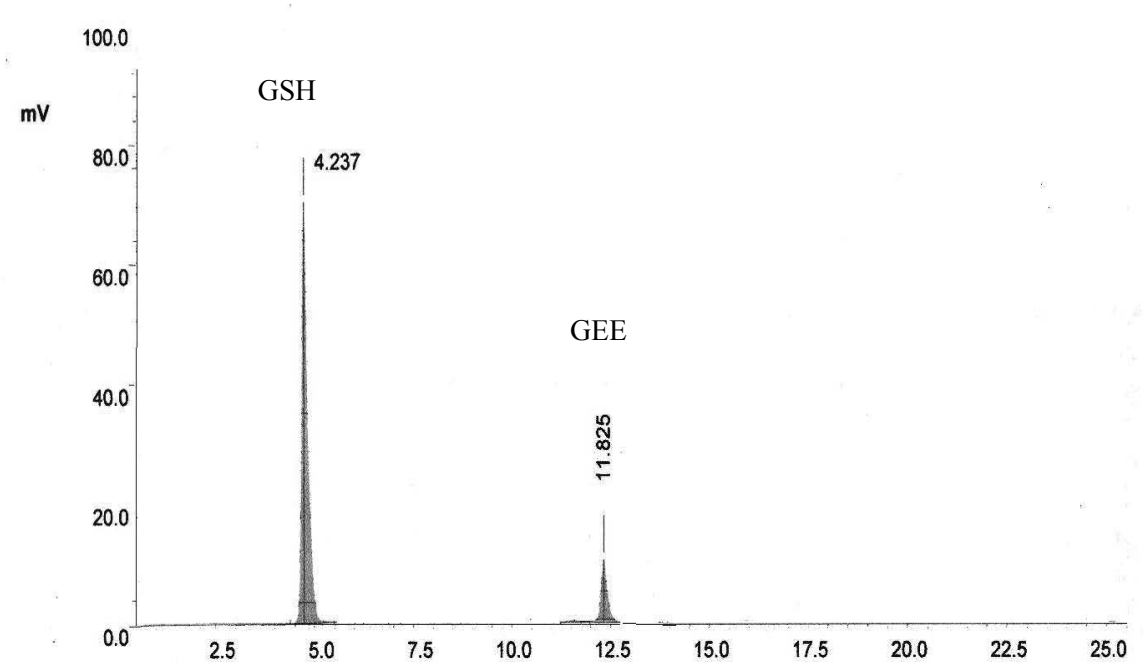


Figure 3.10. Sample chromatogram run with gradient 3. GSH peak from blood at 4.2 min and GEE at 11.8 min.

With the suitable gradient in place, standard calibration lines were constructed over a range of concentrations of GSH (10 mM -1 mM) (Figure 3.11).

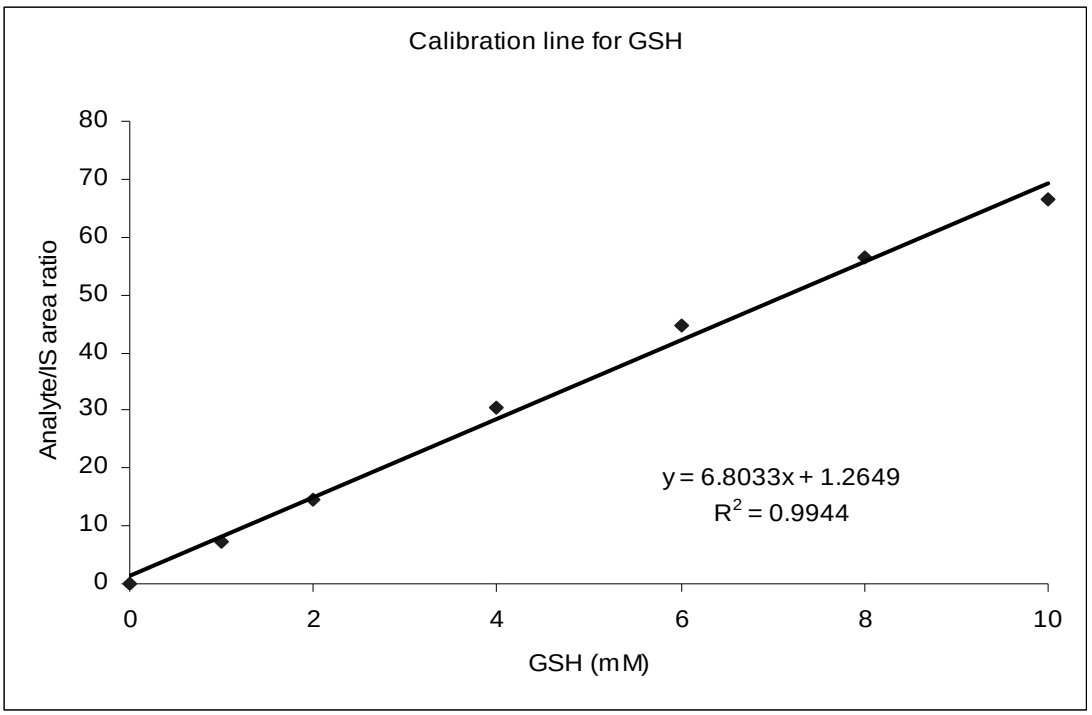


Figure 3.11. Representative calibration line showing linear range for GSH (10 mM- 1 mM).

3.3.5. Practical problems with *o*-phthaldialdehyde reagent

The commercially available OPA reagent showed signs of decomposition in the form of peaks co-eluting with GEE. It was suggested that it could be due to inappropriate storage conditions and manufacturers recommended, that OPA reagent needed to be flushed with nitrogen gas before and after usage. In order to overcome this problem, OPA powder was used. A 5 mg/mL OPA solution was made fresh for every time using 0.4 M potassium borate buffer (pH 9.90).

3.3.6. Analysis of oxidized glutathione (GSSG)

3.3.6.1. Choice of reducing agent

Under normal physiological conditions, the concentration of GSSG in blood is found in micro molar (μM) levels. In order to measure μM levels of GSSG, a calibration line for

GSSG was constructed from a 1 mM solution of GSSG prepared in 0.01M HCl. GSSG does not react with OPA due to the presence of a disulfide bond. Hence, a reducing reagent was needed that would chemically reduce the disulphide bond and free the thiol groups making them available for OPA derivatization. This reduction was performed using 1, 4 dithiothreitol (DTT). Choosing an appropriate DTT concentration was critical for assay performances as incomplete reduction can result in inaccurate data. DTT was preferred as it was the choice of most reports (Paroni et al., 1995, Cereser et al., 2001). It possesses two thiol groups and two hydroxyl groups (Figure 3.12).

Thiols can be kept in reduced state in presence of having excess of another thiol as thiols readily exchange with disulphides (Cleland, 1963). The thiol groups in DTT have a pKa value of 9.2 and 10.1 respectively (Singh et al., 1995). Furthermore, DTT can reduce disulphides rapidly (Cleland, 1963). At certain concentrations above 1 mM, DTT can prevent oxidation of thiols (Kachur et al., 1997, Netto, 1996). With GSH being highly prone to oxidation, a reducing agent like DTT was ideal for reduction reaction. Also, DTT has low redox potential (-0.33 volts at pH 7), it has the capability of maintaining monothiols like GSH in completely reduced state and quantitatively reduce disulphides (Cleland, 1963). Hence, DTT was considered very suitable for the reduction reaction.

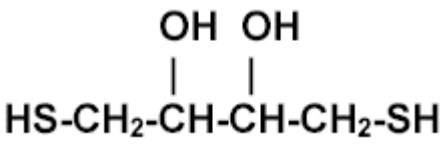


Figure 3.12. Chemical structure of DTT.

3.3.6.2. Choosing appropriate concentration for DTT reaction

In order to identify the optimum concentration of DTT required for reducing the disulphide in GSSG, a dose response curve for DTT with GSSG was performed. A 0.5

mM GSSG solution was made in 0.01M HCl. Various concentrations of DTT were then added (50 mM-10 mM stock solutions). DTT at different concentrations without any standards were run in parallel in order to assess the extent of interference with the assay, as DTT also possess –SH group (See appendix Table 3.2).

On analysing results, fluorescence increased up to a concentration of ≥ 25 mM (15 mM working solution) before reaching a plateau at or after a stock concentration of ≥ 30 mM. Furthermore, at higher DTT concentrations, the -SH group in DTT slowly interfered with the assay components and with GSH-OPA adduct formation. Interferences in the form of unidentified peaks along with peak tailing were also observed. Hence, 15 mM of DTT was chosen for routine analysis (Figure 3.13).

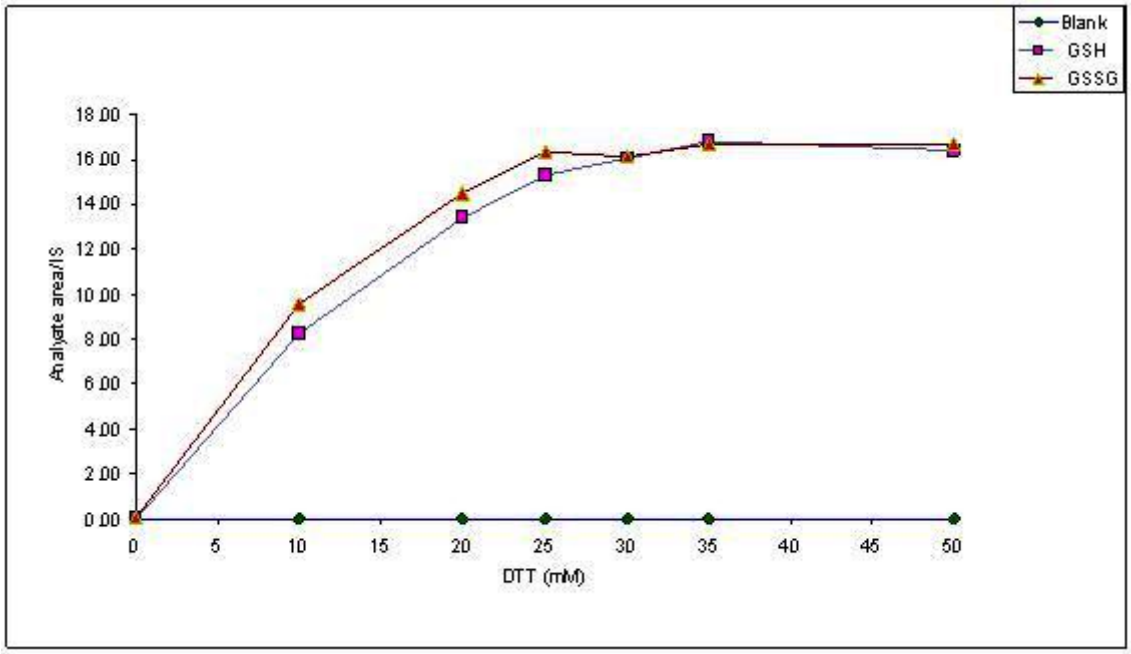


Figure 3.13. Dose dependent study with DTT to select an appropriate concentration for the reduction of GSSG.

Thus, a calibration line for GSSG with 25 mM DTT was constructed at the range of 1 mM -10 µM (Figure 3.14) (See appendix Table 3.3).

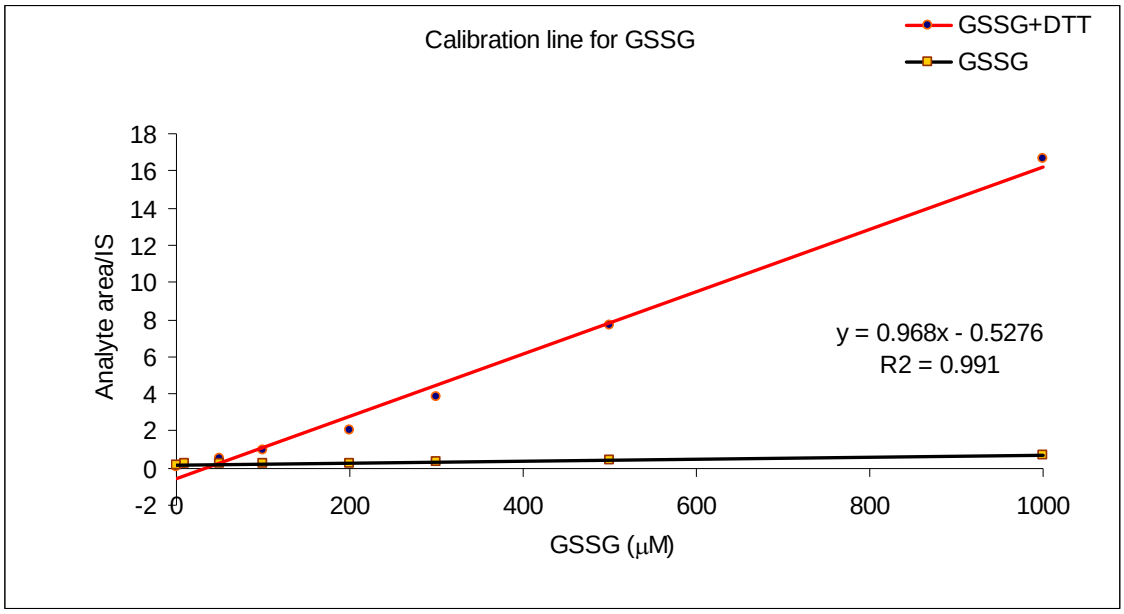


Figure 3.14. Calibration line showing linear range for GSSG in the presence and absence of DTT (25 mM).

3.3.7. Calibration line for total glutathione

As the present method was an approach to determine tGSH and GSSG in whole blood, a calibration line for tGSH was considered. Total glutathione (tGSH) is a mixture of GSH and GSSG with concentration of GSH being greater than GSSG. Determination of tGSH requires DTT reduction of GSSG present in the biological sample. Hence, a calibration line for tGSH was constructed.

A range of different concentrations of standard GSH and GSSG (5 mM - 1 mM) were prepared. Equal volumes of GSH and GSSG were thoroughly mixed, reduced with of 25 mM DTT and derivatized. This mixture of equal volumes of standards was done to mimic the condition in the blood. A sample blank was run with only DTT and without standard mixture (Figure 3.15) (See appendix Table 3.4).

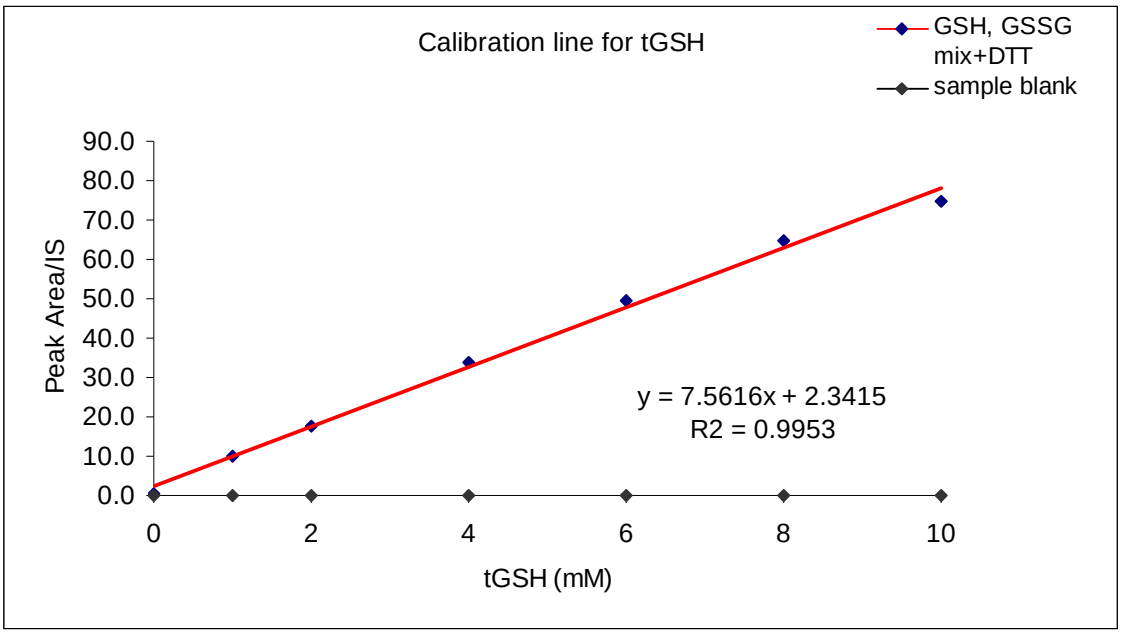


Figure 3.15. Calibration line for tGSH in the presence and absence of DTT.

3.3.8. Choosing a suitable M2VP and NEM concentration for scavenging

In order to achieve a complete scavenging of the thiol moiety, it was critical to choose an appropriate concentration of the scavengers to achieve accurate measurement of GSSG in the biological samples. To achieve complete scavenging of thiol moieties, a series of concentrations of M2VP and NEM were made (1 mM-15 mM). These were later, mixed with a series increasing concentrations of GSH (1 mM-10 mM).

A low concentration of M2VP or NEM mixed with a high concentration of GSH allows determination of the un-quenched GSH. As the concentration of the scavengers was increased proportionally from 1 mM to 15 mM, GSH, was detected which shows the scavenging capabilities of M2VP or NEM.

On analysing the results, a concentration from 5 mM to 15 mM M2VP stock solution was effective in scavenging GSH. Furthermore, increasing concentration of M2VP did not show interferences with other assay components (Figure 3.19) (See appendix Table 3.5).

On the other hand, NEM showed slightly different responses when compared to M2VP. NEM had similar responses as M2VP at low concentration. However, interferences proportionally increased as the concentration of NEM was increased during repeats in two individual occasions. At very high concentration of NEM (15 mM), board unresolved peaks appeared at the same retention time as GSH with several other disturbances in the chromatogram (Data not shown).

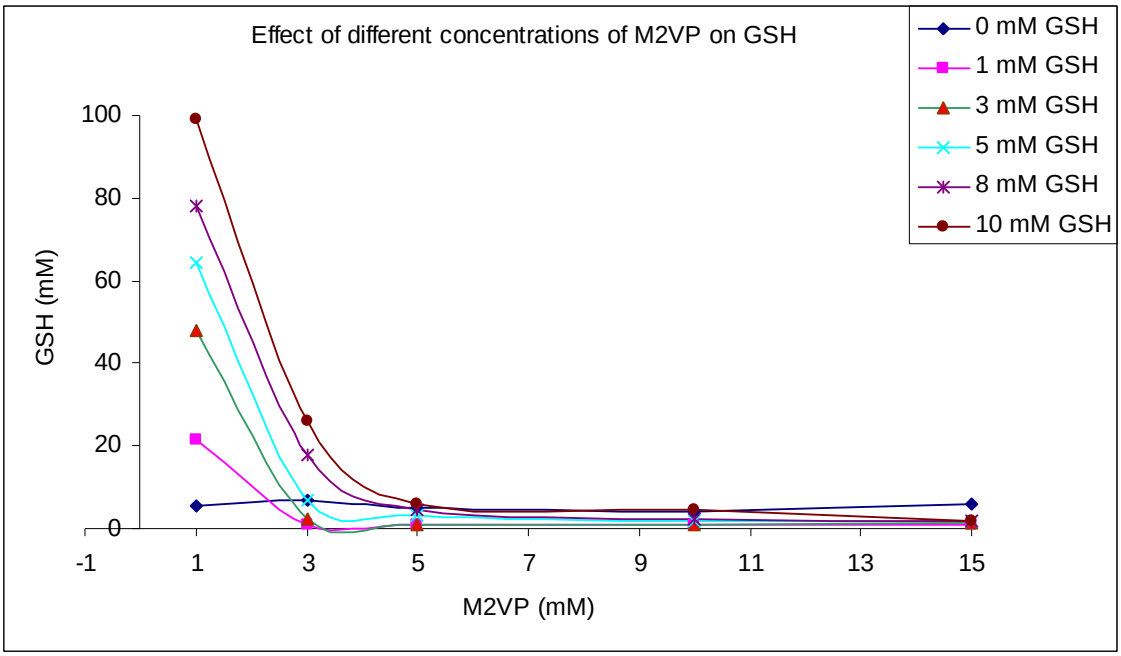


Figure 3.16. Effect of various concentrations of M2VP on GSH.

3.3.9. A comparative study of two scavengers

The present study was aimed determine tGSSG using a novel mercaptan scavenger M2VP. Trapping the thiol moiety of GSH, is considered necessary to measure GSSG. NEM is another trapping agent used for thiol moiety. Hence, M2VP and NEM were studied in comparison.

In order to test the efficiency of the scavengers, an analysis was performed with whole blood to compare the scavenging capabilities of M2VP and NEM. A 3 mM solution of M2VP and NEM were made in water separately. A 100 µL blood sample was collected

in vials containing the scavenger, left for 1 min to for the trapping the –SH groups, followed by protein precipitation and centrifugation. A 100 µL acidic supernatant was mixed with water (to compensate for missing components such as DTT, TRIS) and then taken for derivatization with OPA followed by neutralization with phosphate buffer. The following figure shows the scavenging effect of M2VP and NEM on trapping blood GSH (Figure 3.17).

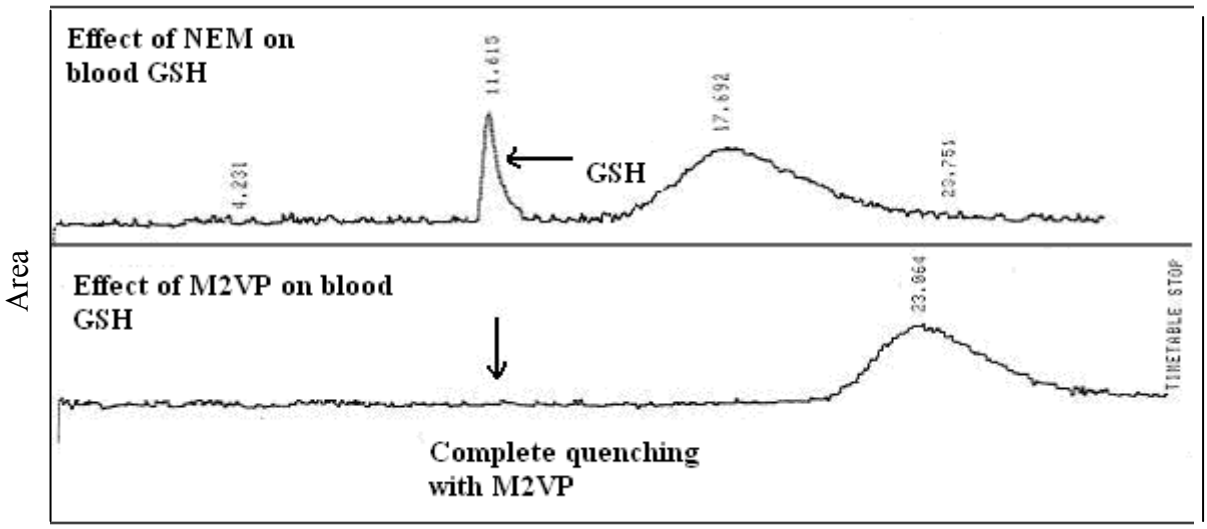


Figure 3.17. Scavenging effect of M2VP and NEM on blood GSH.

Figure 3.17 showed that the scavenging capabilities of M2VP and NEM were different. An assay was designed to compare scavenging capabilities of the two scavengers in standard solutions.

A range of different concentrations of standard GSH and GSSG (500 µM - 10 µM) were prepared. Equal volumes of GSH and GSSG were mixed, reduced and derivatized in the absence of any scavenger to measure tGSH.

A range of different concentrations of GSSG (500-10 µM) was made and mixed with M2VP and NEM separately, reduced with DTT and derivatized to measure GSSG (this assay was done with and without DTT). Finally, a single concentration GSSG (250 µM) was analysed in the absence of any scavenger.

Theoretically, this assay should produce five lines when in graph:

1. tGSH, 2. GSSG in the absence of any scavenger and in the absence/presence of DTT, 3. tGSSG in the presence of M2VP, 4. GSSG in the presence of NEM and 5. A blank (without DTT)

1. Line for tGSH with DTT without any scavenger

Solutions of various concentrations GSH and GSSG with were assayed for tGSH after reduction with DTT. In this assay, there is complete reduction of GSSG to GSH followed by determination of GSH equivalents to form tGSH. Thus, the peak area obtained for tGSH corresponds to whole blood tGSH.

2. Line for GSSG in the absence of scavenger, with and without DTT

A solution of GSSG (250 μM) was assayed for GSSG with and without DTT and in the absence of any scavenger. Theoretically, result obtained, should roughly be half the concentration of tGSH in the absence of any scavenger. The line without DTT is the sample blank.

3. Line for GSSG after scavenging with M2VP, reduction with DTT

Standard solutions of GSH and GSSG at various concentrations were made (500-10 μM) and mixed in equal proportions (the actual concentration is half of the original concentration). M2VP solution (15 mM) was made and mixed with standard mixture, reduced and derivatized. They were then assayed for GSSG. Theoretically, M2VP should scavenge GSH in the mixture resulting in the determination of GSSG.

4. *Line for GSSG after scavenging with NEM, reduction with DTT*

Standard solutions of GSH and GSSG at various concentrations were made (500-10 μM) and mixed in equal proportions (the actual concentration is half of the original

concentration). NEM solution (15 mM) was made and mixed with standard mixture, reduced and derivatized. They were then assayed for GSSG. Theoretically, NEM should scavenge GSH in the mixture resulting in the determination of GSSG.

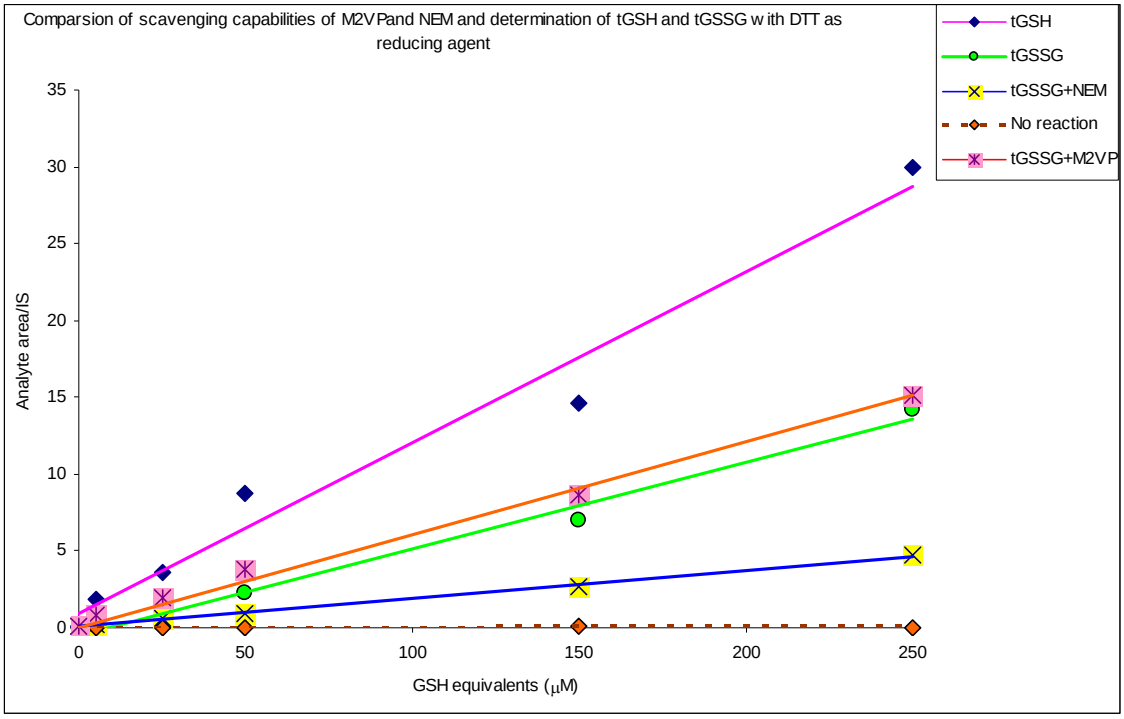


Figure 3.18. Comparison of scavenging capabilities of M2VP and NEM.

It was noted from Figure 3.18, that in an equimolar mixture of GSH and GSSG, assayed for GSSG in the presence of M2VP and NEM, M2VP rapidly scavenges the thiol group of GSH without much interfering with the assay derivatives (red line). This resulted in the estimation of GSSG. The amount of GSSG estimated this way was similar to the GSSG obtained without adding M2VP (green line). Therefore, M2VP allowed the estimation of GSSG and showed complete scavenging of GSH. In addition, M2VP has not shown any interference with OPA or DTT during reduction nor has obscured the estimation of GSSG.

NEM, although a potent thiol group scavenger, showed some interference in the form of unwanted peaks. NEM binds the –SH group in GSH, slows the scavenging process thereby yielding lower quantities of GSSG (blue line) when compared to GSSG

obtained without any addition of scavenger (green line). The closer the blue line is with green line, more efficient the scavenger is. The line tGSH (pink line) shows 100% reduction and a measurement of tGSH.

The line, over lapping/ parallel to the X axis corresponds to GSSG obtained in the absence of any DTT. This is a reference line to show that disulphides cannot be detected in the absence of a reducing agent. As mentioned elsewhere, without DTT reduction, the disulphides are not reduced and there is no derivatization of the disulphide with OPA (Figure 3.18) (See appendix Table 3.6).

3.3.10. Comparison of the scavenging capacities of M2VP and NEM

This assay shows scavenging capabilities of the two scavengers with an increasing concentration of GSH. A 1 mM GSSG standard solution was made. 15 mM M2VP and 15 mM NEM solutions were made separately and mixed with a series of increasing amounts of GSH standard solutions (5- 2000 μM) separately. This reaction mixture was then assayed for GSSG following reduction and derivatization (See appendix Table 3.7).

Theoretically, the recovery of GSSG must be constant and quantitative, indicating complete quenching of GSH. On comparing the data of the recovered GSSG, it was observed that NEM showed a gradual increase in the amount of GSSG detected as the concentration of GSH was gradually increased. This possibly shows an incomplete scavenging of GSH in presence of NEM. Furthermore, it was also noted that NEM interfered with the assay as its concentration was increased. On the other hand, M2VP showed a steady scavenging of GSH (Figure 3.19). This illustrates that the recovery of GSSG with M2VP scavenging is more consistent when compared with NEM scavenging.

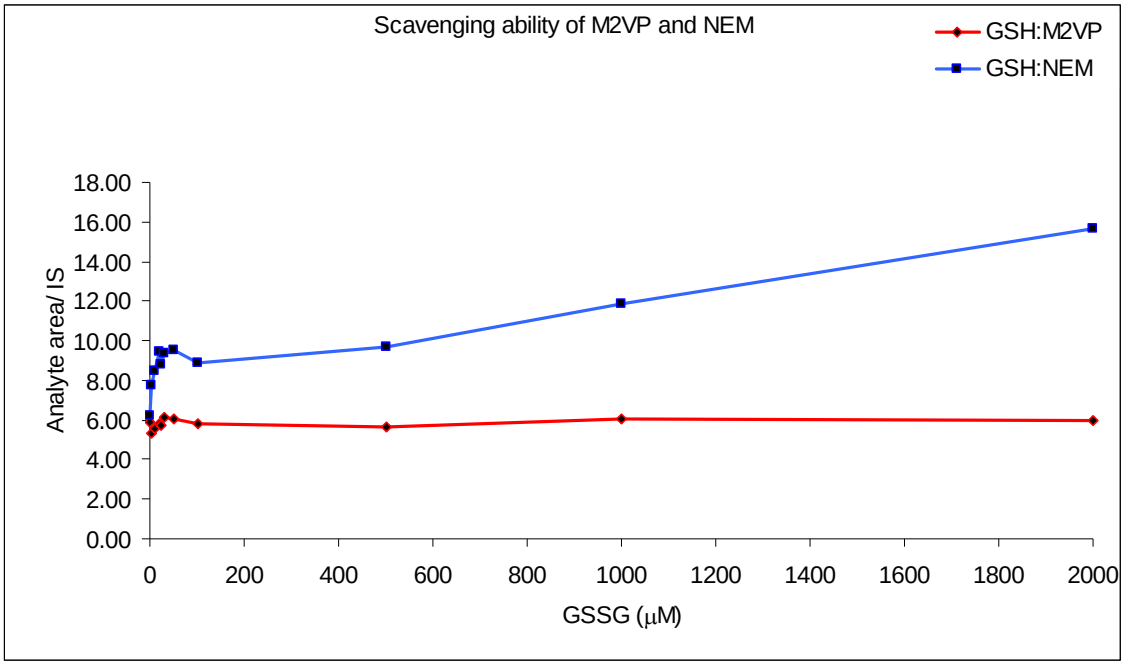


Figure 3.19. Scavenging ability of M2VP and NEM.

3.3.11. Stability of metaphosphoric acid extracts

Blood proteins were precipitated using MPA as reported (Stempak et al., 2001). A 6% MPA solution was used to acidify the samples. Proteins were precipitated after centrifugation. The MPA extracts stored at stored at 4ºC, -20ºC and at -80 ºC, were periodically removed and derivatized with OPA to determine blood GSH for 7 days. The GSH concentration in the acidic supernatant was assessed and considered as 100% just before storage. Results showed that the GSH acidic supernatants were more stable at -80ºC and -20ºC than at 4 ºC for 1week (Figure 3.20).

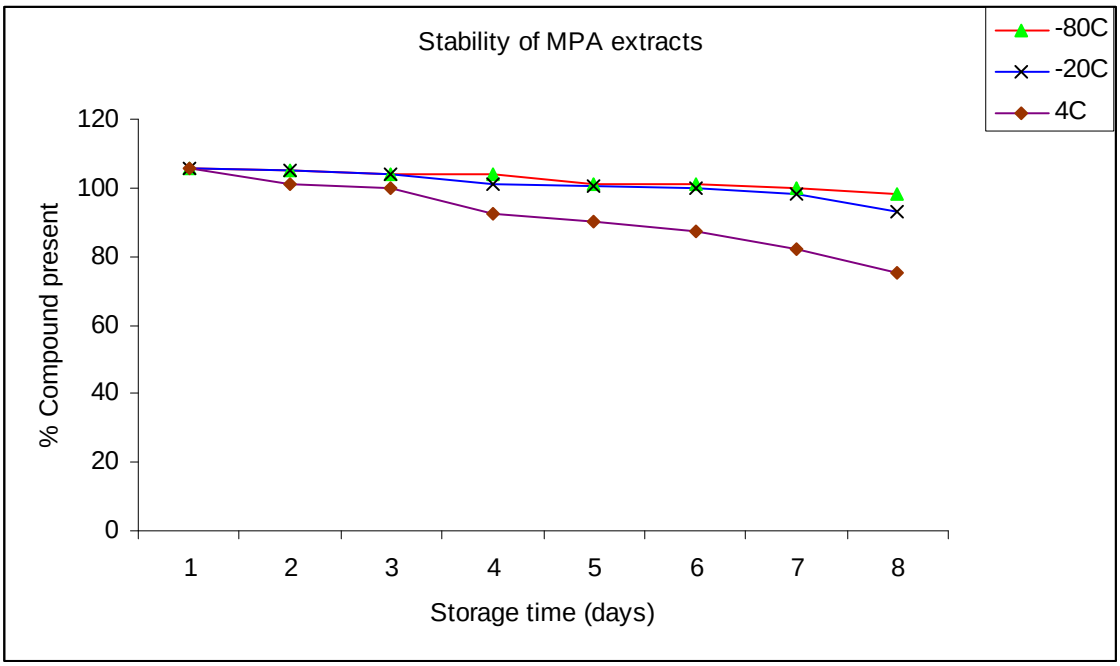


Figure 3.20. Stability of MPA extracts at -80 ºC, -20 ºC and 4 ºC.

3.3.12. Stability of glutathione-*o*-phthalaldehyde adducts at room temperature, 4ºC and at -20 ºC

It was also noted that GEE (internal standard) hydrolysed under inappropriate storage conditions (to be stored at -20ºC with para film). The hydrolysed by-product eluted at the same time as GSH. Hence, it became mandatory to make GEE solution fresh on the day of analysis.

In addition, appropriate stability studies for GSH-OPA adducts were conducted to assess this stability. A standard solution of GSH was prepared immediately before analysis and this was taken as time 0 and day 1. A 5ml aliquot of the standard solution was transferred to pre-labelled eppendroff tubes and then stored under the following conditions: RT, 4^{o}C and - 20^{o}C. Analysis was repeated at intervals of day 1 to day 10. An analysis after 24 hrs revealed derivatized standards stored at room temperature showed significant signs of degradation and were discarded. However, derivatized

standards maintained at 4ºC and - 20^{o}C showed slow degradation as time passed (Figure 3.22). Therefore, derivatization was performed only at the time of analysis.

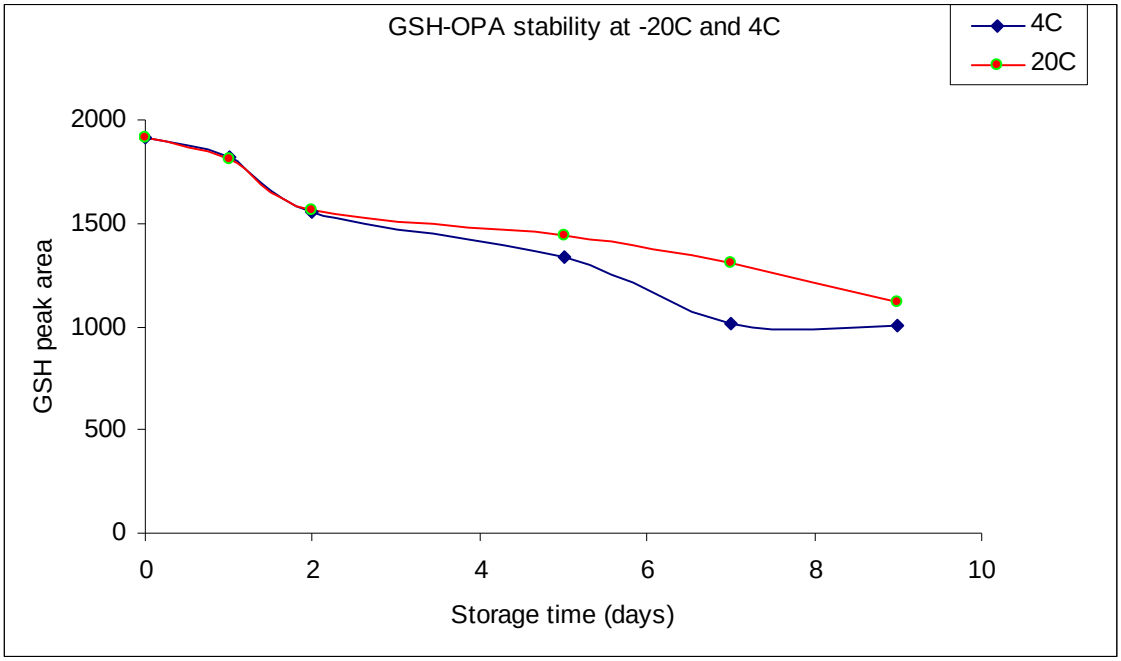


Figure 3.21. Stability of GSH-OPA adducts at -20 ºC and 4 ºC.

3.4. Validation for the analysis of tGSH and GSSG

The assay was validated to determine the linear range, limit of detection (LoD), limit of quantitation (LoQ) and the co-efficient of variation. In developing a bio-analytical method, it is important to make sure that the method is specific for the objective considering the interfering substances in whole blood. Further, with the use of OPA it became imperative to select a suitable volume or concentration of components for the intended analysis. The specificity of the assay was assessed by carrying out the analysis and thoroughly looking for any interfering substances. The retention time of any unwanted peak were compared with standards (GSH and GSSG).

Linearity

The linearity in this validation study involved the analysis of six calibration points prepared for tGSH and GSSG that covered the entire range of expected concentrations

including the limit of quantitation. Most importantly, sample blanks were also analysed along with calibration points. The assay illustrated excellent linearity from 10 mM to 1 mM for tGSH (Figure 3.20a) and 1mM -10 µM for GSSG assay (Figure 3. 20b) with correlation co-efficient of 0.9953 and 0.991 respectively. The response was directly proportional to the analyte concentration as shown in the Figures 3.20a and 3.20b.

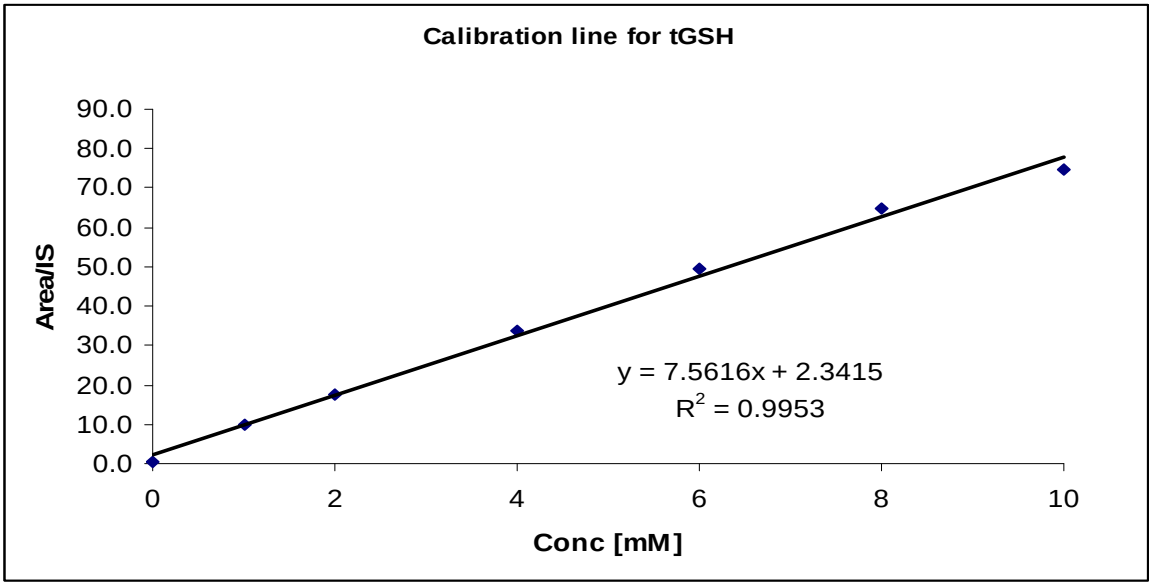


Figure 3.22a. Sample calibration line showing the linear range for tGSH from 1 mM to 10 mM.

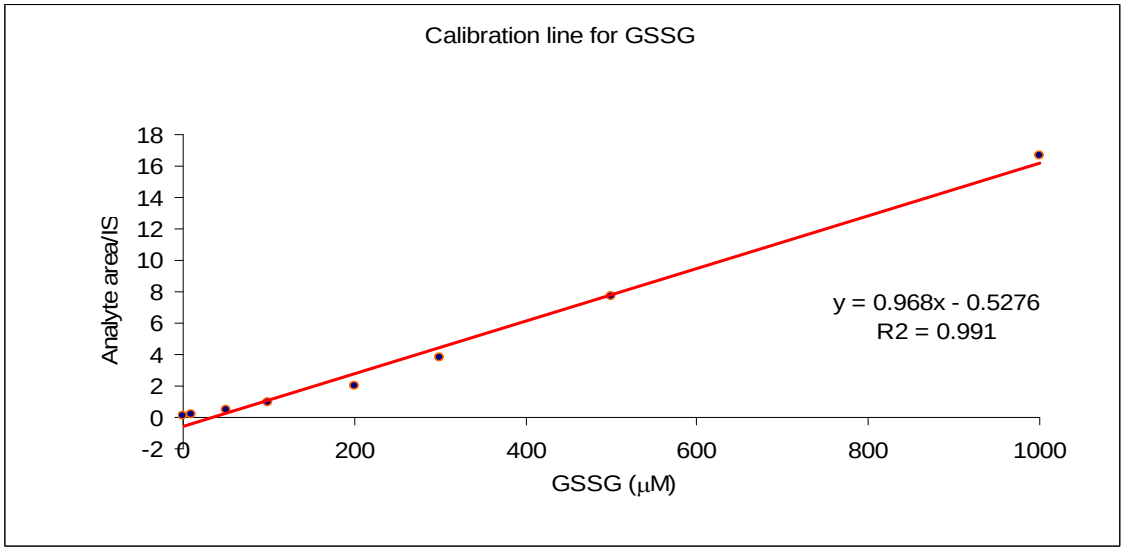


Figure 3.22b. Sample calibration line showing the linear range for GSSG from 10 µM to 1 mM.

Limit of detection and quantitation

The limit of detection (LoD) and limit of quantitation (LoQ) were established through the signal-to-noise (S/N) ratio. Serial dilutions of the GSH solution were prepared, derivatized and analysed. The smallest observable peak area of the GSH-OPA derivative was identified. The limit of detection (LoD) was calculated using a signal to noise (S/N) ratio of 3. The limit of quantitation (LoQ) was considered as the lowest concentration of an analyte measured and is determined by using an S/N ratio of 10. The limit of detection and quantitation was established at 1µg and 5µg respectively for tGSH while that for GSSG was established at 5 ng and 10 ng.

Reproducibility

The co-efficient of variation (CV%) or relative standard deviation was determined using the 500 µM/mL standard GSH. This mid-range standard was injected a total of five times in succession and the co-efficient of variation was calculated by dividing the standard deviation of the five replicates by the overall mean area and converting the value to a percentage. This was measured both intra-assay and inter-assay. The CV/ mean RSD were calculated to be less than 5%.

Precision

The precision was expressed as intra-day co-efficient of variation and inter-day co-efficient of variation. To assess the precision of this assay method, within day (n=10) and between day (n=10) calibration curves with various concentrations of the GSH equivalents were calculated. The precision was expressed as within day and between days of variation and was expressed as RSD (%). The % RSD for within day precision was ±3.51% and ±2.42% for between day precision.

No particular experiments were conducted to assess robustness. However, during the course of validation experiments unidentified variations in the mobile phase were introduced. These variations did not have any conclusive experimental results.

3.5. Application of the assay using whole blood samples

Blood samples were analysed in duplicates. Calibration points for tGSH and GSSG were run in the beginning, in between and at the end of sample runs to assess the performance of the assay and the instrument on the day of analysis. The following flow chart provides detailed information of the assay (Figure 3.21). The results are summarized in Table 3.1. The averages of tGSH and GSSG levels in whole blood from 10 healthy volunteers were as follows: the mean total glutathione was 2.79±0.9 (mM/L); the mean values for total oxidized glutathione resulting from the scavenging effect of M2VP and NEM were 0.9±0.1 (mM/L) and 3.16±1.88 (mM/L) respectively.

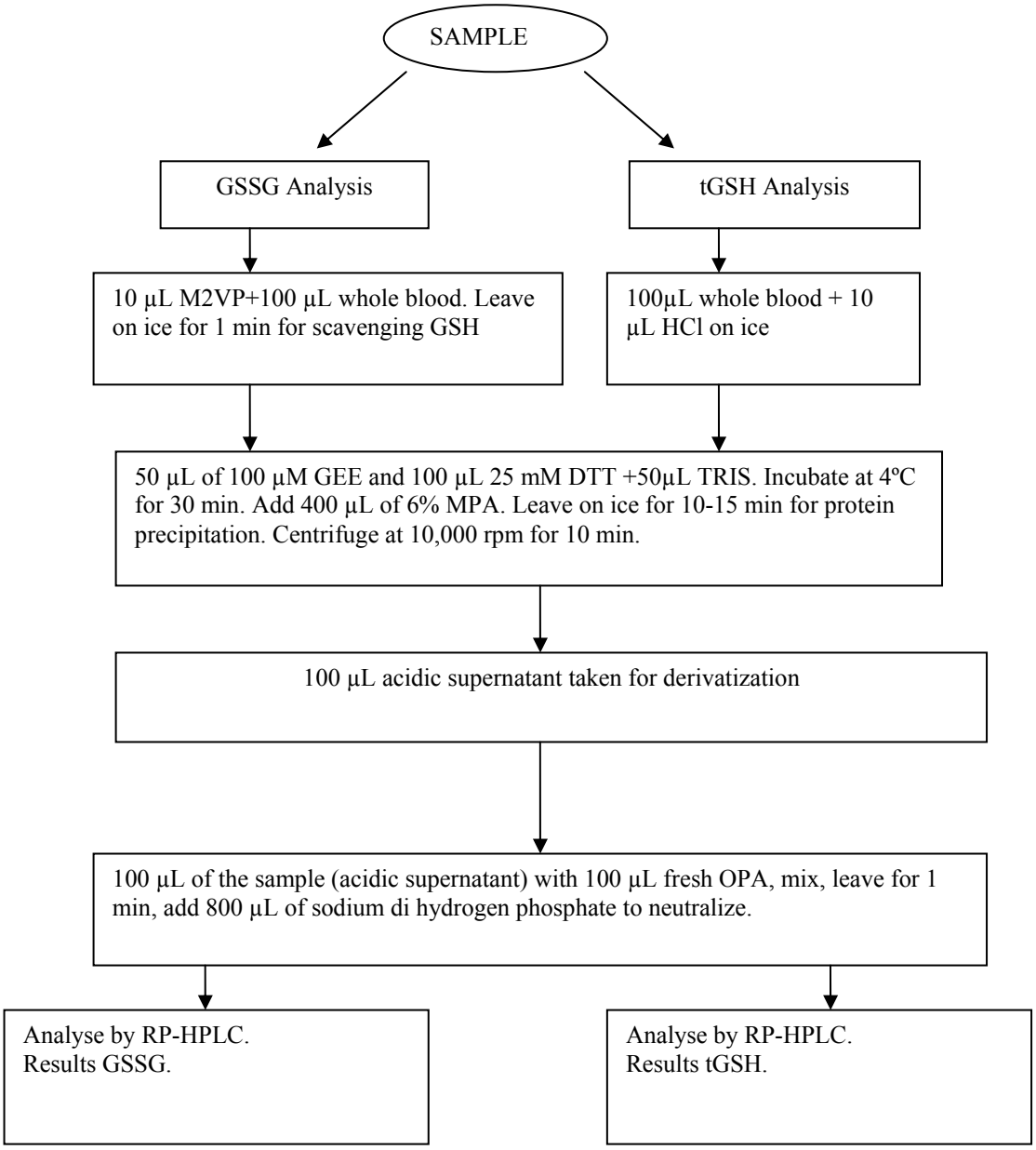


Figure 3.23. A flow chart showing the experimental procedure for GSSG and tGSH in blood sample determination.

Table 3.1. Whole blood GSSG and tGSH levels in healthy human subjects (results expressed as mmol/L)

Subject (n=10)	tGSH	GSSG (M2VP)	GSSG (NEM)	[GSH] [a] (tGSH-GSSG)	[GSH] [b] (tGSH-GSSG)	Ratio GSH:GSSG [a]	Ratio GSH:GSSG [b]
1	2.83	0.93	2.26	1.9	0.57	2.04	0.25
2	4.75	0.97	2.24	3.78	2.51	3.90	1.12
3	3.48	1.2	2.15	2.28	1.33	1.90	0.62
4	3.21	1	2.61	2.21	0.6	2.21	0.23
5	3.14	0.73	2.23	2.41	0.91	3.30	0.41
6	1.88	0.79	5.79	1.09	3.91	1.38	0.68
7	3.79	0.66	2.45	3.13	1.34	4.72	0.55
8	2.63	0.81	2.14	1.82	0.49	2.25	0.23
9	2.19	0.82	2.49	1.37	0.3	1.67	0.12
10	1.35	1.08	2.53	0.75	0.7	0.69	0.28
Mean±SD	2.79±0.9	0.90±0.1	3.16±1.88	2.07±0.91	0.28±1.72	2.41±1.22	0.23±0.50

a- Ratio obtained after M2VP scavenging

b- Ratio obtained after NEM scavenging

3.6. Discussion

The present work focussed on developing a method to determine tGSH and GSSG in whole blood, subtracted the two to get the concentration of GSH, and calculated the ratio GSH/GSSG. A gradient system was used to separate GSH and GEE. As changes in the pH of the buffers produced no practical results in terms of separating GSH from

GEE, the concentration and the compositions of the buffers were changed. In order to achieve an acceptable degree of separation of all the components of interest, a highly polar buffer with 50 mM sodium acetate buffer was used with pH 6.20.

Although, the pKa of amine group in GSH was 9.2, was counter-balanced by bringing down the pH with the use of acetic acid. The acetate ions in sodium acetate buffer acts as a counter ion for $R\text{-}NH_3^+$, avoiding any further ionisation in the column. Further, to favour this, GSH was prepared in 0.01M HCl to protonate the thiolate ions, thus making the conditions favourable for appropriate resolution.

Acetate buffers have a pKa of 4.75 and are the best buffers at a pH close to pK. Further, acetate buffers are used within the pH range of 3.0 - 6.0. Low concentration of acetate buffers tends to give weak and drifting effect of the peaks. This also helped the column performance and avoids column damage. Hence, 50 mM sodium acetate was optimum for good peak shape. Acetonitrile was used as an organic modifier as it is relatively less polar and suitable for minor adjustments of chromatographic profiles. In the absence of any literature support to employ a suitable gradient using GEE as a internal standard and acetonitrile as an organic phase, various percentages (v/v) of acetonitrile were mixed with acetate buffer with pH 6.2 to a get a good separation of GSH from GEE.

The study focussed in determining GSSG by using a mercaptan thiol scavenger- M2VP. The use of thiol scavengers is not a novel approach, as thiol group blockers like NEM, 2-VP, IAA have been used previously to trap the thiol moiety in GSH (Guntherberg and Rost, 1966, Griffith, 1980b). Although NEM, a reactive thiol alkylating agent, can effectively react with free thiol group (Camera et al., 2001, Piccoli et al., 1994), the reaction rate is quiet slow (Shaik and Mehvar, 2006) allowing GSH oxidization in the biological samples and resulting in overestimation of GSSG. Owing to the fact that redox

changes can potentially happen during sample collection, precautions were taken to minimise auto-oxidation of GSH. Samples were collected in EDTA coated vials as EDTA, apart from serving as an anticoagulant, serves as a heavy metal complex that can possibly catalyse oxidation of GSH (Rabenstein and Saetre, 1978).

M2VP and NEM share the same principle of scavenging the thiol moiety (-SH) in GSH. However, no previous HPLC assay for the determination of GSSG using M2VP as a scavenger has been reported. Analyses for tGSH and GSSG in whole blood were performed using a HPLC pre-column derivatization with OPA. OPA was an attractive choice due its mild reactive conditions with both sulfhydryl and primary amino group with less derivatization time. Further, HPLC separation of the derivatized compound enhanced with improved hydrophobicity with a fluorescence compound, is performed in most cases in reverse phase mode (Camera and Picardo, 2002).

Accurate measurement of GSSG has always been complicated as the amount of GSSG in whole blood is low under normal physiological conditions. The determination of GSSG is directly dependent upon the efficiency of the scavenger used. In order to demonstrate the scavenging capabilities of M2VP over NEM, a standard mixture solutions (500-10 μM) were assayed for tGSH and GSSG (with and without scavengers). It was found, that M2VP scavenging was more efficient and quicker than NEM scavenging. The superimposition of GSSG obtained as a result of M2VP scavenging with GSSG obtained without any scavenger suggests that there was no possible oxidation of GSH in blood during the experimental procedure.

NEM, although a potent thiol group blocker is non-specific with thiols (Asensi et al., 1994, Rossi et al., 2002, Giustarini et al., 2003). No doubt, NEM binds the –SH group in GSH but also has a potential to slowly bind to the amino group in the glutamyl moiety in

GSH, thus making the scavenging process slow. There are also possibilities that NEM can bind with the amine groups, which in turn can contribute to the loss of NEM making it less available for scavenging thiol group. Thus, NEM directed GSSG estimation from the standard mixture (500-10 μM) seems to be low when compared to GSSG estimated without any scavenger.

DTT was used as a reducing agent. DTT reduces the disulphide, followed by OPA derivatization. DTT, can react with OPA and interfere with GSH-OPA adduct formation (Cereser et al., 2001). Thus, selecting a suitable DTT concentration is a compromise between the DTT concentration and effective disulphide reduction. The experiment conducted in the present study gives way to select a suitable DTT concentration that can completely reduce GSSG without interfering with side reactions with OPA or forming unidentified peaks. At 25 mM DTT concentration the disulphide reaction is complete thus facilitating disulphide reduction.

Quantitation of GSH has always been subject to studies, aiming to prevent its auto-oxidation. In order to prevent thiol-disulphide exchange reactions and improve the thiol scavenging, it is very crucial to choose an appropriate concentration of scavenger. There are studies done using various concentrations of NEM (Giustarini et al., 2003, Kand'ar et al., 2007).

M2VP is used in a commercially available scavenger in a assay kit and although easy to use are expensive and are not good value for money for routine analysis of large number of samples. To choose an appropriate M2VP concentration, a series of GSH concentrations were allowed to quench with varying M2VP concentrations. A final concentration of 3 mM completely quenched GSH. The present study also includes evidence that concentration from 3 mM to 15 mM M2VP did not seem to interfere with

GSH/GSSG assay. However, it has been reported that a concentration above 50 mM may show some interference (Fu and Murray, 2001).

Results from the present study have shown interference of NEM as the concentration was increased. Furthermore, trapping the thiol group with NEM although prevents oxidation, it has been reported that this trapping makes the GSH unavailable for derivatization process (Cereser et al., 2001, Cohn and Lyle, 1966). No doubt NEM is considered a better scavenger among other thiol blocking agents such as 2-VP and IAA, however, NEM directed scavenging in the present study has shown higher GSSG (3.16±1.88) levels in whole blood when compared to the literature (Gough, 1990).

In conclusion, we have developed a simple, rapid and sensitive method for tGSH and GSSG determination in whole blood using M2VP as scavenger for GSH. Sample collection plays an important role in the determination, as oxidation of GSH can be very rapid. Collecting samples in tubes held on ice does not prevent oxidation of GSH. Thiol trapping agent like M2VP prevents further oxidation. An ideal thiol trapping agent should scavenge almost all the thiol and aid in GSSG determination. Therefore, the present method could be used for clinical analysis. Data obtained from the this assay was compared with various reported studies (Camera et al., 2001, Cereser et al., 2001, Paroni et al., 1995, Pastore et al., 2001, Sakhi et al., 2006) and were found to be comparable. The method could be used for the determination of tGSH and GSSG to assess redox imbalance in under normal and diseased conditions.

CHAPTER 4

Sulfur amino acids in acute pancreatitis

CHAPTER 4. Sulfur amino acids in acute pancreatitis

4.1. Introduction

The sulfur containing amino acids - Cys, Hcy, Cys-Gly and GSH have been implicated in various functions in the body including redox homeostasis via transsulfuration. Briefly, transsulfuration reactions (Refer to Figure 1.3) result in the transfer of an Hcy–SH group to a serine residue to form cystathionine, following an irreversible reaction to form Cys. Therefore, Cys can be synthesized from Met and serine. Hence, Cys is not an essential amino acid, but is conditionally essential due to limited production rate of Met and serine under certain circumstances (Laidlaw and Kopple, 1987), when Cys requirement is increased. It is reported that cells such as rat hepatocytes favour Cys directed GSH synthesis rather than its precursor Met (Stipanuk et al., 1992).

Under normal physiological conditions, Cys regulates the main step of GSH biosynthesis and is considered to be the limiting amino acid for GSH synthesis (Meister and Anderson, 1986). Under certain conditions like in oxidative stress, Cys biosynthesis is altered and hence may affect the synthesis of important downstream metabolites (Yu et al., 1993, Finkelstein, 1990, Malmezat et al., 2000). Several hypotheses have supported altered amino thiol metabolism in human pathology (Boushey et al., 1995, Kang et al., 1992, Ueland, 1995). The association between amino thiol concentration and oxidative stress in AP has received much attention (Yuzbasioglu et al., 2008). It is stated that despite an increased Cys flux it appears that this does not compensate for the enhanced GSH turnover (Finkelstein, 1990, Vina et al., 1992, Malmezat et al., 2000). Hence, it appears that the GSH biosynthetic pathway may be defective at a number of levels during the course and severity of inflammatory insult. It is reported that severe GSH depletion is a consistent feature of AP and this is also associated with progression

to severe attacks (Rahman et al., 2004). Therefore, to further investigate GSH biosynthesis during AP, the temporal relationship between the plasma amino thiols Cys, Hcy and Cys-Gly, total reduced erythrocyte GSH concentrations were evaluated and related with the severity of attack. The plasma from these patients was collected soon after the onset of abdominal pain (Day 1 or 24 hrs) upon admission to the hospital. Subsequently, the plasma samples were also collected consecutively on day 3 (72 hrs). Not all diseases in a clinical setting present all the clinical elements and / or symptoms at once. Some disease states (including AP) evolve over time. The time points 24 and 72 hours in the present study were chosen according to clinicians recommendations.

Research into plasma amino thiols has been receiving growing interest ever since their importance in cell chemistry has been interpreted and altered metabolism implicated in human pathology. Assaying the total amino thiols or individual amino acids is increasingly becoming a clinical practice in laboratory medicine (Andersson et al., 1993, Bald and Glowacki, 2005, Deyl et al., 1986, Walker and Mills, 1995). Amino thiol levels in the plasma and/ or in the urine reflect the metabolic and/ or the nutritional status of total or individual amino acids.

Hence, quantification of individual amino acids is important in monitoring the therapeutic intervention by way of dietary manipulations and/ or vitamin supplement therapy. A comprehensive profile of sulfur amino acids is of immense significance in clinical and diagnostic laboratories. The importance of –SH group containing amino acids and their relation in various disorders/ pathologies, has led to a spurt of interest in the development of analytical methods. Immuno, (Frantzen et al., 1998), enzymatic assays (Refsum et al., 1989), electrochemical (Robenstein and Yamaahita, 1989) have limited use to detect a given amino acid or any –SH group containing amino acid. RP-HPLC is a frequently used analytical technique for the determination of amino thiols with fluorescent detection after its reduction with trialkylphosphines followed by derivatization with 7-flurobenzo-

2-oxa-1,3-diazole-4-sulfonate (SBD-f) (Krijt et al., 2001). Trialkylphosphines are powerful reducing agents. They stoicmetrically and irreversibly reduce disulfides in an aqueous solution;

$$R'_3P + RSSR + H_2O \longrightarrow R'_3P{=}O + 2RSH$$

The phosphine nucleophilic attack on the disulfide bond determines the rate of the reaction (Burns et al., 1991).

For brevity, experimental design involved, treatment of the plasma sample from the patients prior to application on the analytical column included a reduction reaction with TBP, protein precipitation with TCA followed by derivatization with SBD-f and incubation. A modified version of reverse phase-HPLC–fluorescence assay (Fortin and Genest, 1995, Vester and Rasmussen, 1991) was employed to profile Cys, Hcy, Cys-Gly and GSH in these patients.

In an attempt to explore the effects of sulfur amino acid deficit in these patients, the sulfur amino acid levels were correlated with the results obtained for plasma C-reactive protein (CRP) acute physiology score (APS) and γ-GT. CRP is an acute phase protein and a marker of inflammation that is synthesised by hepatocytes. Serum levels of CRP have been known to rise within 24 hours of onset of AP and reach peak levels at about 72-hours (Yadav and Agarwal, 2002). APS is a subcomponent of APACHE-II scoring system (See section 2.3.1.1 for details). The clinicians determine APS at 48 hrs from the onset of abdominal pain in order to estimate the magnitude of the systemic inflammatory response (Larvin and McMahon, 1989). γ-GT is present in the cell membranes of many tissues including the kidneys, bile duct, pancreas, liver, spleen, heart, brain, and seminal vesicles (Goldberg, 1980). The enzyme is involved in the transfer of amino acids across the cellular membrane (Meister, 1974). Abnormal activity of the enzyme appears to be specific for liver, biliary tract and pancreatic diseases .

Therefore, the aim of this study was to experimentally address the temporal relationship between plasma amino thiols in these patients and correlate with the physiological and biochemical parameters such as APS, CRP and γ-GT levels.

4.2 Materials and methods

The materials for this study are fully listed in section 2.1.1. The equipment conditions are described in section 2.2.2. The sampling procedure has been described in method section 2.3.1 and methodology in 2.5.

4.3. Results

4.3.1. Sulfur amino acid assay

Sulfur amino acids in the plasma from the patients were derivatized with SBD-f. Separation of the four sulfur amino acids and the internal standard were achieved using a gradient method (See section 2.5.3.7 for details). Injecting single standards of each amino acid and comparing the retention time confirmed the sulfur amino acid identities.

Cys eluted at 4.6 min, Hcy at 5.8 min, Cys-Gly at 6.6 min, GSH at 8.3 min and the internal standard (MPG) at 19.8 min. Quantification of sulfur amino acids was achieved using calibration lines of known concentrations (120-3.75 μM). Further, at least three different concentrations of the calibration line were derivatized and run on each day of plasma sample analysis. The concentration of sulfur amino acids from the patients were directly read from the chosen three-point calibration line of the day. The peak-area ratio of every amino acid to the internal standard (MPG) were calculated and plotted against the concentration of the calibration standards (120-3.75 μM) (Figure 4.1). All results are expressed as μM /mL. Typical chromatograms of a combined standard of S-amino acid (7.5 μM) along with a plasma control sample are shown in Figures 4.2a and 4.2b.

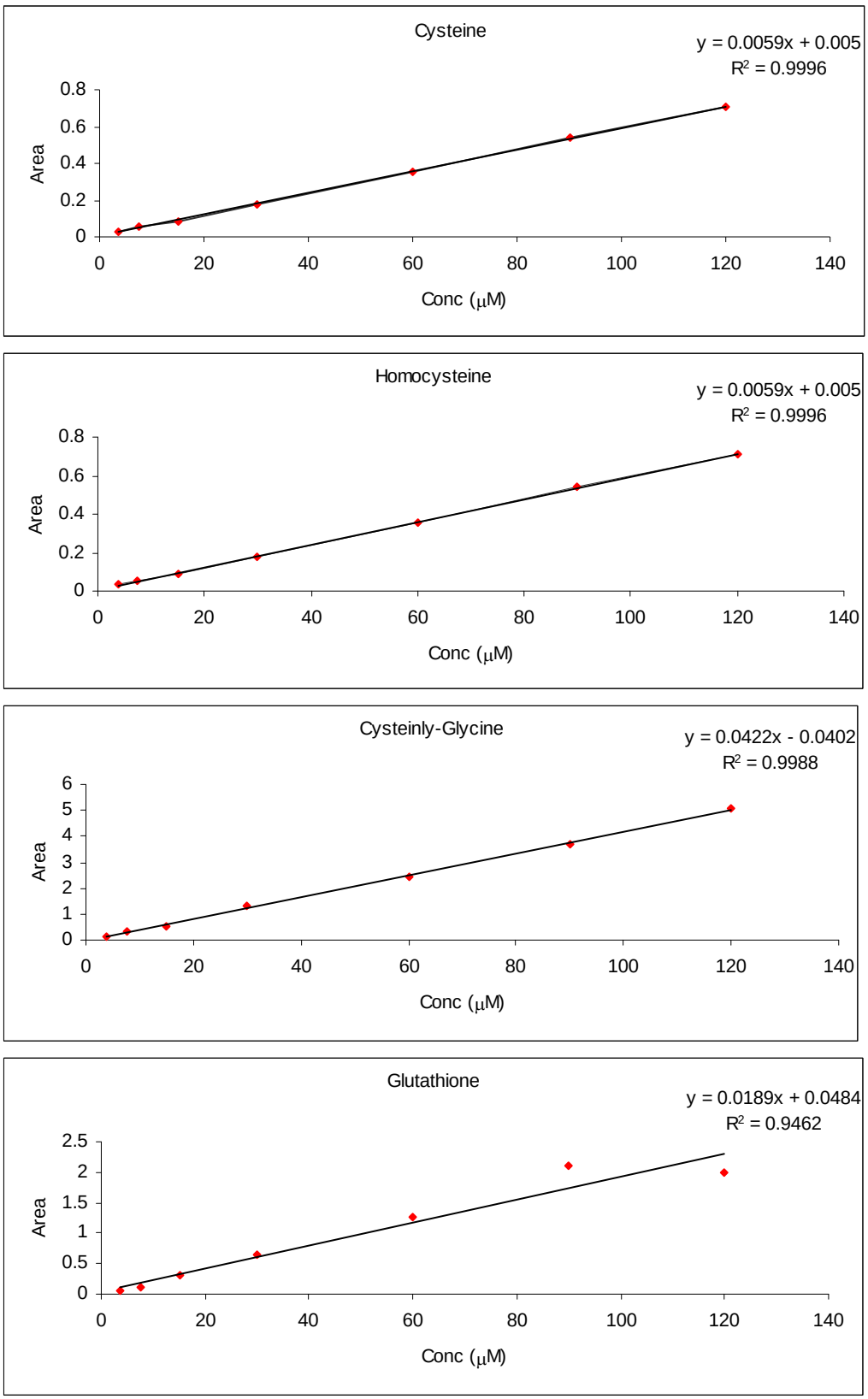


Figure 4.1. Calibration lines for Cys, Hcy, Cys-Gly and GSH.

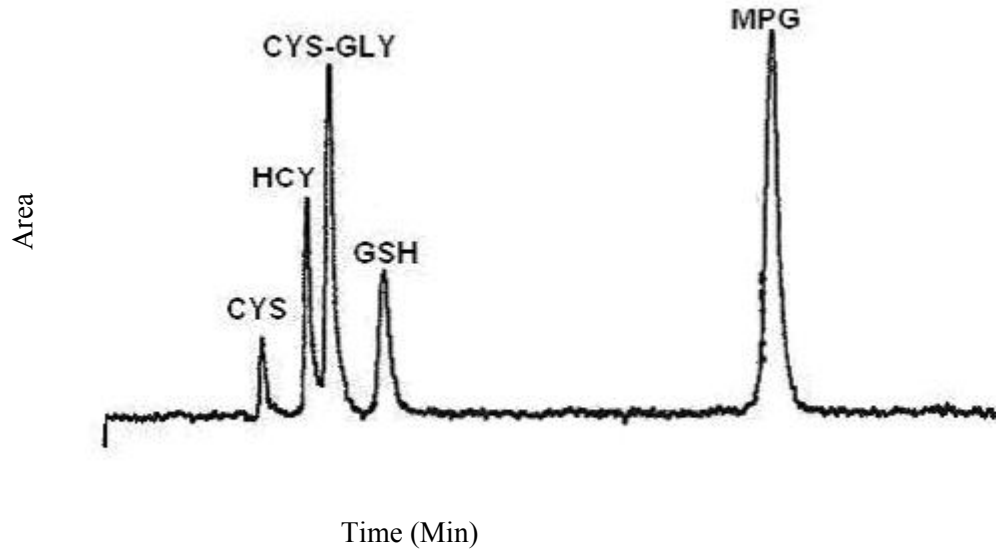


Figure 4.2a. A typical chromatogram of the HPLC analysis of S-amino acid combined standard (7.5 µM).

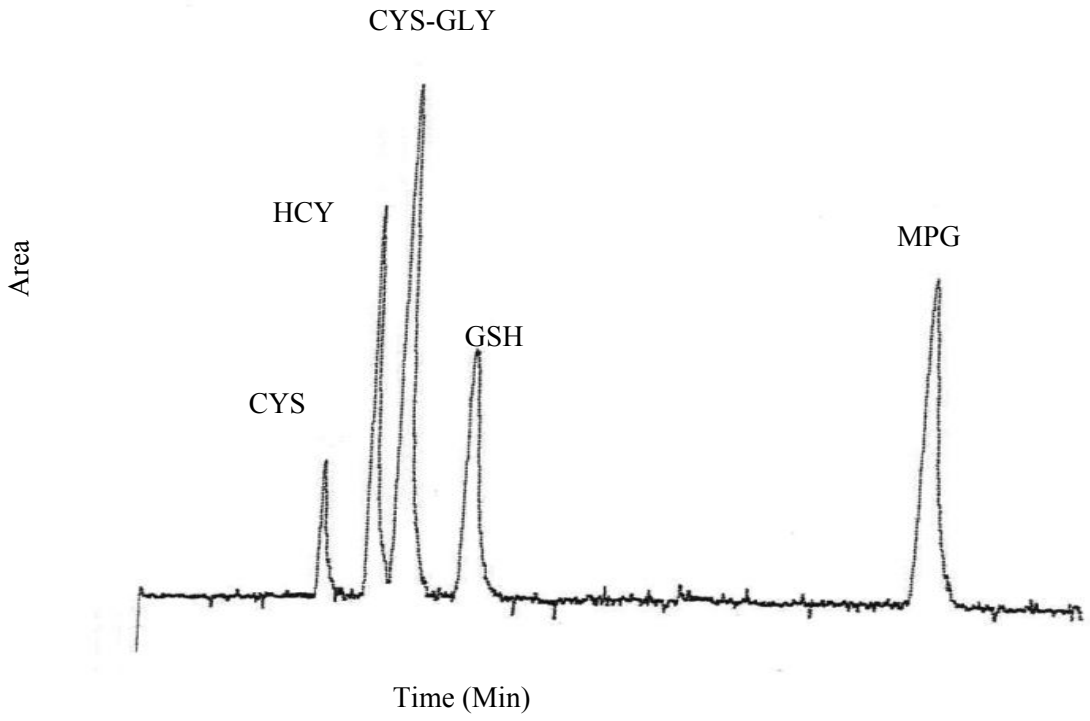


Figure 4.2b. A typical chromatogram of the HPLC analysis of S-amino acid from plasma control.

Cysteine (Cys), Homocysteine (Hcy), Cysteinyl-Glycine (Cys-Gly), glutathione (GSH) and mercaptopropionyl glycine (MPG) (internal standard).

4.3.2. Sulfur amino acid profile in patients with acute pancreatitis

In total 42 patients with AP (30 mild and 12 severe) and 12 healthy controls were studied. Attacks of AP were classified as “mild” and / or “severe” retrospectively in accordance with the Atlanta criteria of 1992 (Bradley, 1993), however within this frame work, a severe attack was that which was associated with organ failure, pancreatic necrosis, and / or sepsis. Mild disease was defined as that associated with minimal organ dysfunction and an uneventful recovery.

Age and gender were similar in all the groups (Table 4.1) (Rahman et al., 2009). The Acute Physiology and Chronic Health Evaluation (APACHE-II) (Larvin and McMahon, 1989, Yeung et al., 2006) scores at 48 hrs were found to be significantly higher in patients with severe attack compared to those with mild attacks ($p< 0.001$) (Table 4.1) (Rahman et al., 2009). The cause of the mild attack of AP was gallstones in 29 patients (69%) and ethanol in 9 patients (21.4 %) respectively (Table 4.1).

In the remaining 4 patients, AP was either due to hyperlipidemia (n=1), pancreas divisum (n=1) or idiopathic (n=2) and these were collectively referred to as “Other” in Table 4.1. Amongst the patients with severe attack, 7 had persistent organ failure, 2 with pancreatic necrosis without organ failure and 3 with both pancreatic necrosis and organ failures. Overall mortality in the severe group was 2 of 12 patients (18%).

Table 4.1. Clinical and demographic details of the study group**.** (AP: Acute pancreatitis: CRP: C-reactive protein, APACHE- Acute Physiology and Chronic Health Evaluation).

Parameters	Control N=12	Mild AP N=30	Severe AP N=12	All AP N=42
Alcohol	-	20%	25%	21.4%
Gall stones	-	66%	75%	69%
Other	-	14%	0%	9.6%
Median age (range)	58.5 (18-92)	55.5 (17-95)	65 (20-91)	57.5 (17-95)
Male: Female ratio	1.0	0.9	1.0	0.9
APACHE-II Score	-	5 (0-10)	9 (5-26)*	6 (0-26)
Peak (0-72 h) CRP (g/dL) Median (range)	-	195 (3-357)	339 (132-444)*	221 (3-444)
24h APScore	-	1(0-5)	5.5(3-20)*	-
72h APScore	-	0(0-4)	3.5(1-12)*	

*Significant difference between patients with mild and severe attacks of acute pancreatitis. $p<0.001$. Mann-Whitney U-test.

4.3.3. Sulfur amino acid and clinical disease severity

In order to determine individuals with high/low levels of plasma sulfur amino acid metabolite concentrations, the distribution of data for the all the four plasma sulfur amino acids was analysed at 24 and 72 hrs respectively. The Met metabolites: Cys, Hcy, Cys-Gly and GSH showed varied difference from 24 and 72 hrs between mild and severe attacks (Table 4.2)(Rahman et al., 2009).

After the onset of abdominal pain, plasma Hcy was found to be significantly increased in patients with mild attack at 24 hrs ($p=0.002$) (Figure 4.3), however no significant difference was observed in the plasma Cys pool at 24 hrs mild attack. However, as the disease progressed from 24 to 72 hrs after a mild attack, plasma Hcy decreased while Cys levels increased ($p=0.03$) (Figure 4.3 and 4.4). These increased levels in Cys however, remained within normal range (Table 4.2). Analyses for correlation between amino thiols Cys and Hcy seemed positive ($r=0.77$, $p<0.001$). The ratio Hcy: Cys was elevated at 24 hrs ($p <0.001$) appearing to normalise reaching control values at 72 hrs.

Table 4.2. Plasma Hcy, Cys, Cys-Gly and total erythrocyte glutathione (GSH) concentration, γ-glutamyl transferases (γGT) activity and disease severity markers (APS=acute physiology score; CRP= C-reactive protein) in patients with AP and healthy controls. Values represent medians and (range). # $p<0.05$, ## $p<0.001$ comparing data to control; * $p<0.05$, ** $p<0.01$ comparing 72 h to 24 h. Mann-Whitney U-test.

	Control N=12	Mild AP N=30		Severe AP N=12	
		24 h	72h	24h	72h
Plasma HCy (μM)	10.5 (6-15)	13.4# (3-23)	10.6* (7-21)	11.7 (8-40)	12.9#* (7-14)
Plasma Cys (μM)	164 (111-222)	150.9 (28-204)	172* (96-306)	161.5 (110-241)	174 (83-223)
Plasma Cys-Gly (μM)	25.9 (16-39)	38.1## (18-63)	112.9##** (54-162)	40.8## (29-101)	154###* (110-225)
Erythrocyte GSH (nmol/g protein)	3685 (2460-6262)	420## (305-3560)	639##** (344-978)	423## (168-942)	372## (168-539)
Serum γGT (IU/L)	28 (12-43)	239## (40-1504)	238## (50-1171)	338## (42-506)	186##* (60-314)
APS Score	0	1## (0-4)	0.0#** (0-4)	5## (3-20)	3## (1-11)
Plasma CRP (mg/L)	<5.0	118## (3-324)	152##* (10-226)	309## (161-416)	325## (238-339)

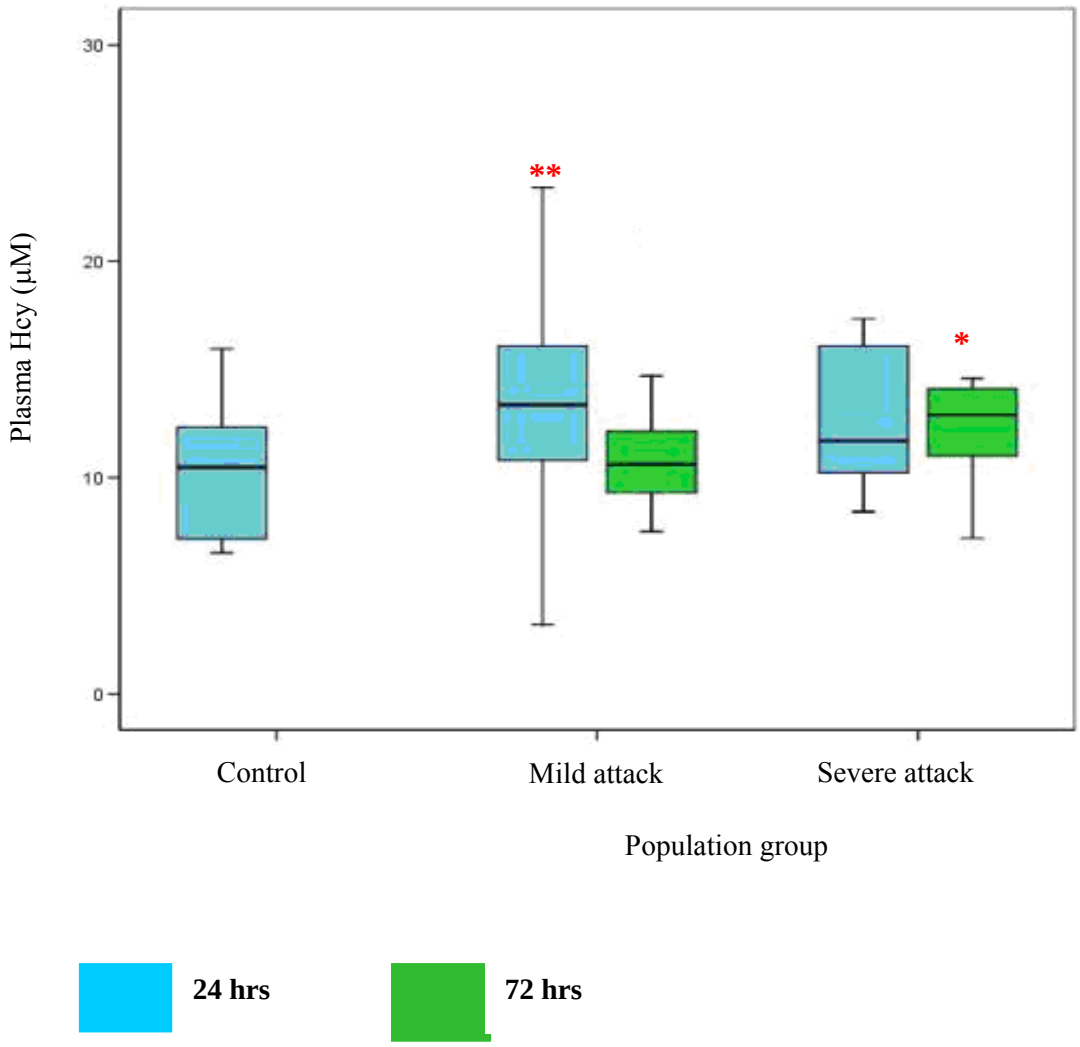


Figure 4.3. Hcy levels after the onset of abdominal pain in mild and severe attacks at 24 and 72 hrs corresponding to control * $p<0.05$, **$p<0.001$.

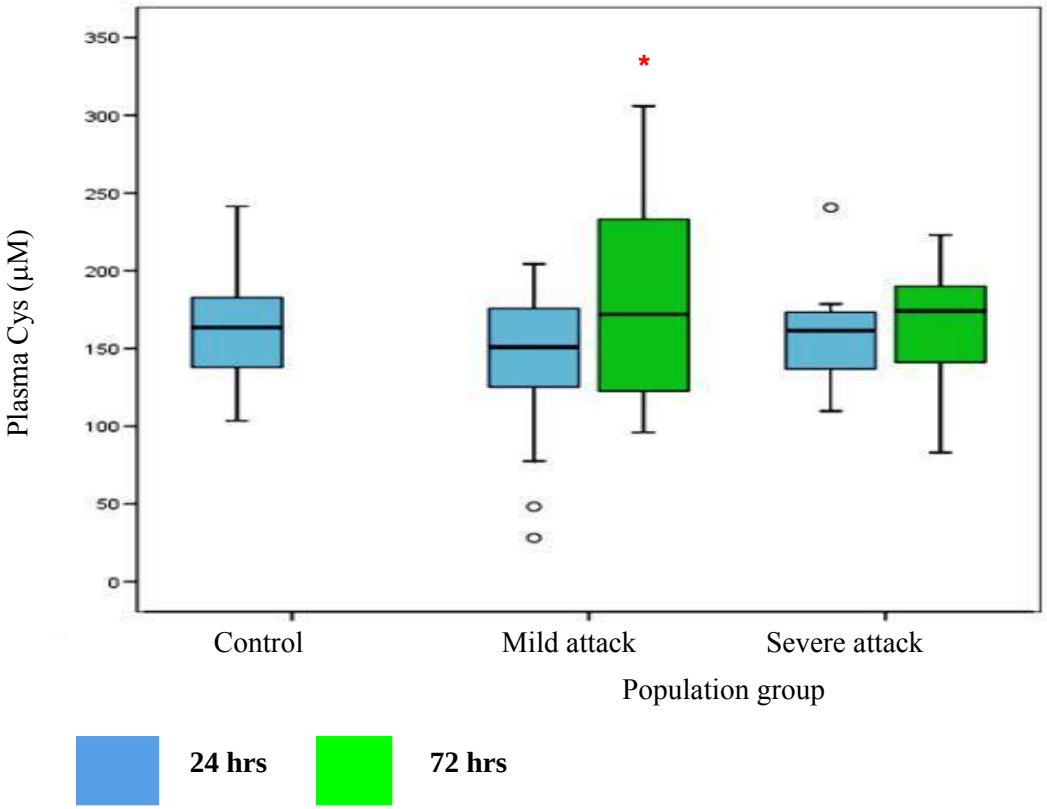


Figure 4.4. Cys levels after the onset of abdominal pain in mild and severe attacks corresponding to control at 24 and 72 hrs. *p<0.05.

Plasma Cys and Hcy levels seemed slightly higher compared to healthy controls in the early stages of severe AP at 24 hrs. However, as the disease progressed to 72 hrs following a severe attack there seemed no further changes in Cys levels but an increase in Hcy levels was apparent, possibly indicating Hcy flux through the transsulfuration pathway. Hcy flux through transsulfuration concurs with an increase in Hcy levels from 24 to 72 hrs as disease progressed.

An analyses for correlations between Hcy and Cys was strong and positive at 24 hrs in severe attacks ($r=0.86$, $p<0.001$) although this became less significant at 72 hrs. Following severe attacks, the ratio Hcy: Cys was elevated above healthy control values at both 24 ($p<0.001$) and 72 hrs ($p=0.049$) respectively (Figure 4.5).

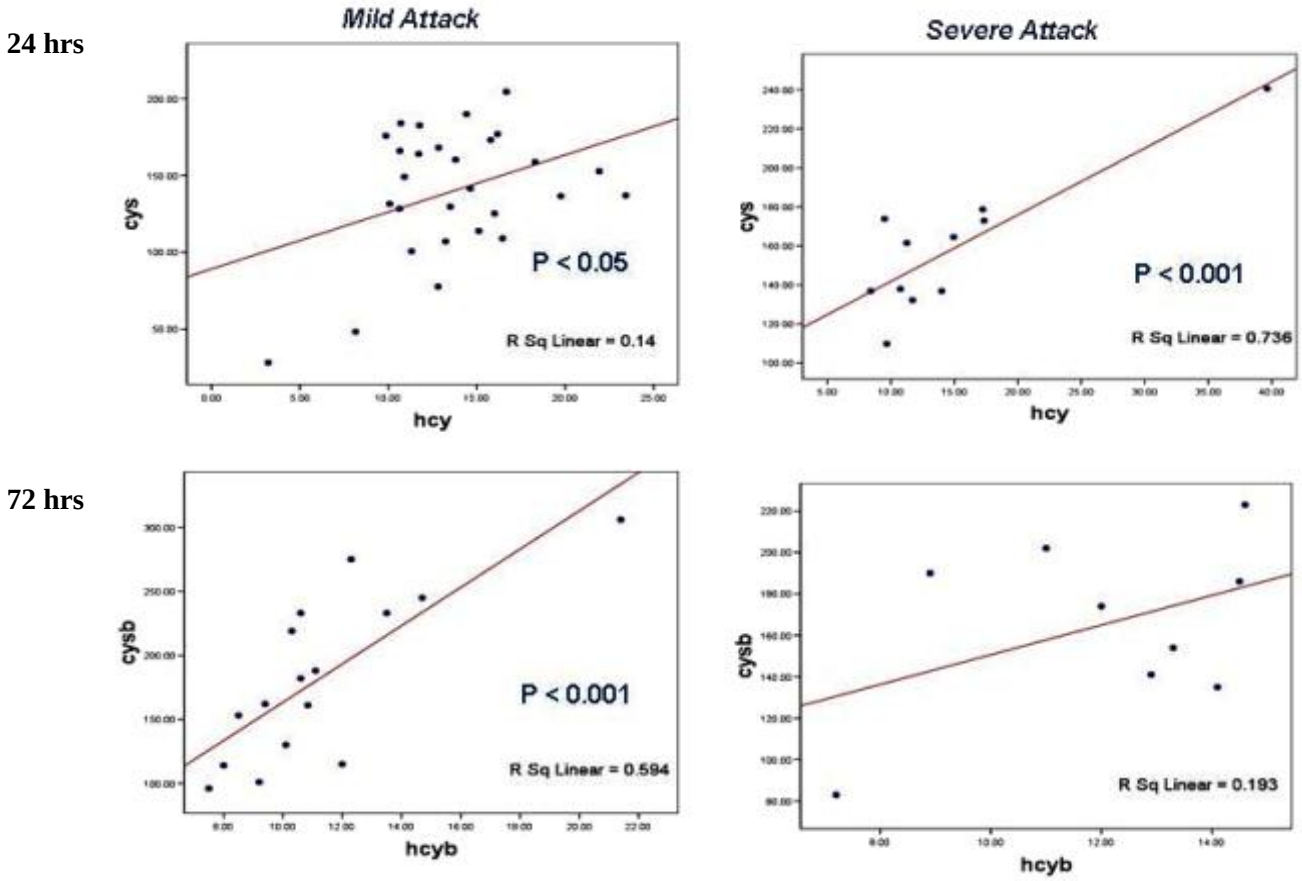


Figure 4.5. Correlation Hcy: Cys in mild and severe attacks at 24 and 72 hrs.

Hcy, Cys and Cys-Gly are all metabolically inter-related (Mansoor et al., 1992). Plasma Cys and Cys-Gly are of interest in understanding the biological interrelationship for the disease progression in AP (Bates et al., 2002). In the present study, all the measured and calculated fractions of plasma Cys-Gly, a dipeptide metabolite of GSH.Cys-Gly, which re-synthesise GSH in many intracellular reactions, were elevated in both mild and severe AP attacks ($p<0.001$) and increased further as the disease progressed (Table 4.2). Levels of Cys-Gly were significantly higher in patients with severe attacks compared to the mild attacks ($p=0.002$) (Figure 4.6). In addition, Cys-Gly and total glutathione levels were significantly different at 72 hrs between two attacks ($p=0.03$). A scatter plot showed an inverse relationship of the amino thiols Cys-Gly with GSH ($r= -0.99$, $p=0.01$) respectively (Figure 4.7).

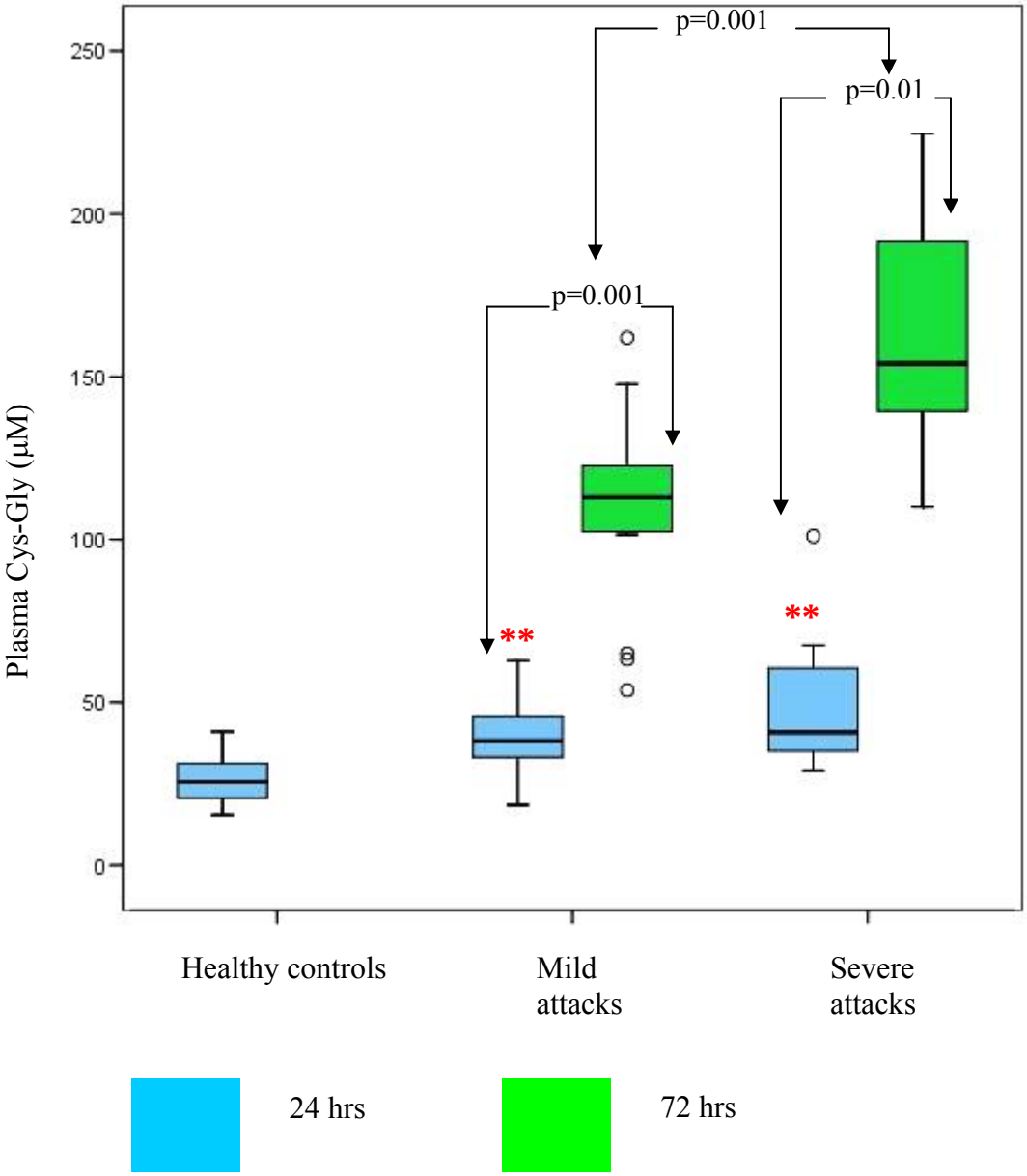


Figure 4.6. Cys-Gly levels in mild and severe attacks at both 24 and 72 hrs compared to control. (**p<0.001).

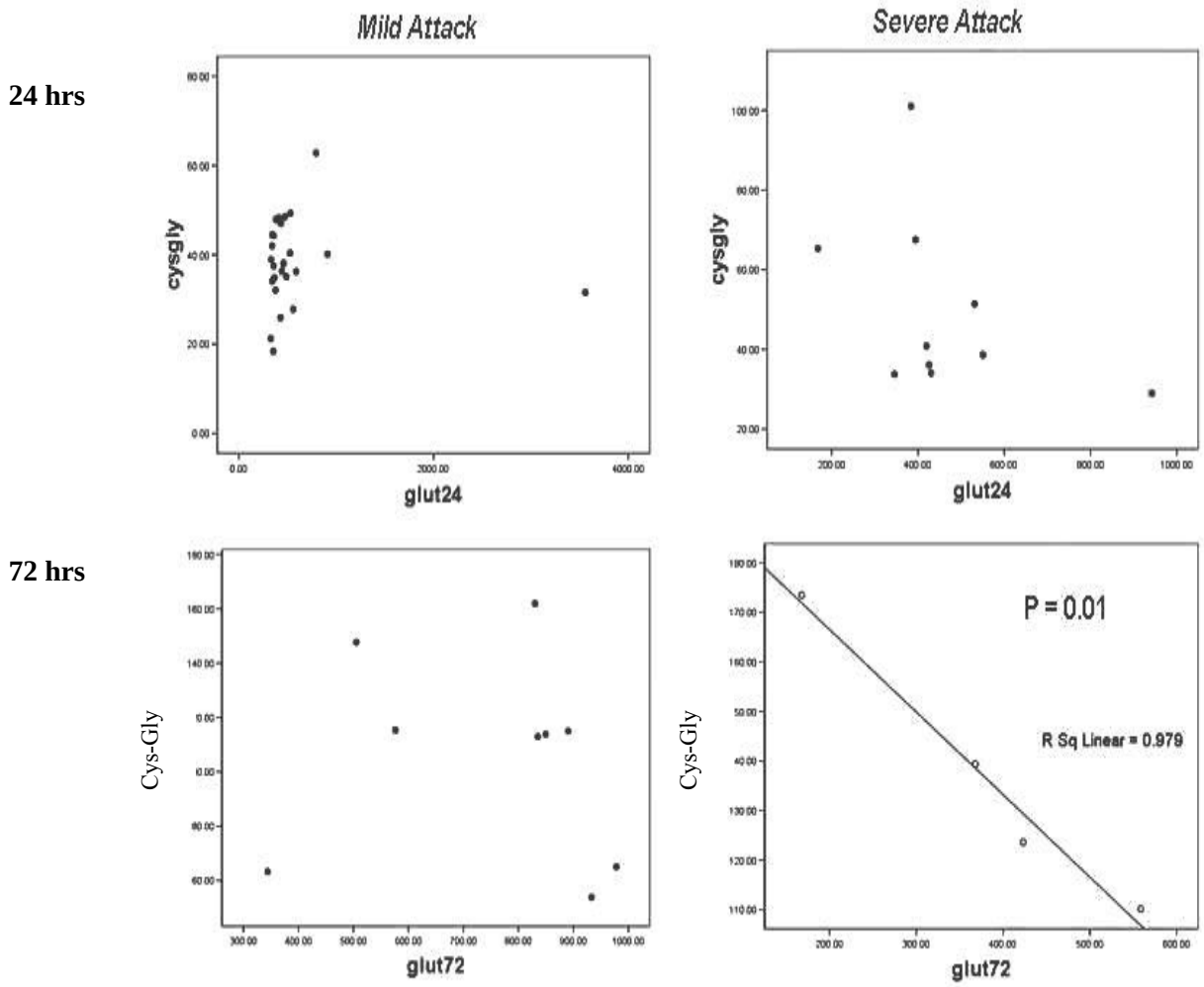


Figure 4.7. Correlation between Cys-Gly and GSH in mild and severe attacks at 24 and 72 hrs.

4.3.4. Erythrocyte glutathione levels and disease severity

Marked changes were observed in the erythrocyte glutathione concentrations in the patients suffering from acute pancreatitis. The median erythrocyte glutathione concentration in healthy controls was shown to be 3685 nmol/g of total erythrocyte protein (Table 4.2).

At 24 hrs after the onset of abdominal pain, the erythrocyte GSH concentration was found to be significantly reduced in patients with mild (median: 420 nmol/g [Range: 305 – 3560 nmol/g, $p < 0.001$], and severe attacks (median: 423 nmol/g [Range: 168 –

942 nmol/g, $p < 0.001$], compared to the control group. However, there were no observed differences in GSH concentration between mild and severe attack (p = N.S.).

At 72-hrs, erythrocyte GSH concentration continued to be significantly lower in patients with mild (median: 639 nmol/g [Range: 344 – 978 nmol/g] and severe attacks (median: 372 nmol/g [Range: 168 – 539 nmol/g], compared to healthy controls ($p < 0.001$) (Figure 4.9) (Table 4.2).

At 72 hrs, erythrocyte GSH concentration was significantly higher in patients with mild attack compared to those who subsequently developed severe attacks ($p = 0.006$). The 72 hrs GSH increased in patients with mild attacks relative to 24 hrs GSH ($p = 0.012$), but no significant change was observed among patients with a severe attack.

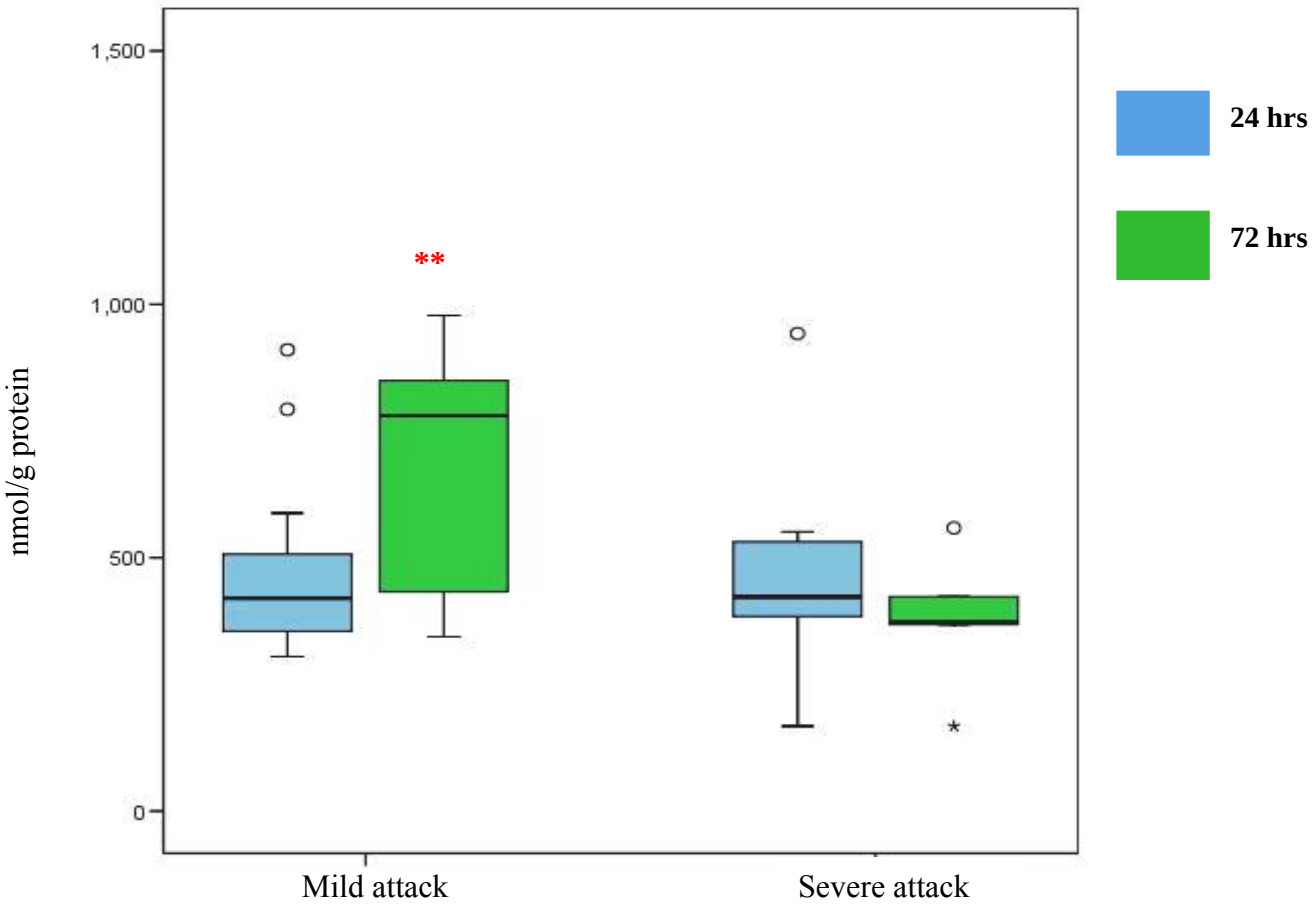


Figure 4.8. Comparison of erythrocyte GSH concentration (nmol/ g protein) in mild and severe acute pancreatic attacks at 24 and 72 hrs compared to normal (** p<0.001).

Cys is the limiting amino acid for GSH synthesis. The ratio Cys: GSH was significantly higher in both mild and severe attacks ($p<0.001$) when compared with the healthy volunteers. Although levels were similar at 24 hrs in mild and severe diseases ($p=0.74$), at 72 hrs this ratio tended to decrease over the course of mild attacks ($p<0.05$) while it remained elevated after severe attacks.

It should also be noted that, according to UK recommendations, severity stratification is done on all the patients on admission with AP (Anon, 2005, Anonymous, 1998). Hence, the plasma sulfur amino acid levels from the present study were compared with some of the physiological (APS) and biological (CRP) parameters, to validate the severity of the disease referring only to S-amino acids.

Many potential markers for predicting the severity of acute pancreatitis act as systemic inflammatory markers. CRP is a well-established marker of inflammation produced in the liver by cytokines from the site of inflammation (Johnson et al., 2004). Values of 150 mg/L at 48 hrs after the onset of symptoms are now accepted as a proven indicator of severity of pancreatitis (Yadav and Agarwal, 2002). To determine whether the individuals with low or high amino thiol levels also had relatively low or elevated levels CRP, a comparison was carried out to relate values provided, among the different individuals at 24 and 72 hrs. Elevated levels of CRP can possibly reflect the disease progression/ severity in AP. CRP acts as a part of systemic inflammatory response marker (Anon, 2005). Stratifying the data to disease severity, it was observed that there was significant changes in C-reactive protein ($p=0.43$) (Table 4.2). Further, an increase in Hcy levels, also increased the CRP significantly ($p<0.05$)

The physiological scores (based on the acute physiology and APACHE II scoring systems), a correlation of Met metabolites was carried out at respective time points to stratify disease severity. The amino thiol Cys-Gly ($p=0.025$) correlated with APS ($r=0.37$, $p=0.025$) and serum CRP at 24 and 72 hrs ($r= 0.59$, $p=0.003$). However, an

inverse correlation was demonstrated with GSH at 72 hrs suggesting that low GSH levels (r= - 0.49, p=0.046) concurs with high APS and hence more physiologically the unwell the patient is. The erythrocyte glutathione also showed an inverse correlation with APACHE-II score (r= - 0.50, p=0.026) respectively.

4.3.5. γ-Glutamyl transpeptidase

The plausible potential cause of pancreatic GSH loss was addressed by the study of the enzymes of γ-glutamyl cycle, most importantly γ-glutamyl transpeptidase (γ-GT), the only known enzyme to initiate the breakdown of GSH. It is well established that γ-GT is essential for GSH precursor uptake and involved in amino acid reabsorption and Cys transport (Hanigan and Ricketts, 1993, Hanigan, 1995). However, γ-GT directed catabolism of GSH is the only major source of circulating Cys (Lieberman et al., 1996). This infers that, without γ-GT initiated reutilization of Cys, a dietary intake or cleavage of cystathionine forming Cys are insufficient to compensate for its loss as GSH (Lieberman et al., 1996)

In the present study, the serum γ-GT levels was compared in both mild and severe pancreatic attacks. The serum γ-GT activity was increased in both mild and severe attacks at both time points when compared with healthy control ($p< 0.001$) (Table 4.2). As already mentioned, there was an immediate change in the GSH pool at 24 hrs (decrease) mild and severe attacks ($p<0.001$). Concomitantly, there was no significant increase/ decrease in serum γ-GT levels at 24 hrs mild attacks. However, there was a significant decrease in serum γ-GT levels following severe attacks at 72 hrs (p=0.012). This decrease in serum γ-GT directly reflects GSH levels at 72 hrs (p=0.002) (Figure 4.11).

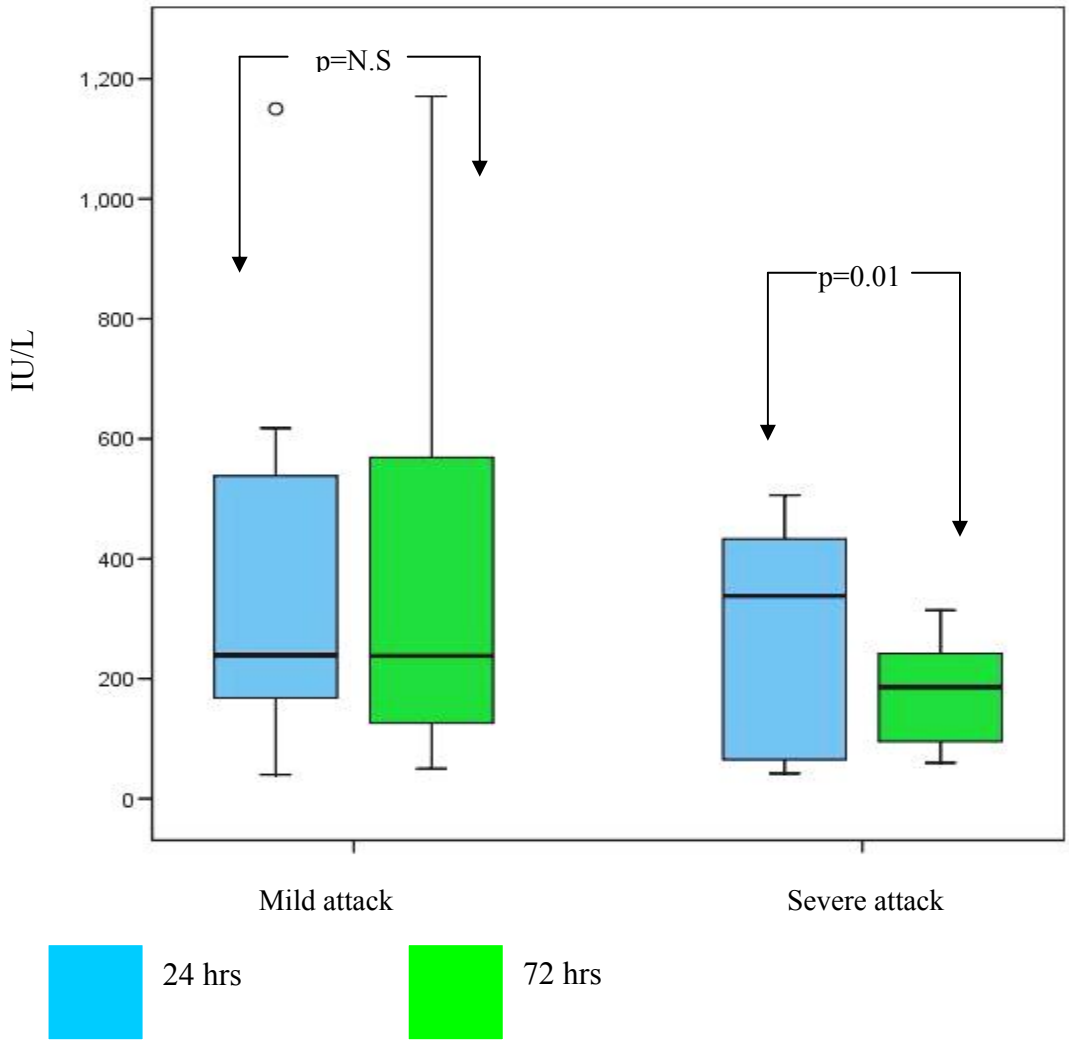
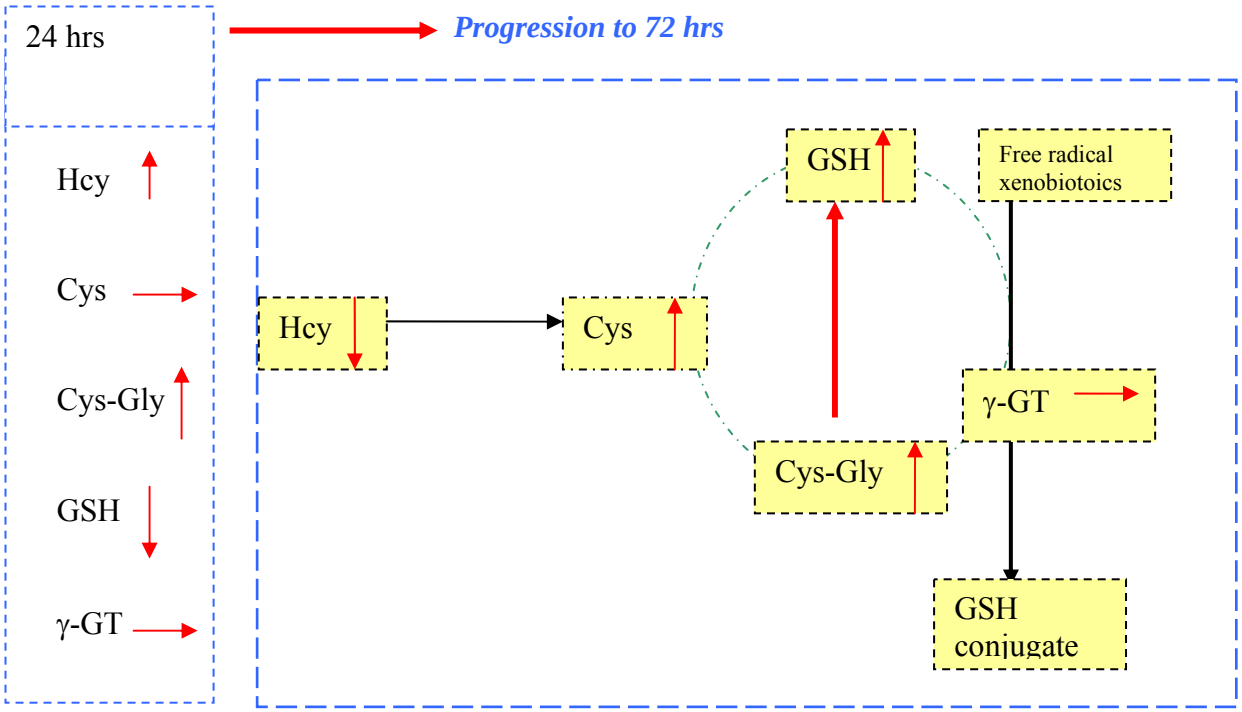


Figure 4.9. Serum γ-glutamyl transpeptidase levels in patients with acute pancreatitis. **(***p<0.05, ****p<0.001).

4.4. Discussion

The overall outcome of the sulfur amino acid profiling in patients suffering from AP in both mild and severe attacks from 24 hrs to 72 hrs can be can be summarized as follows;

1) Mild attack



2) Severe attack

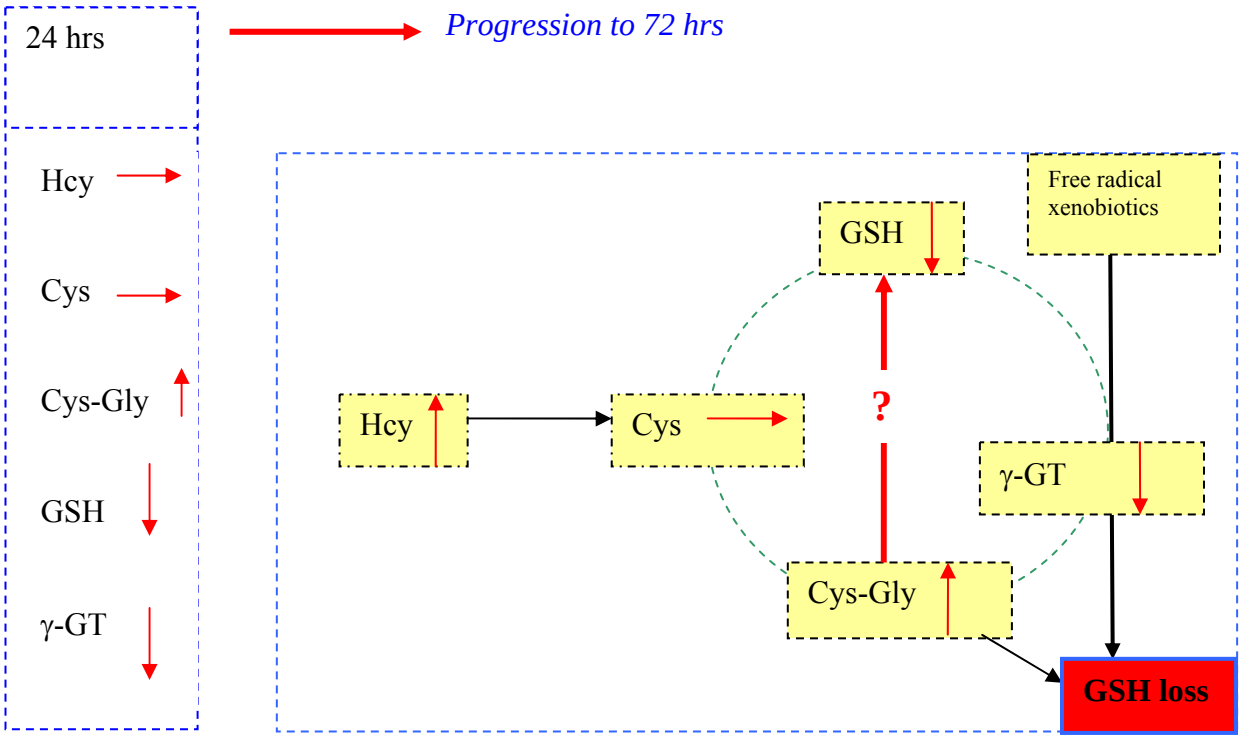


Figure 4.10. Summary of sulfur amino acid profile at 24 and 72 hrs in patients suffering from AP.

The Met transsulfuration pathway supplies the key amino acid Cys (source of GSH), through the metabolism of Met via Hcy. It is reported that Hcy flux through transsulfuration increases during oxidative stress conditions (Mosharov et al., 2000), certain systemic injuries and /or trauma like sepsis and burns (Yu et al., 1993). Hcy is an important key intermediate in both transmethylation and transsulfuration pathways. Hence, Hcy plays a key role in the maintenance of the cellular GSH pool.

The results from the present study provide an interesting insight into the differences in transsulfuration capacities of certain cells in an event of pathophysiology such as AP. At 24 hrs during a mild attack, an increase in Hcy possibly provides a meaningful correlation for an elevated plasma Hcy. This metabolic demand for transsulfuration under certain pathological conditions directly or indirectly reflects oxidative stress and demonstrates, probably, a diminishing pool of GSH. Hence, transsulfuration can be described as an auto corrective response leading to an increased GSH synthesis via Cys in the cells challenged by oxidative stress.

It must be noted, that Cys elevated slightly during mild or severe attacks. Plasma Cys concentrations were surprisingly similar to healthy controls in both mild and severe AP. A fall in plasma Hcy concentration was noted at a later stage of the disease. Elevated levels of Hcy reported in the present study correlated with another study who also showed changes in plasma Hcy levels in patients with AP (Yuzbasioglu et al., 2008). The latter study suggested that hyperhomocysteinemia was due to disturbed methylations.

The transsulfuration pathway supplies the key amino acid Cys for GSH synthesis. It has been shown in rat pancreatic acinar cells, that the exocrine pancreas synthesizes GSH from precursor amino acids and that the transsulfuration pathway is functionally intact

in the pancreas and may serve as an important source of pancreatic Cys (Neuschwander-Tetri et al., 1997). In the present study, despite elevated levels of Hcy, its conversion to Cys was limited. Although, there can be many potential riposte for this, the present hypothesis contemplated on an insufficient utilization of Hcy for Cys synthesis in response to an increase in demand. This insufficiency has probably led to GSH deficit within 24 hrs of onset of the disease.

Nevertheless, given that Hcy and Cys concentrations were maintained within the normal range irrespective of disease severity, the results suggest that Cys bioavailability does not appear to be impaired in AP. It is cited that there could be Met flux among patients with acute inflammatory disorders, without an apparent change in serum Hcy or Cys levels (Vina et al., 2001). Yu *et al* (1993) studied a group of patients with severe burns and showed that rates of Hcy remethylation and Met oxidation all increased without changes in Cys levels (Yu et al., 1993). This reflected in the current study as demonstrated by the increased Hcy: Cys ratio in both mild and severe attacks without an apparent change in Cys concentration.

Ostensibly, in mild attacks the Cys: GSH ratio fell significantly ($p<0.05$) as the disease progressed, despite increases in the Cys concentration. This can be attributed to an increase in erythrocyte GSH, suggesting that intra and extra-cellular recycling of GSH is sufficient to meet the demand for Cys in mild attacks. However, following a severe attack, the Cys: GSH ratio was unchanged and the Hcy: Cys ratio remained elevated despite an increase in plasma Cys-Gly levels. This suggests that in severe AP attacks defects in GSH biosynthesis may occur downstream of Cys biosynthesis, and are unlikely to be attributed to altered cystathionine activity (Vina et al., 1992, Rao et al., 1990).

A persistent observation has come from the array of data, which has allowed uncovering the anticipated decrease in GSH levels within 24 hrs after the onset of a pancreatic attack. This decrease has been consistent and continued to 72 hrs following severe attacks. This perhaps explains a common thread connecting the progression of the disease and oxidative stress, which through free radical generation and/ or accumulation leads to depletion of GSH levels.

Reduced cellular GSH levels have also been observed in critically ill and septic patients and has shown to correlate with increased organ damage and mortality (Rahman et al., 2004, Mishra et al., 2005, Lyons et al., 2001, Hammarqvist, 1997). It appears therefore, that oxidative stress is associated with the progression of clinical AP, and this is highlighted by the observed inverse correlation between GSH concentration and the systemic physiological response to AP.

This result has in fact served to underscore the intimate coupling between GSH and the enzyme involved in GSH metabolism viz γ-GT. Hypothetically, γ-GT activation intracellularly can lead to inappropriate GSH degradation (Stark, 1991). A diminished extracellular activity of γ-GT can result in decreased GSH precursor uptake (Neuschwander-Tetri et al., 1992). As mentioned elsewhere, the enzyme is expressed and exposed to the extracellular milieu. At 24 hrs mild attack, the cells, despite depleted levels of GSH try to export GSH into the extracellular milieu to combat oxidative stress and prevent cell damage. However, as time progresses, the cells unable to divert Hcy through transsulfuration have depleted GSH levels.

GSH catabolism by the membrane bound γ-GT enzyme at 24 hrs yield Cys-Gly, a dipeptide metabolite of GSH. A concomitant decrease in GSH with an increase in Cys-Gly levels at 72 hrs concurs with decreased levels of γ-GT. Despite increased Cys-Gly levels, the membrane bound enzyme is unable to transport Cys back to the cell for the reasons unrecognized.

The enzymes of the transsulfuration pathway are vulnerable to oxidative stress as shown by studies on hepatocytes poisoned by paracetamol (Davis et al., 1986). The two penultimate rate limiting enzymes in GSH biosynthesis, namely γ-glutamyl cysteine synthetase and glutathione synthetase participate in the downstream biosynthesis of Cys (Biolo et al., 2007, Dickinson and Forman, 2002). Mitochondrial dysfunction and depletion of ATP have been linked to intracellular antioxidant (reduced GSH) depletion in critically ill patients (Liaw et al., 1980, Tresadern et al., 1988, Gasparetto et al., 1983) and this may further directly affect the activity of γ-glutamyl cysteine synthase and glutathione synthase, which are both ATP dependent rate-limiting enzymes subjected to feedback inhibition by GSH.

Furthermore, reduced levels of GSH could result in lack of feedback inhibition of γ-glutamyl cysteine synthase leading to accumulation of γ-glutamyl cysteine that may be metabolised to glutamic acid via the γ-glutamylcyclotransfereses and oxoprolinase reactions, thereby potentiating cellular acidosis (Benedetti et al., 1980, Vander Jagt et al., 1997, Kaplowitz, 1980). Similarly, impaired GR activity or the excessive production of GSSG in the face of oxidative stress or xenobiotic challenge, may impair protein function by reacting with protein sulfhydryl via a mixed disulfide reaction (Walker and Gilbert, 1995). GSSG and glutathione-S-conjugates may in such circumstances be removed from cells through ATP-dependent processes mediated by the multidrug-resistance (MRP) family (Singer and Brealey, 1999). Therefore, increased GSH oxidation may affect the cellular capacity to recycle GSH (Vina et al., 2001, Rao et al., 1990, Singer and Brealey, 1999).

CHAPTER 5

Analysis of bile salts in cholestatic hepato-biliary diseases

CHAPTER 5 Analysis of bile salts in cholestatic hepato-biliary diseases

5.1. Introduction

Bile acids are a family of molecules derived from cholesterol and form the major components of the bile. They are a complex group of acidic steroids forming a large heterogeneous group of water-soluble compounds characterized by steroid scaffolding with a carboxylic acid group located in the side chains (Figure 5.1). The most abundant primary bile acids in humans are cholic acid (CA) and chenodeoxycholic acid (CDCA). All primary bile acids appear to have three common features; i) they are the major end products of cholesterol metabolism, ii) secreted in to the bile in a conjugated form, iii) the conjugates are membrane impermeable, water-soluble, amphiphilic molecules (Danielsson, 1973, Hoffman, 1994). Briefly, the steps leading to the formation of the primary bile acids involve hydroxylation at C_7 in the steroid ring of cholesterol or at C_{24}, C_{25} or C_{27} position thus shortening the steroid ring. The terminal step in bile acid biosynthesis, involves conjugation with the amino acids glycine and taurine and that is aided by the hepatic microsomal, mitochondrial and lysosomal enzymes. Their conjugation increases the aqueous solubility of the bile salts under physiological conditions. The primary bile acids are further metabolized by the aid of intestinal bacterial flora that remove the hydroxyl group at position 7 to form the secondary bile acids: lithocholic acid (LCA) is formed from chenodeoxycholic acid (CDCA) and deoxycholic acid (DCA) is formed from cholic acid (CA) and ursodeoxycholic acid (UDCA) (Perwaiz et al., 2001). The latter two secondary bile acids constitute 70-80% of total bile acid pool in humans.

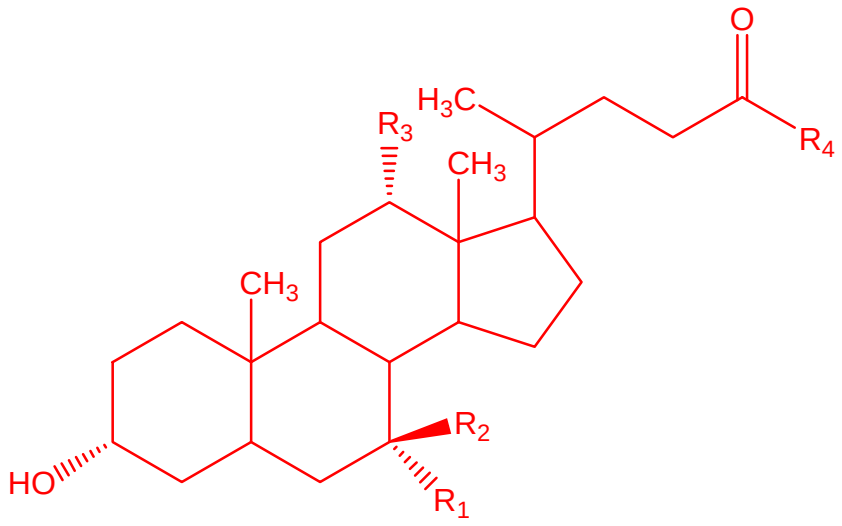
These five bile acids namely CA, CDCA, DCA, UDCA and LCA form the most abundant bile acids in the human body. These acids differ from each other by the number and position of hydroxyl or keto groups and by the presence of double bonds in the steroid ring (Figure 5.1). They can either occur as free bile acids (unconjugated forms) or

as amide conjugates of carboxylic groups with glycine ($NH_2CH_2CO_2H$) and taurine ($NH_2CH_2CH_2SO_3H$) via amidation on C_{24} forming taurocholic and glycocholic acids (Perwaiz et al., 2001, Tagliacozzi et al., 2003).

Oxygen on the sulfur of the taurocholic acid and the terminal carbon of the glycocholic acid are ionized at physiological pH. Together with planar structure of the bile acid and the ample hydroxyl groups on the rings renders the bile salt (the ionized form) very amphipathic. Further, conjugation also reduces the pKa, for example: cholic acid binding with glycine reduces the pKa from 6.4 to 4.4. This change in the pKa ensures complete ionization and increases solubility. However, the hydrophobicity of the bile acids is inversely related to the number of –OH groups and linked with the intrinsic toxicity of the bile acids. For example: LCA has only one –OH group and is highly hydrophobic and toxic (Little et al., 1990).

Upon their release into the small intestine, the majority of the bile acid conjugation involve re-conjugation of the unconjugated bile acids of previously secreted bile acids (Combes etal 1999). They are then used to solubilise dietary lipids such as cholesterol and triacylglycerides, aiding their absorption into the bloodstream. Thereafter, bile acids are reabsorbed from the portal blood by hepatocytes and re-excreted into bile for reuse.

Thus, both primary and secondary bile acids are reabsorbed by the intestine and re-delivered back to the liver via the portal circulation for uptake and re-secretion into the bile thus, recycling about 95% of the bile acids via the enterohepatic circulation. Any loss in the bile acid pool (through faeces), is compensated by the *de novo* synthesis to maintain the bile acid pool size in the body (Heaton, 1972, Hoffman, 1977).



	R_1	R_2	R_3
CA	OH	H	OH
CDCA	OH	H	H
UDCA	H	OH	H
DCA	H	H	OH
LCA	H	H	H

R_4	
OH	**Unconjugated**
$NHCH_2CO_2H$	**Glycine conjugates**
$NHCH_2CH_2SO_3H$	**Taurine conjugates**

Figure 5.1. Chemical structure of the parent bile acid along with side chain structure of six major bile acids with their taurine and glycine conjugates**.**

R1, R2, R3 and R4 are shown with their respective chemical groups in the glycine and taurine conjugates. CA-cholic acid, CDCA-chenodeoxycholic acid, UDCA-Ursodeoxycholic acid, DCA-Deoxycholic acid, LCA-Lithocholic acid.

In normal physiological conditions, only small quantities of bile acids are found in peripheral circulation (Perwaiz et al., 2001). The bile acid level in the peripheral circulation is in the micromolar (μM) range (Burkard et al., 2005). However, blood bile acid levels rise when bile flow is reduced or blocked as in cholestasis thereby escaping into the bloodstream.

Circulating bile acids levels in plasma are rapidly cleared by the liver. Decreased liver function results in elevated plasma bile acid concentrations (Salen and Batta, 1999), thus there is less efficient removal of bile salts from circulation causing the bile acid levels to rise in the peripheral circulation. Bile acid levels in pathological conditions can be efficient in early diagnosis of many diseases associated with liver and intestine (Roda et al., 1998, Scalia, 1995). Further, it has been investigated that altered bile acid levels are more sensitive indices in the diagnosis of liver disease when compared to conventional liver function tests (Kaplowitz, 1973).

Hepatobiliary, intestinal dysfunction and related gastro-intestinal diseases disturb the bile acid synthesis, metabolism and its clearance by the liver. Hence, the bile acid pools in various compartments in the body like serum, gall bladder, liver, urine and faeces are affected quantitatively. A comprehensive analysis of bile acids in various biological matrices requires their isolation and separation from the given sample. Primary or conjugated bile acid levels vary in their concentration dependent on the source they obtained from.

As bile acids differ broadly in their physiochemical properties such as lipophilicity and polarity, a complete and accurate separation requires the use of chromatographic techniques (Roda et al., 1983). Major advances have been made in analytical methodologies for the determination of bile acids in various biological matrices and physiological fluids.

Gas chromatography (GC) was extensively used for bile acid analysis before any other analytical assay was developed. However, extensive samples clean up prior GC analysis have ostensibly reduced the use of GC in bile acid analysis. GC coupled with mass spectrometer (MS), has been more useful in the elucidation of bile acid profile in biological fluids. Nevertheless, a routine GC-MS analysis of bile acids mainly in plasma required a laborious sample preparation involving enzymatic and/ or chemical deconjugation, prior derivatization (Bootsma et al., 1999)

Mass spectrometry is a valuable method for providing qualitative information about metabolite identification. LC-MS methodologies for bile salts, both primary and conjugated, acids are promising. Specifically, LC-MS with an ESI interface, offers rapid and efficient methodology amenable for the both conjugated and non-conjugated bile acids in biological samples (Alnouti et al., 2008)

Although several analytical methods have been described to determine bile acids levels, only a few publications have specified individual bile acid concentrations in a single run. Thus, the aim of the present study was to develop a LC-MS technique to assay the profile of 15 bile acids in a cohort of patients suffering from various degrees of cholestatic hepato-biliary diseases. Based on the chemistry of the bile acids and in agreement with the literature (Grattagliano et al., 2005), electrospary ionization (ESI) was used as the method for ionisation.

5.2. Materials and methods

The materials used for this study are fully listed in section 2.1.3. The equipment conditions are described in section 2.2.3. The sampling procedure has been described in method section 2.3.3, whilst the extraction procedure is explained in detail in section 2.7.

5.3. Results

5.3.1. Optimisation of conditions for ionisation

ESI with a combination of high voltage and heat was used to generate ions at atmospheric pressure from the biological sample prior its transfer to the mass analyser. Furthermore, ESI was operated in both positive $[M+H]^+$ and negative $[M-H]^-$ modes for the bile standards. Positive-ion mode analysis resulted in a spectrum with low signal strength and with ample background noise. Hence, based on the signal strength and the dominant ionic species present, negative mode $[M-H]^-$ was selected for further optimisation. The negative ion formation has enabled low concentration detection. Further, the presence of –COOH (acid group) and abundant –OH groups in almost all the bile acids favoured negative ion selectivity in the detection process.

In order to optimise the conditions for ionisation in ES^-, all the 15 bile acid standards were prepared in MeOH at a concentration of 1 mg/mL (stock solution) individually and stored in -80ºC. 10 µL of the individual sodium salt of bile acid standards were diluted with 990 µL MeOH (10 µg/mL, working standard). Optimization was achieved by both direct infusion into the mass spectrometer through a syringe pump (10 µL/mL) and with corresponding HPLC mobile phase (0.2 µL/ min). The coupling of LC allowed the separation of isobaric components.

Full scan spectra for all the 15 bile standards were acquired over the mass range of *m/z* 300-550. The operating parameters of the ESI source were optimised with regard to maximum signal intensity with source and desolvation temperature of 120ºC and 200ºC. The capillary voltage was maintained at 3.50 kV, the dwell time was 0.2s with an interscan delay of 0.1s. The optimised conditions for the formation, detection and recording of a single ion species forms the basis of an analytical protocol using single

ion recording (SIR). All relevant information is summarized in Table 5.1 A and B. The corresponding ESI spectra for bile acids are shown in Figures 5.2 a to o.

Table 5.1 A and B. Summary of the optimized mass spectrometer conditions for the bile acid standards.

A)

Sodium salts of bile acid standards	Relative molecular mass	Parent-ion $[M-Na]^-$	Cone voltage (V)
TCA	537	514	82
TUDCA	521	498	60
TDCA	521	498	90
TCDCA	521	498	60
TLCA	505	482	90
GCA	487	464	60
GUDCA	471	448	60
GDCA	471	448	60
GCDCA	471	448	60
GLCA	455	432	60

B)

Bile acids	Molecular weight	Parent-ion $[M-H]^-$	Cone voltage (V)
CA	408	407	60
CDCA	392	391	60
DCA	392	391	60
UDCA	392	391	60
LCA	377	376	80
CA-*d*4*	412	411	92

***Internal standard

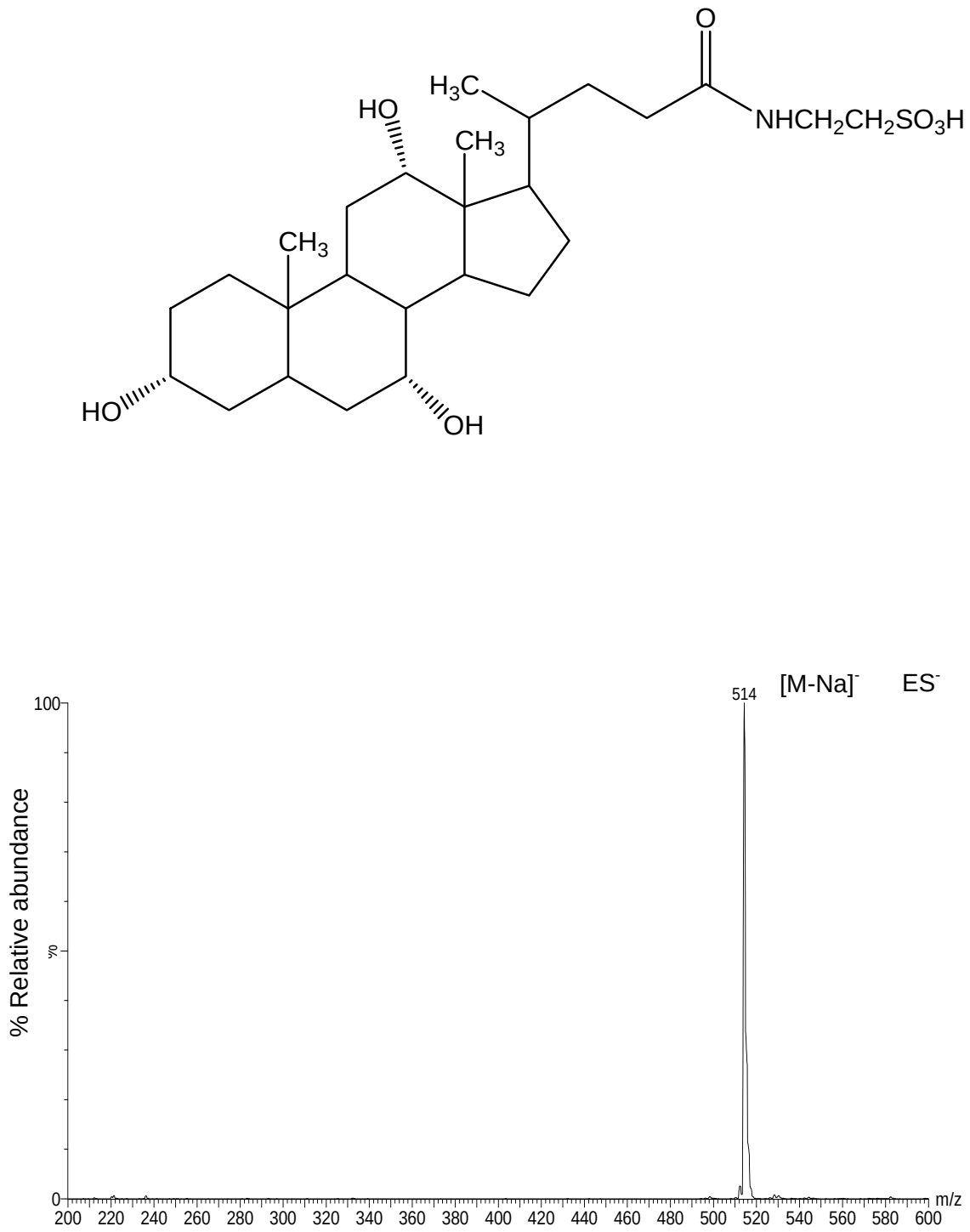


Figure 5.2a) Structure and ESI-MS spectra for sodium taurocholate (TCA) (10 μg/mL).

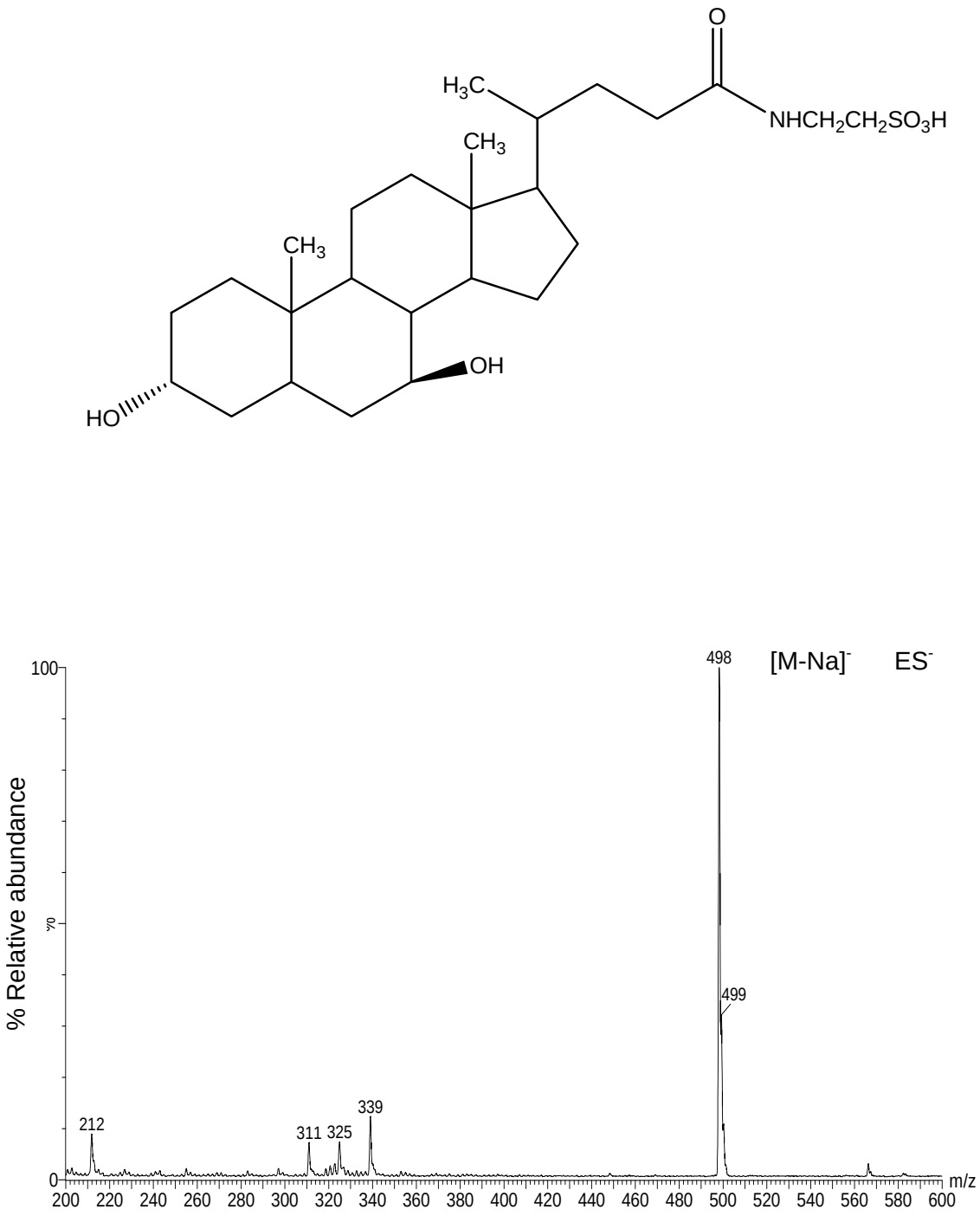


Figure 5.2b). Structure and ESI-MS spectra for sodium tauroursodeoxycholate (TUDCA) (10 μg/mL).

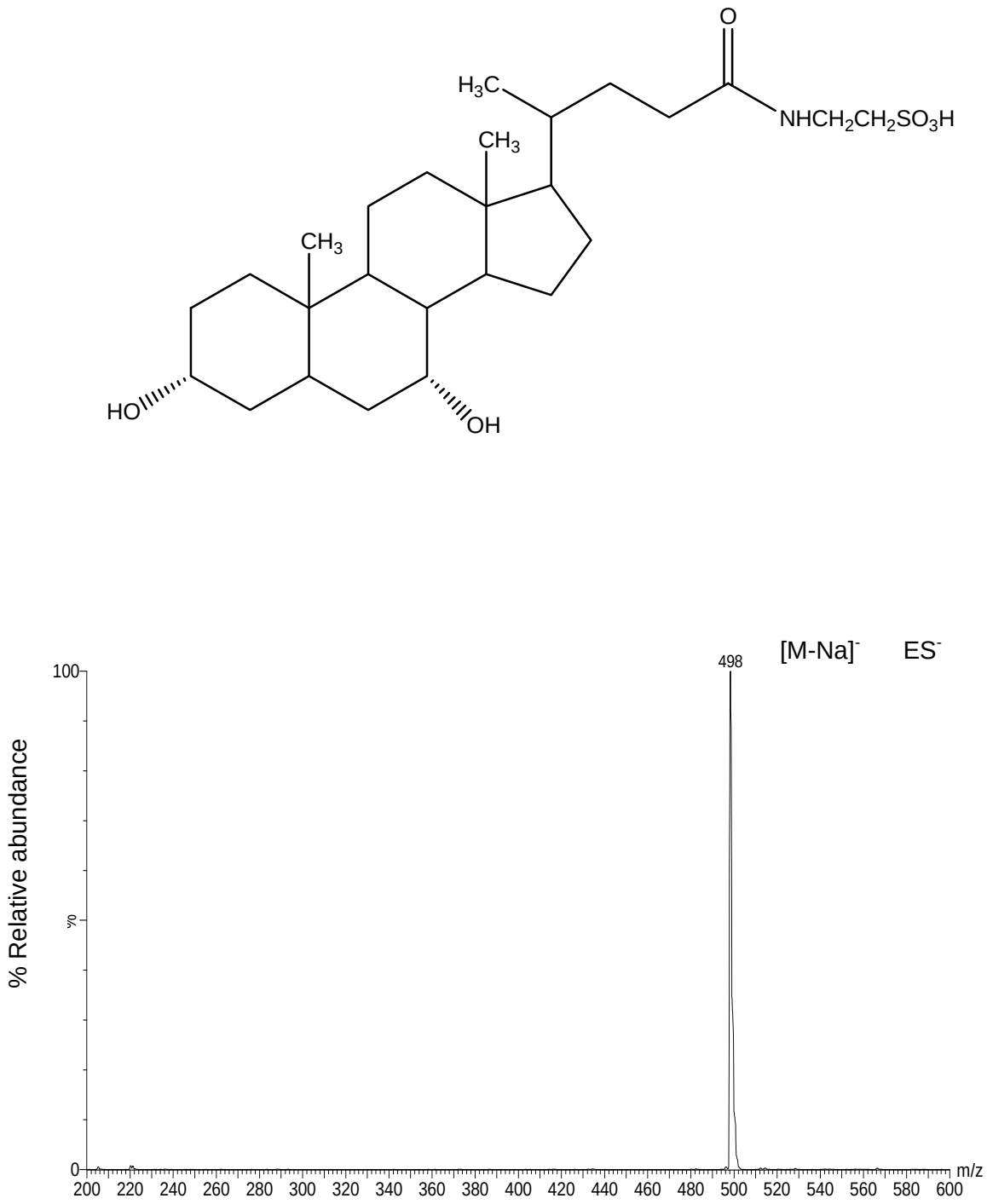


Figure 5.2c) Structure and ESI-MS spectra for sodium taurochenodeoxycholate (TCDCA) (10 μg/mL).

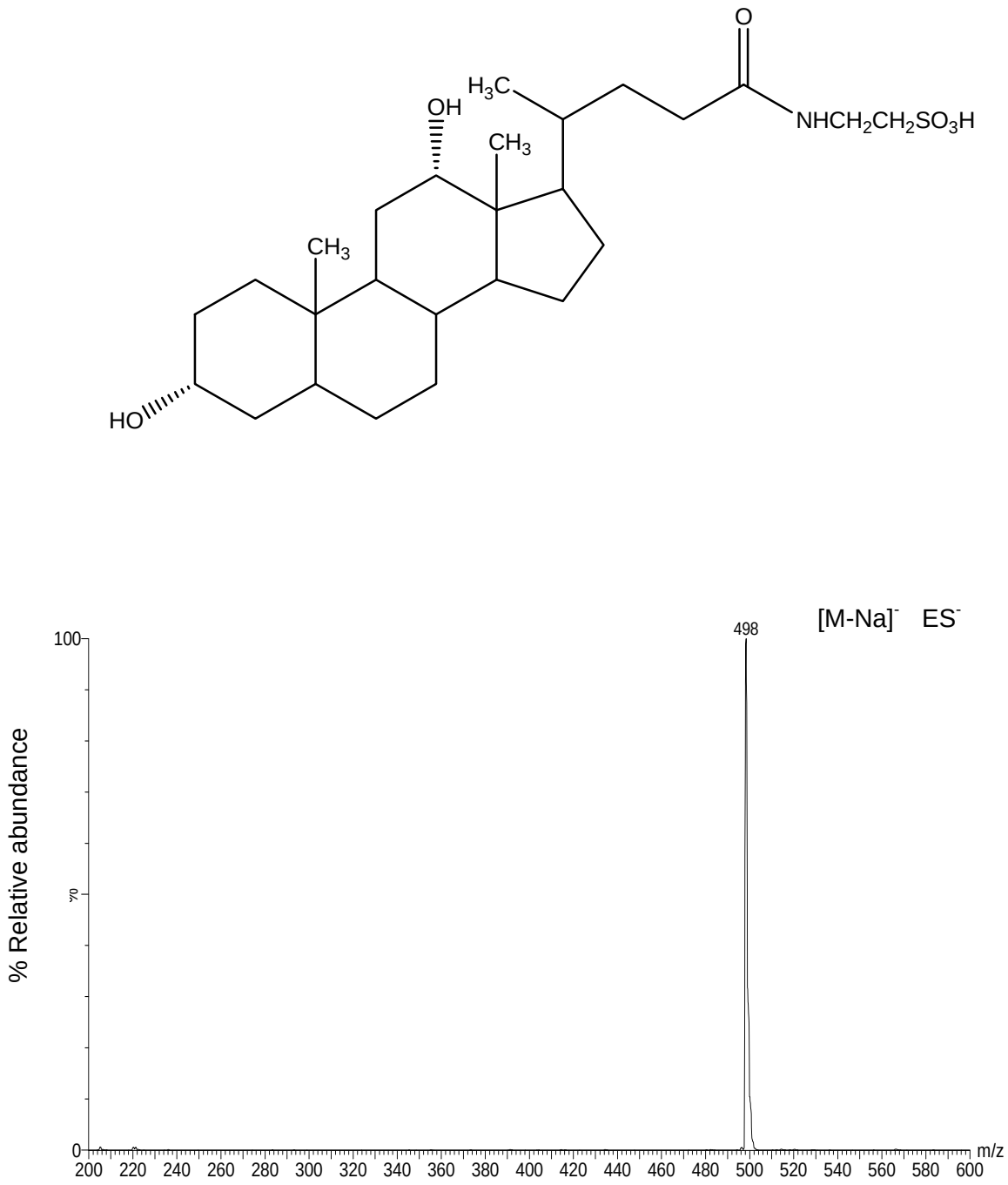


Figure 5.2d) Structure and ESI-MS spectra for sodium taurodeoxycholate (TDCA) (10 μg/mL).

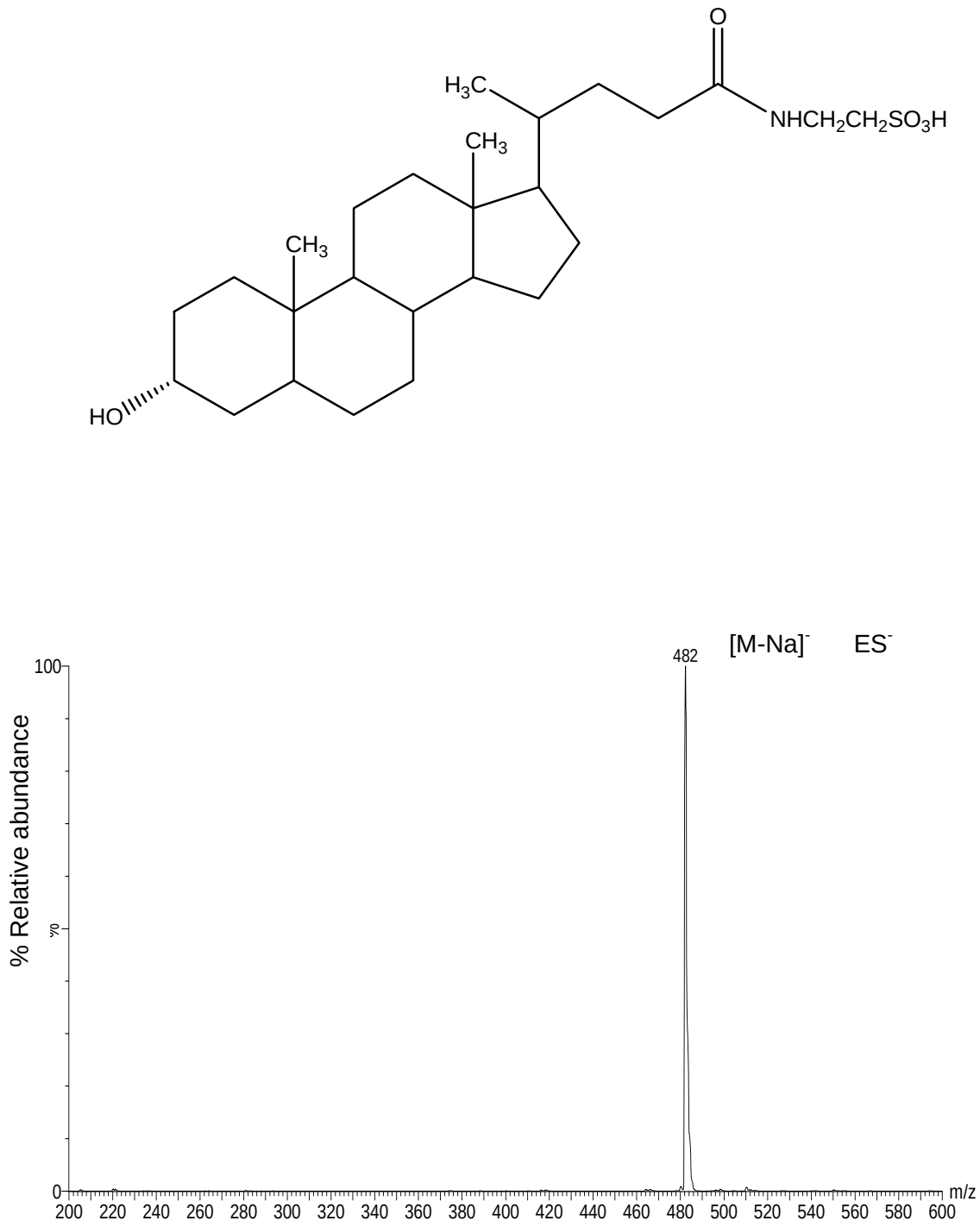


Figure 5.2e) Structure and ESI-MS spectra for sodium taurolithocholate (TLCA) (10 μg/mL).

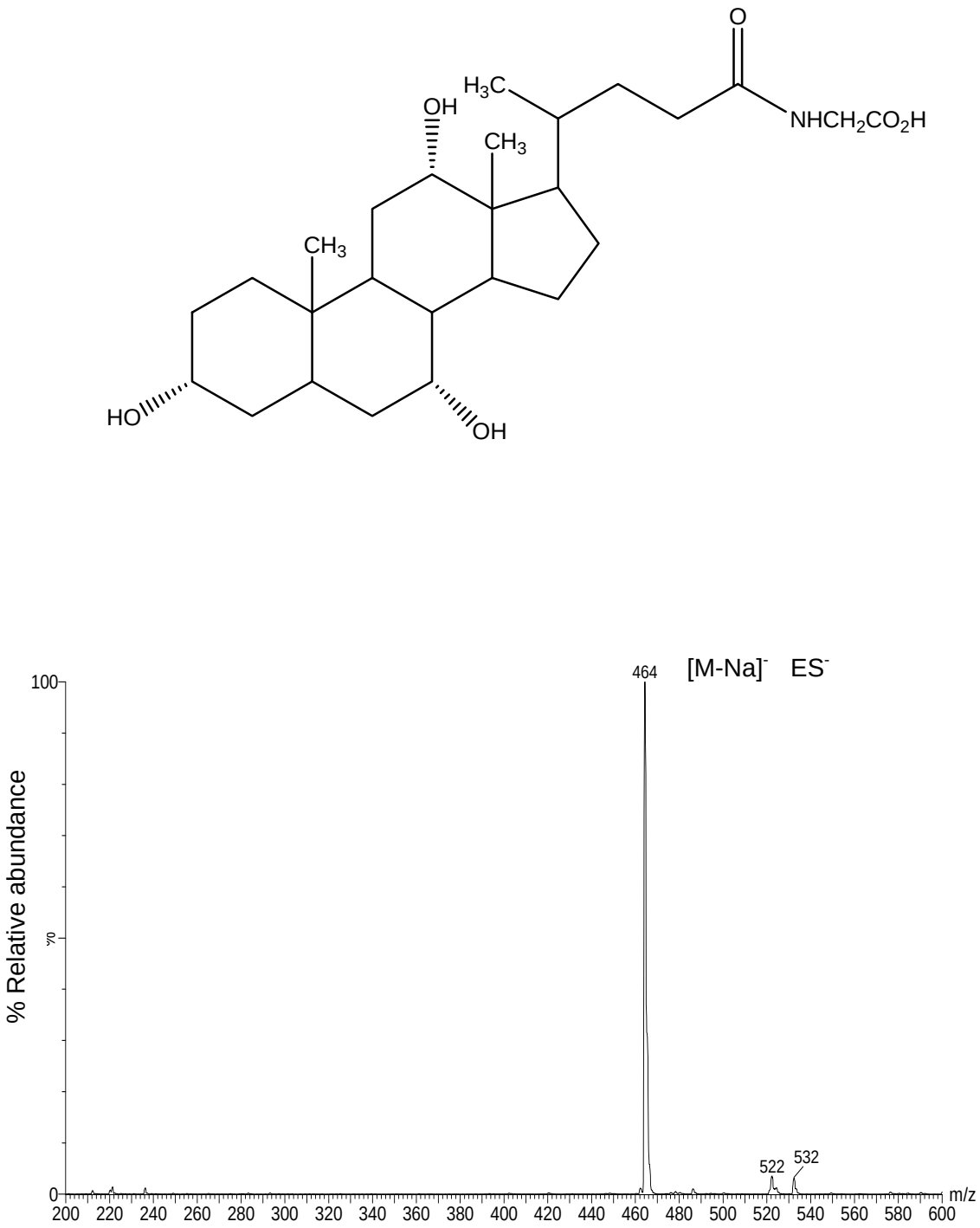


Figure 5.2f) Structure and ESI-MS spectra for sodium glycocholate (GCA) (10 μg/mL).

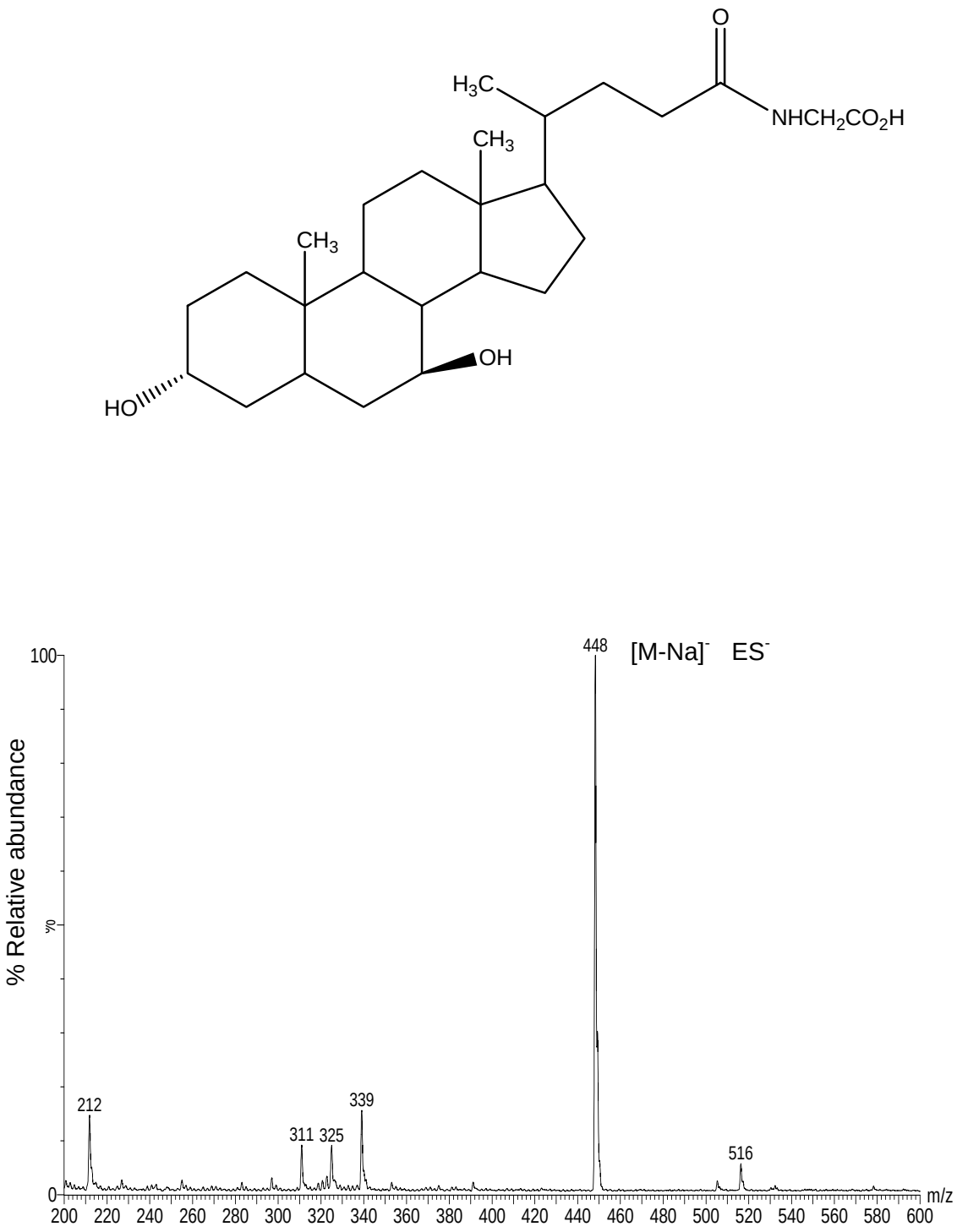


Figure 5.2g) Structure and ESI-MS spectra for sodium glycoursodeoxycholate (GUDCA) (10 μg/mL).

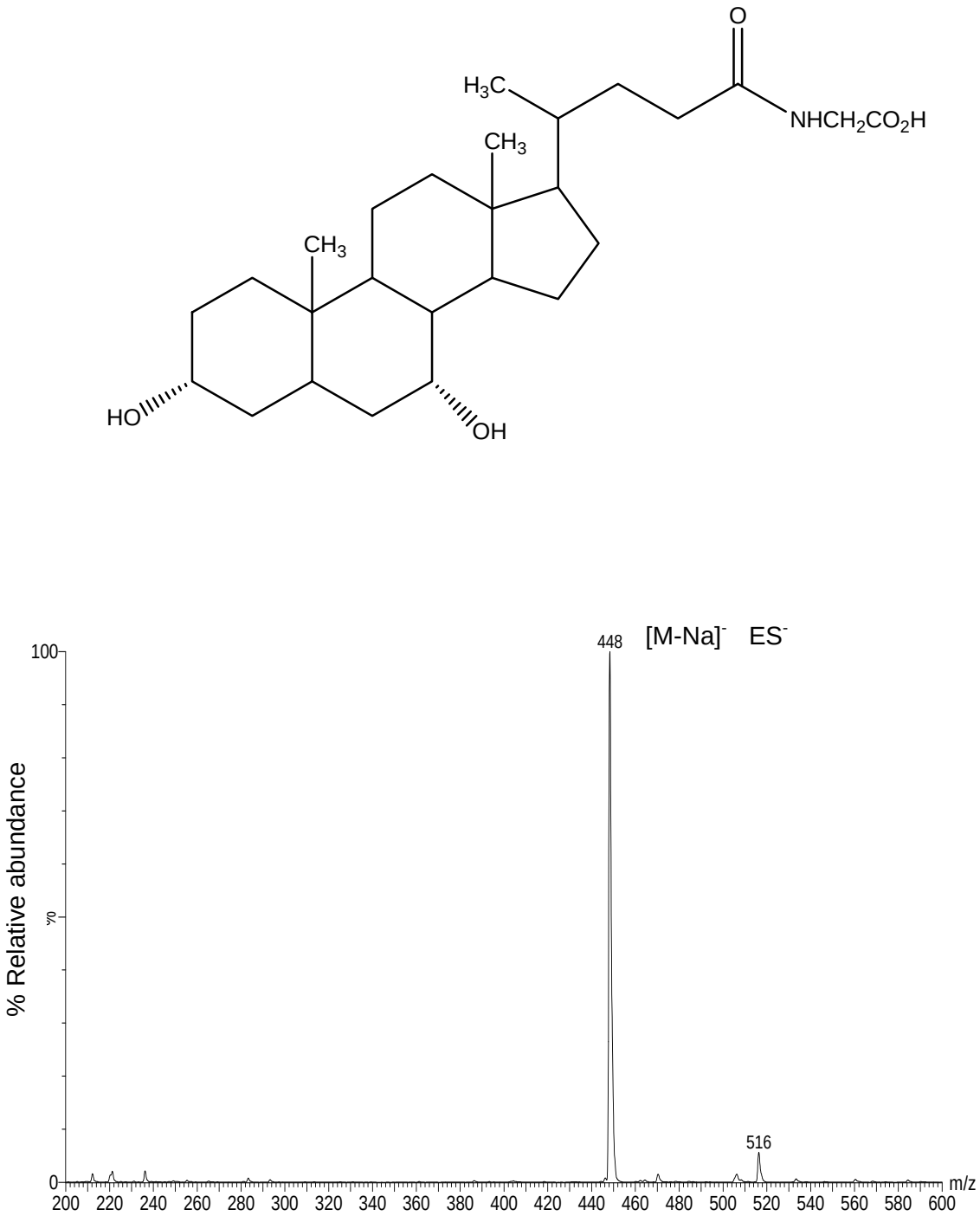
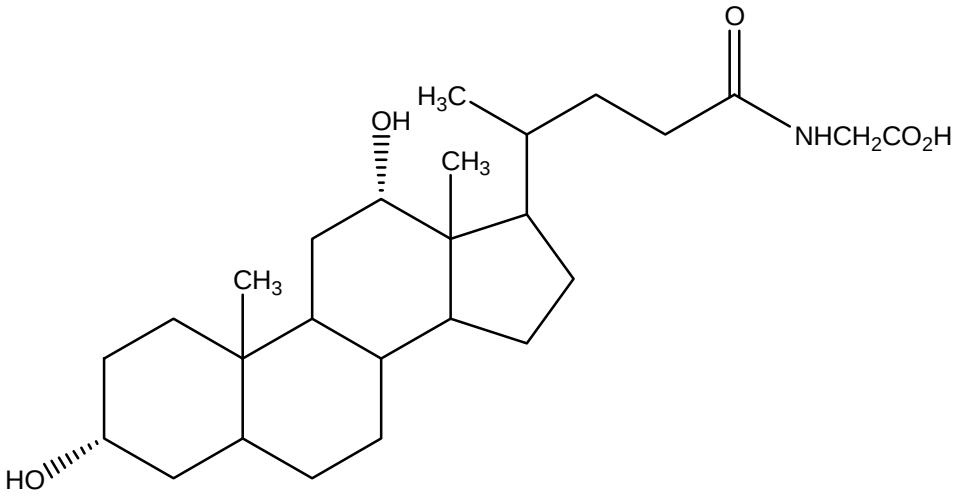


Figure 5.2h) ESI-MS spectra and structure for sodium glycochenodeoxycholate (GCDCA) (10 μg/mL).



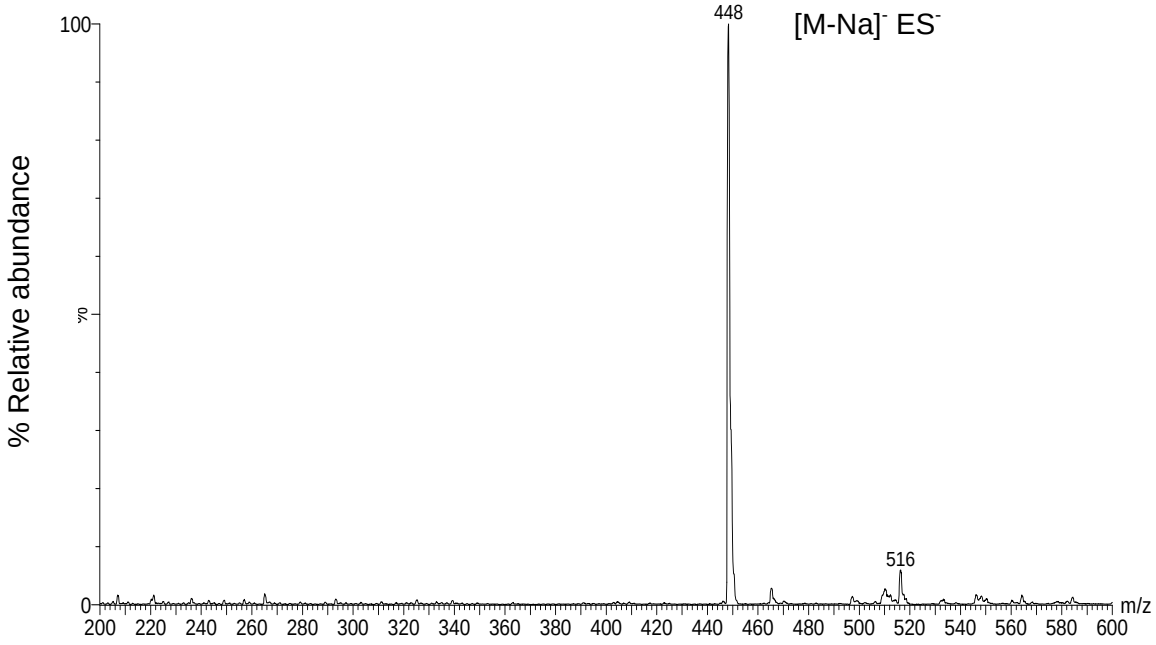
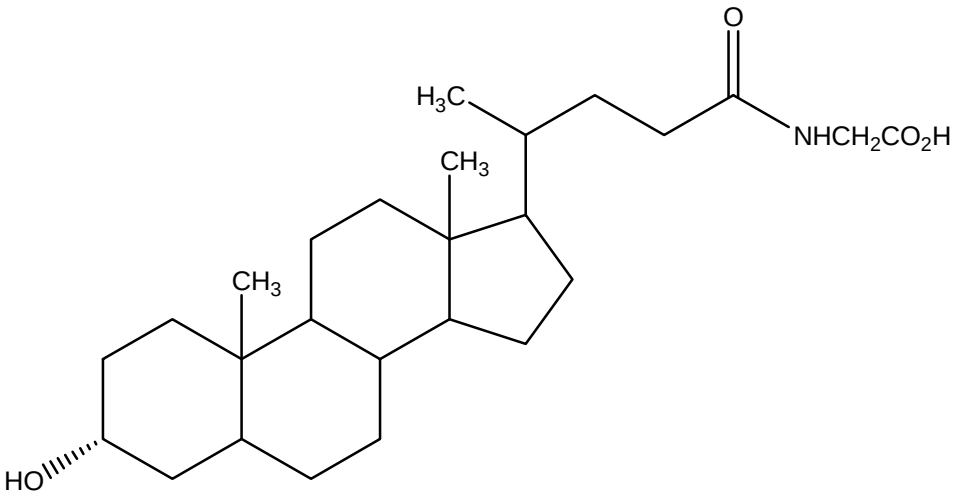


Figure 5.2i) ESI-MS spectra and structure for sodium glycodeoxycholate (GDCA) (10 μg/mL).



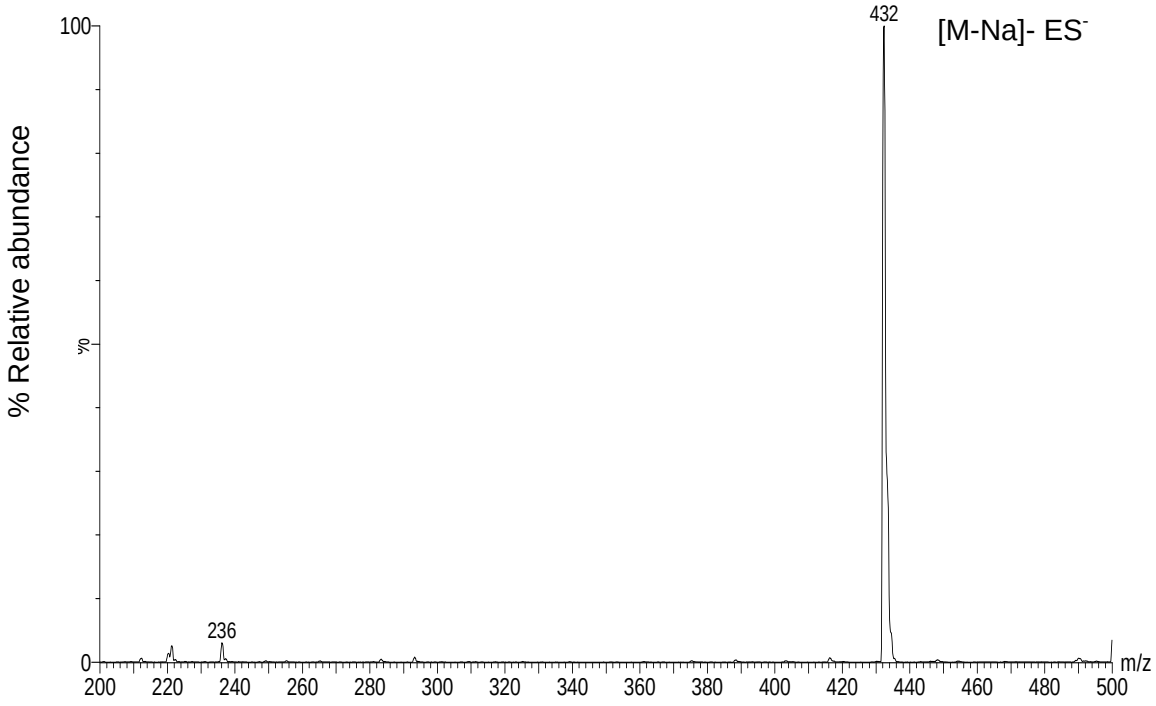
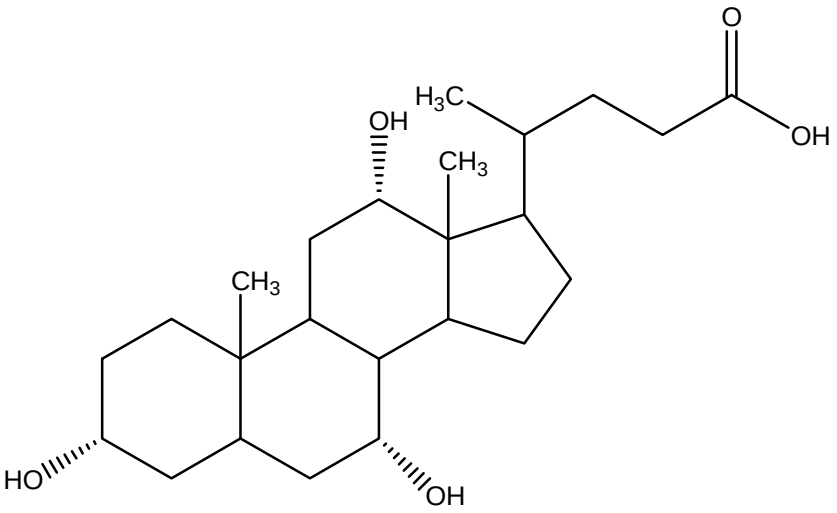


Figure 5.2j) ESI-MS spectra and structure for sodium glycolithocholate (GLCA) (10 μg/mL).



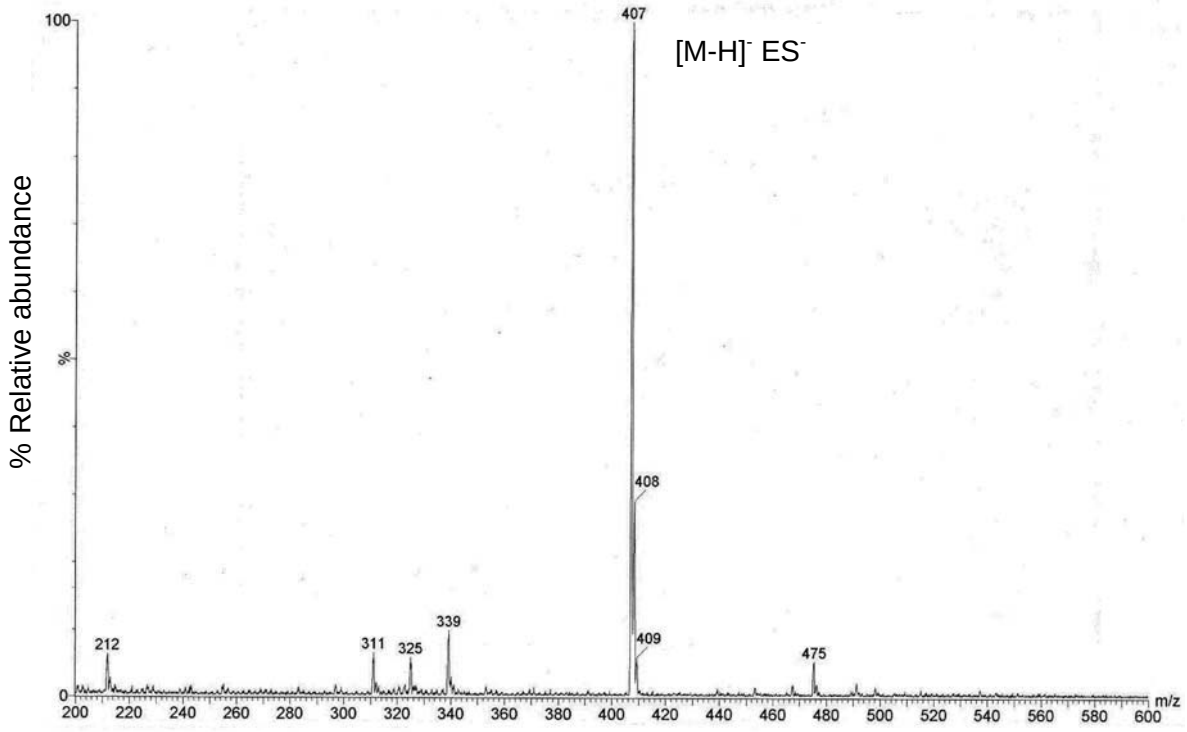


Figure 5.2k) ESI-MS spectra and structure for cholic acid (CA) (10 μg/mL).

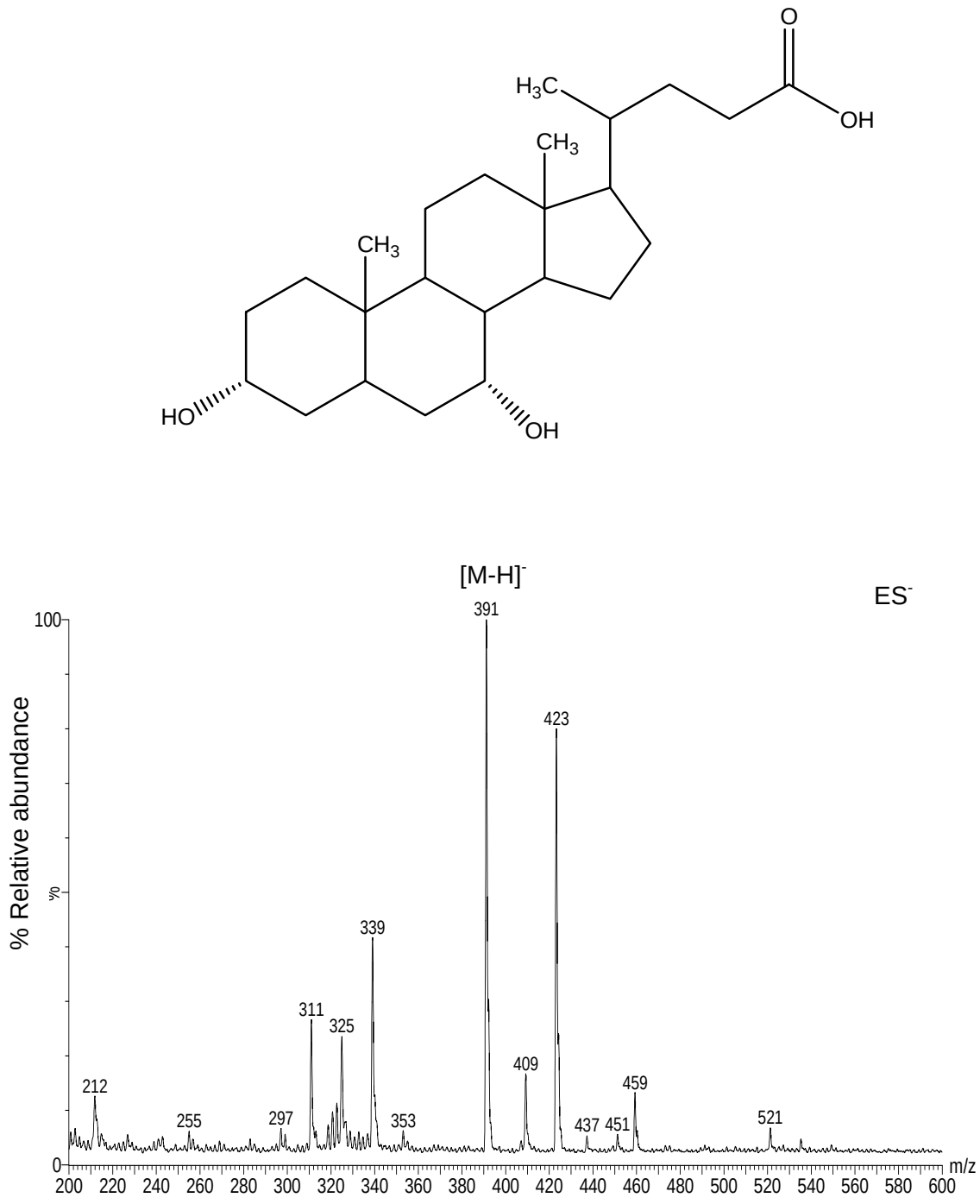


Figure 5.2l) ESI-MS spectra and structure for chenodeoxycholic acid (CDCA) (10 μg/mL).

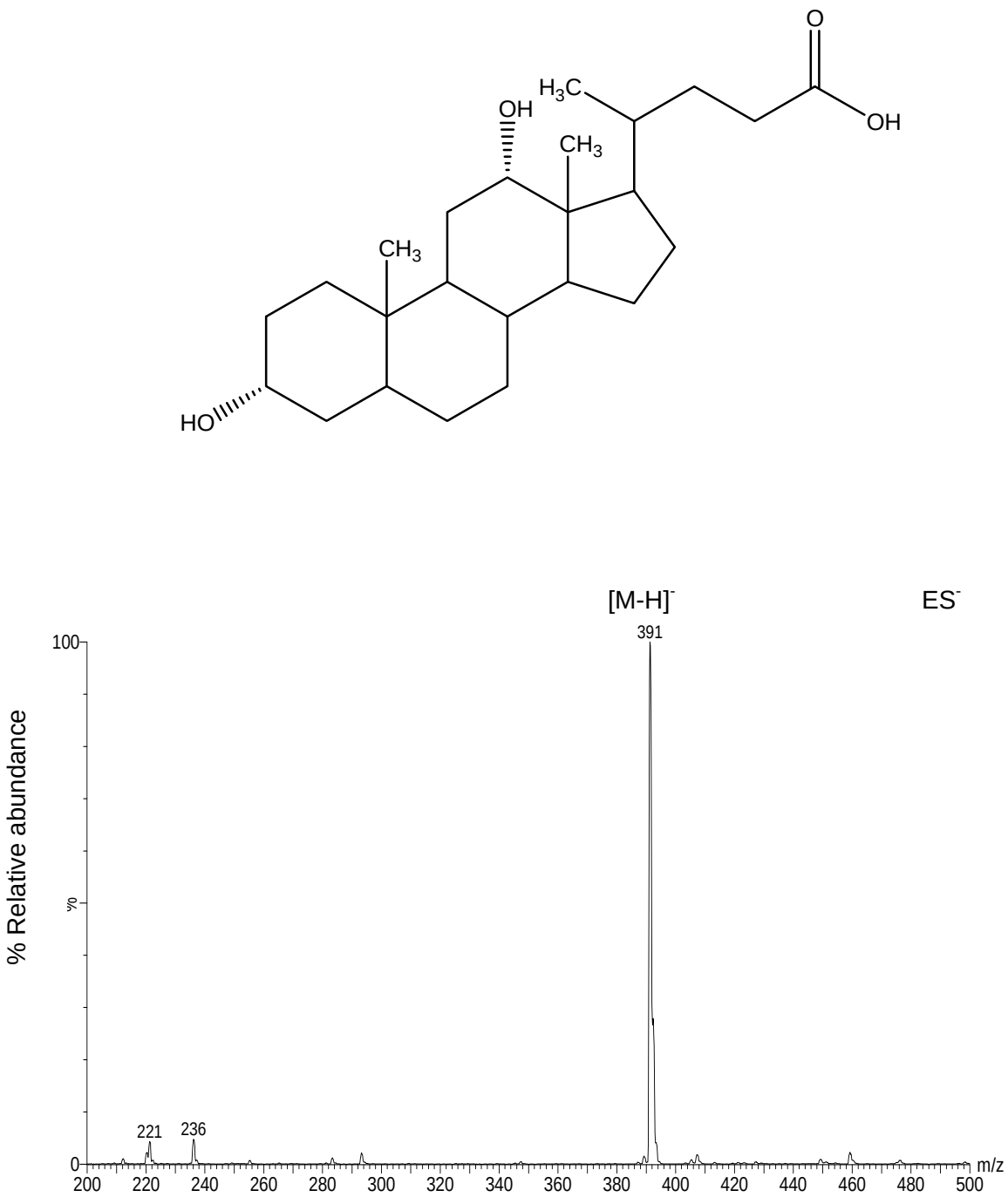
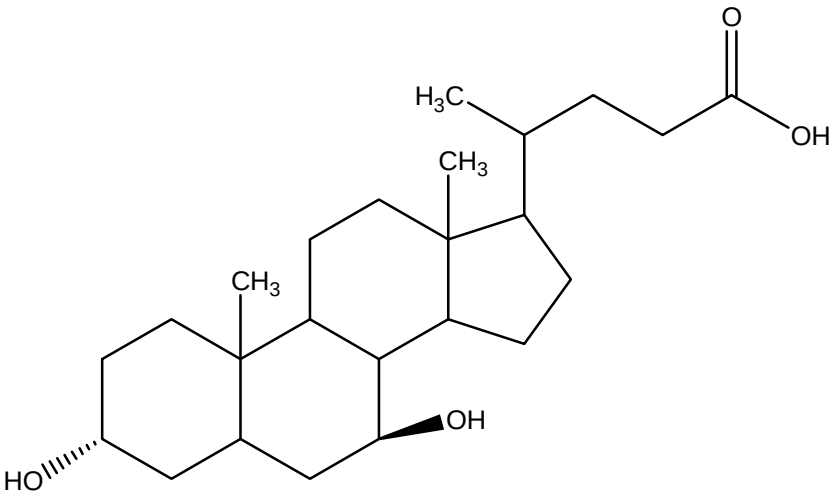


Figure 5.2m) ESI-MS spectra and structure for deoxycholic acid (DCA) (10 μg/mL).



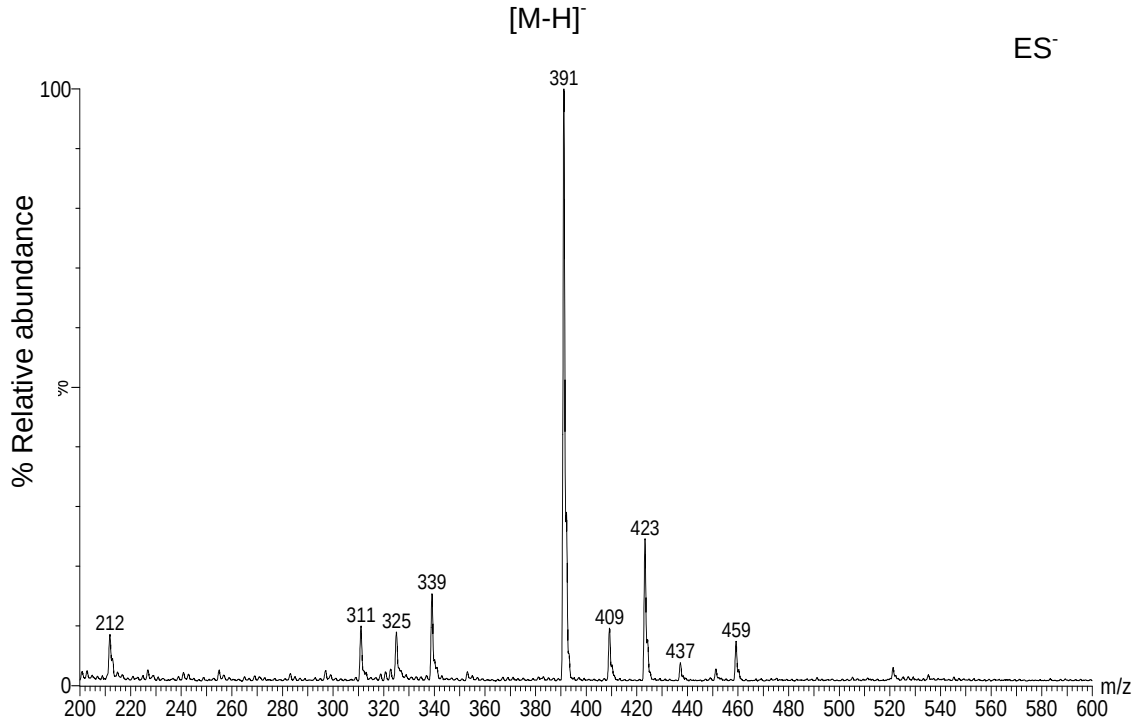


Figure 5.2n) ESI-MS spectra and structure for ursodeoxycholic acid (UDCA) (10 μg/mL).

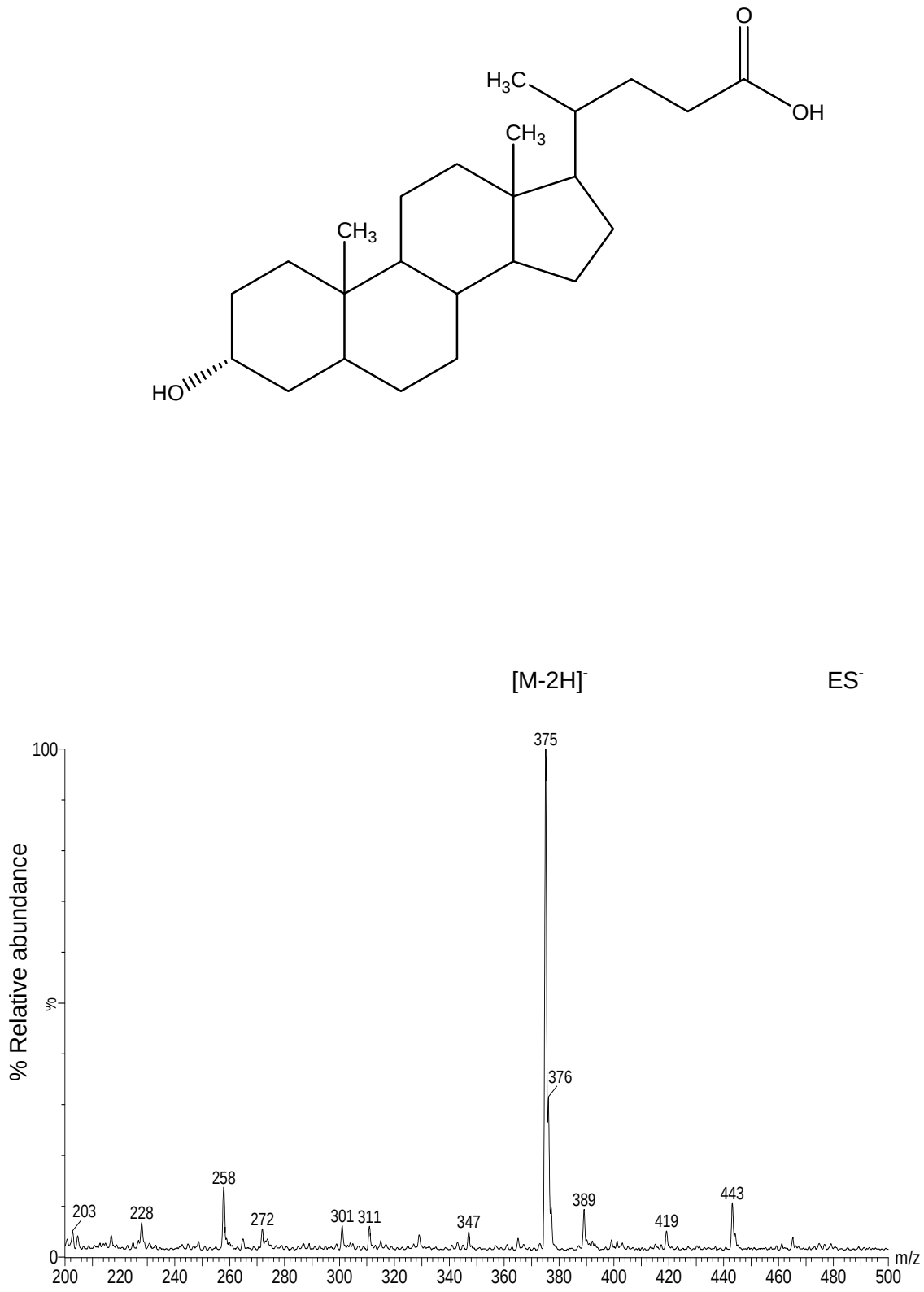
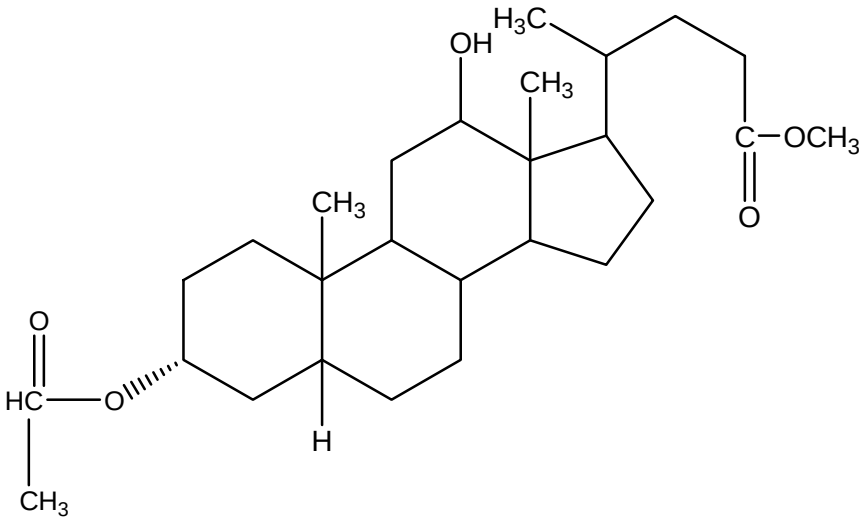


Figure 5.2o) ESI-MS spectra and structure for lithocholic acid (LCA) (10 μg/mL).

5.3.2. Choice of internal standard

The compound 5-β cholanic acid, 3α, 12α-diol 3 acetate methyl ester was tested as a possible internal standard. The compound showed weak signal in both negative mode and in positive mode. However, when the standard was made in ammonium acetate: MeOH solution, it showed a strong signal in positive mode and no signal in negative mode (Figure 5.3).



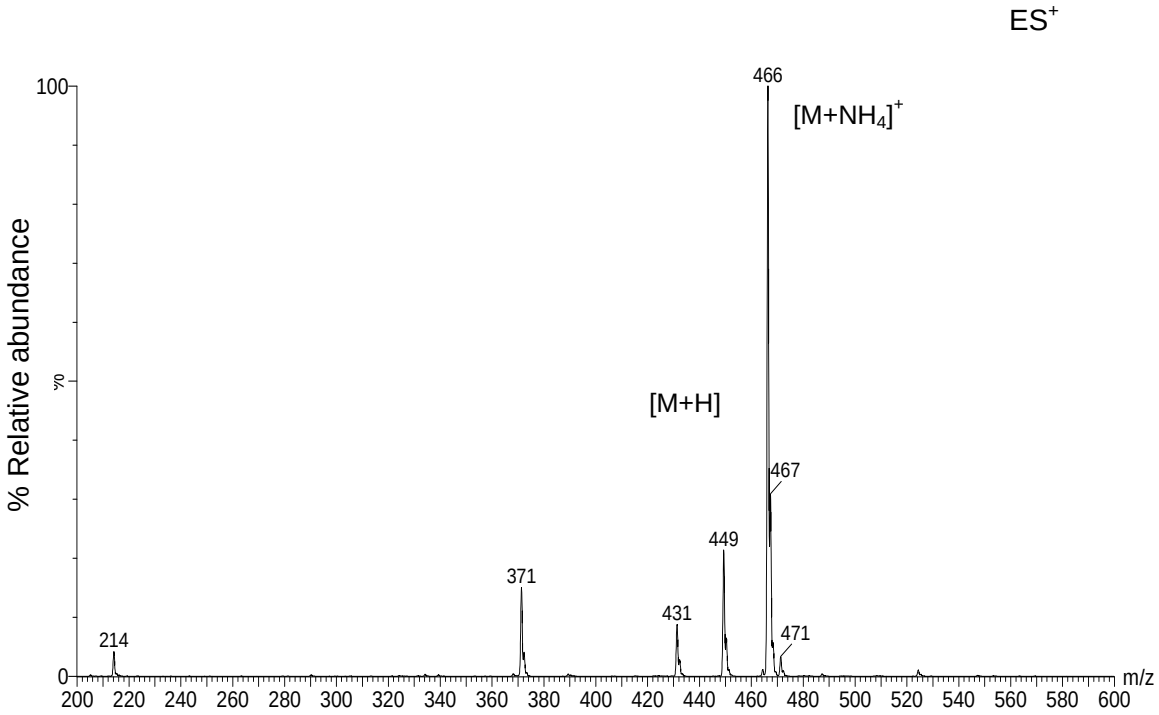


Figure 5.3. Structure and ESI-MS spectra of 5-β cholanic acid, 3α, 12α-diol 3 acetate methyl ester (10 µg/mL).

Deuterated analogues of bile acids behave almost identically to the corresponding non-labelled analytes differing only in their mass to charge ratio. Thus, cholic-2, 2, 4, 4-d_4 acid was tested. A stock solution of 1 mg/mL was made by dissolving 1 mg of the internal standard in 1 mL MeOH. A 10 µg/mL working solution was then prepared. The internal standard was monitored by the formation of the ion. Parameters for the SIR assay and a sample spectrum are shown in Figure 5.4 and Table 5.1B.

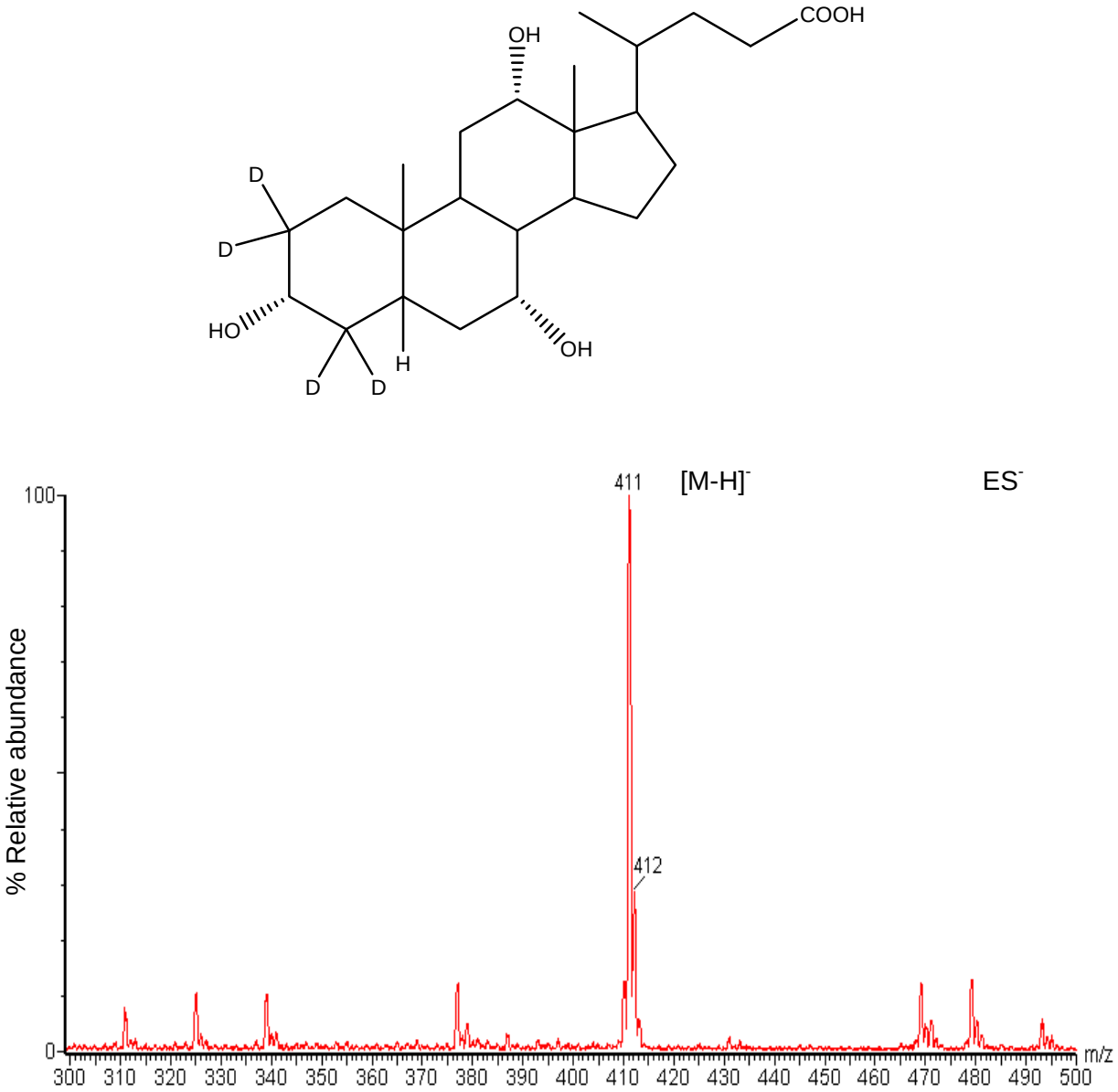


Figure 5.4) ESI-MS spectrum and structure for internal standard cholic-2, 2, 4, 4-d4 acid. (10 μg/mL).

5.3.3. LC-MS method development

Individual bile standards were serially diluted (50 μg-100 ng/mL) and analyzed chromatographically. The column was maintained at ambient temperature. The sample injections were performed with the sample chamber temperature set at 8^{o}C. This temperature was suitable to avoid sample evaporation as all the samples were dissolved/ reconstituted in MeOH. A needle wash was programmed in between each runs to avoid any cross-contamination in the injector system. A sample chromatogram of 15 resolved bile acids (66 μg/mL) is shown (Figure 5.5).

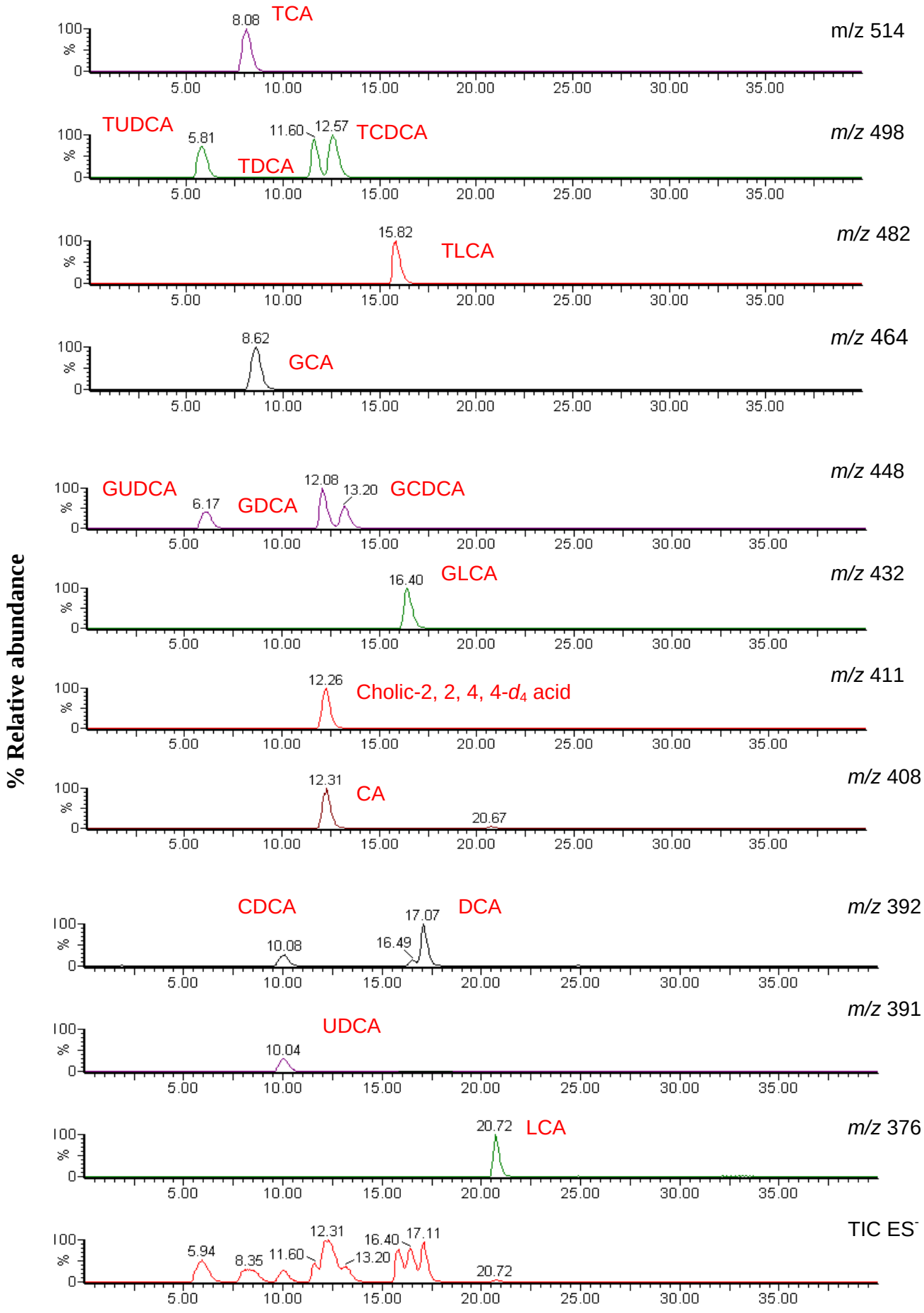
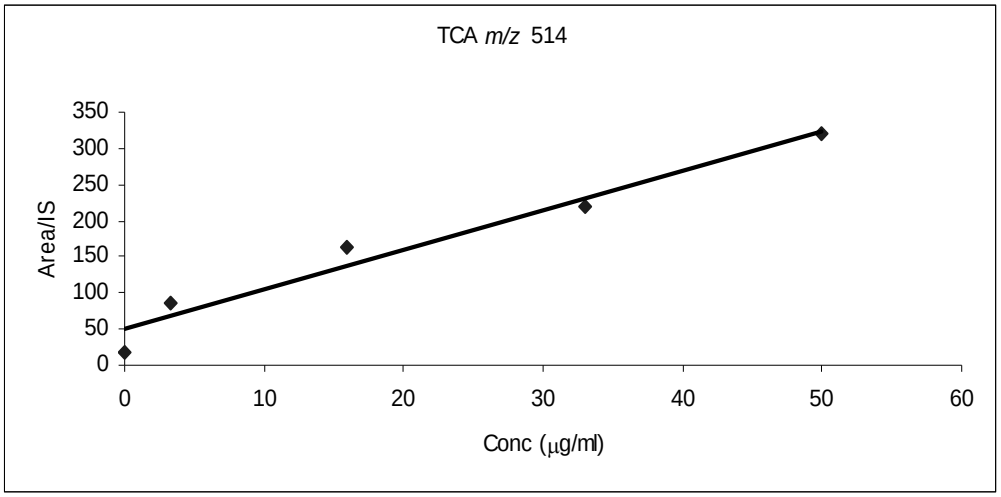


Figure 5.5. Representative chromatogram of ESI-LC/MS analysis of bile salt standards (33 µg/mL).

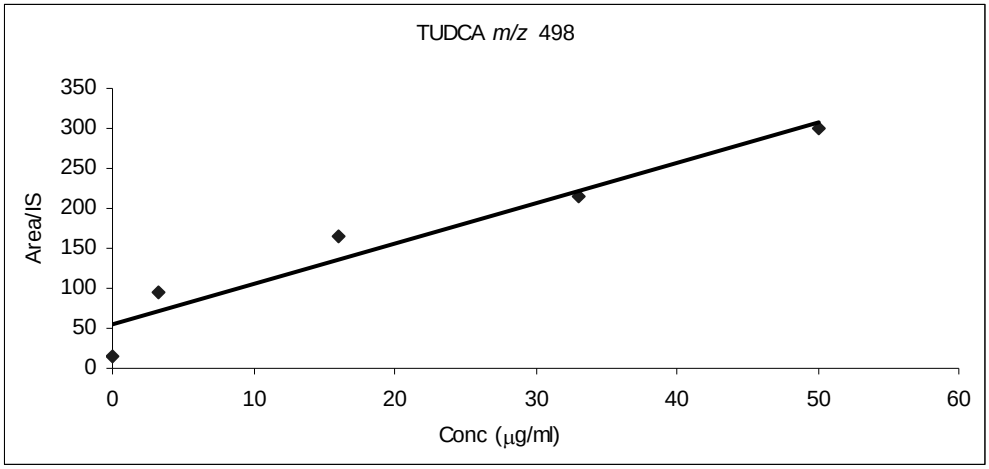
1. Taurocholic acid (TCA), 2. Tauroursodeoxycholic acid (TUDCA), 3. Taurochenodeoxycholic acid (TCDCA), 4. Taurodeoxycholic acid (TDCA), 5. Taurolithocholic acid (TLCA), 6. Glycocholic acid (GCA), 7. Glycoursodeoxycholic acid (GUDCA), 8. Glycochenodeoxycholic acid (GCDCA), 9. Glycodeoxycholic acid (GDCA), 10. Glycolithocholic acid (GLCA), 11. Cholic 2, 2, 4, 4-d4 acid (internal standard) 12. Cholic acid (CA), 13. Chenodeoxycholic acid (CDCA), 14. Deoxycholic acid (DCA), 15. Ursodeoxycholic acid (UDCA), 16. Lithocholic acid (LCA).

5.3.4. Calibration lines, linearity, detection and quantitation limits

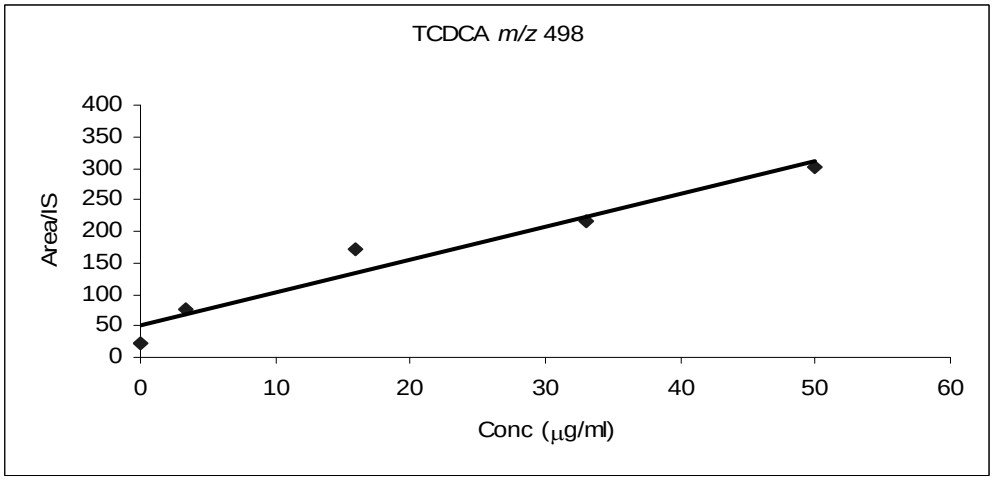
The composite standard was used to obtain a six point calibration curve with internal standard at the range of 25 μg-2.5 μg/mL. Linear regression and was estimated using the response ratio of the analyte to internal standard. These were then plotted against the concentration of the calibration standards (Figures 5.6 a to o). Indicative retention times, linear regression equation, correlation coefficient (R^2), limit of detection (LoD) and limit of quantitation (LoQ) for each standard is shown in Table 5.2.



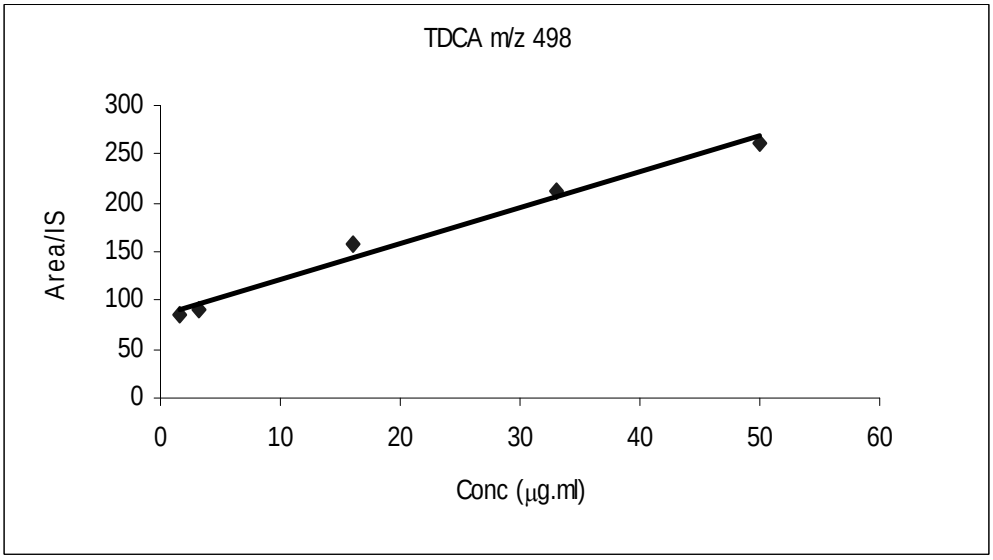
a)


TUDCA *m/z* 498
Area/IS
350
300
250
200
150
100
50
0
0
10
20
30
40
50
60
Conc (μg/ml)

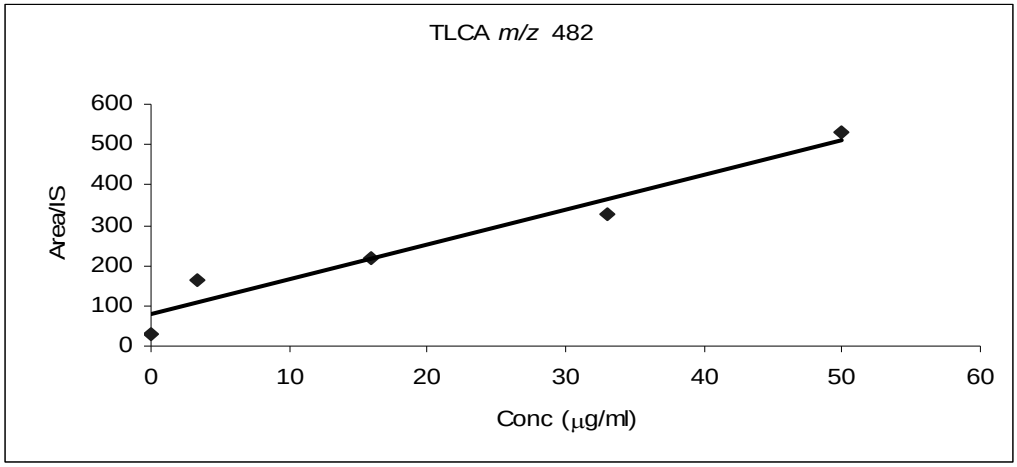
b)


TCDCA *m/z* 498
Area/IS
400
350
300
250
200
150
100
50
0
0
10
20
30
40
50
60
Conc (μg/ml)

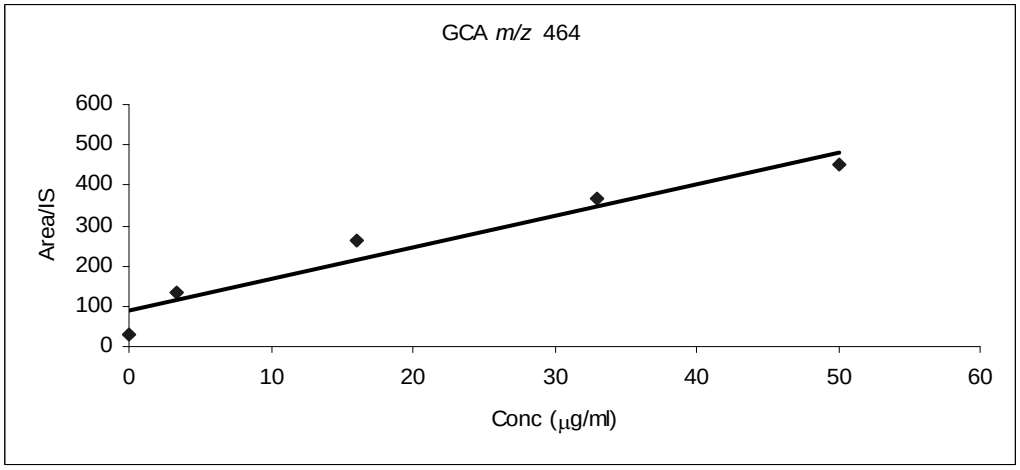
c)


TDCA m/z 498
Area/IS
300
250
200
150
100
50
0
0
10
20
30
40
50
60
Conc (μg.ml)

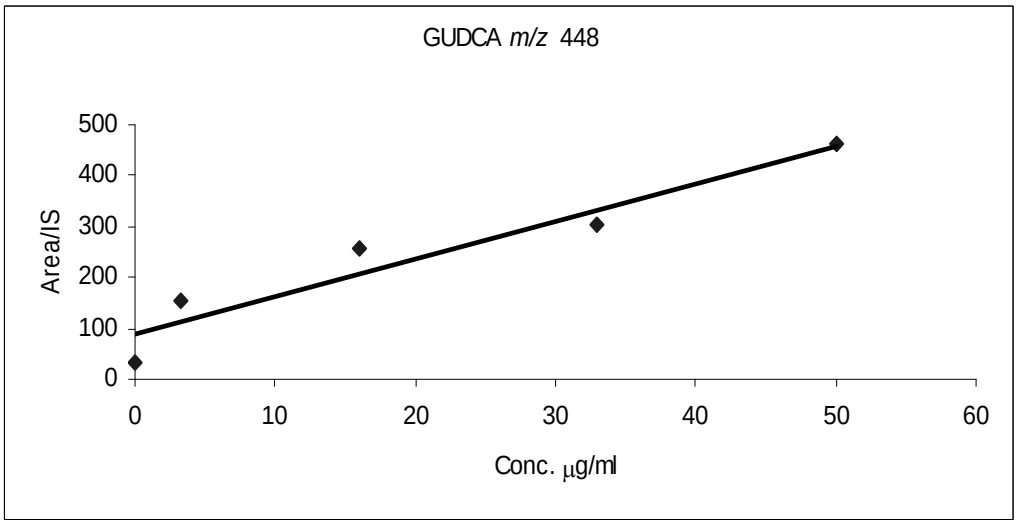
d)


TLCA *m/z* 482
Area/IS
600
500
400
300
200
100
0
0
10
20
30
40
50
60
Conc (μg/ml)

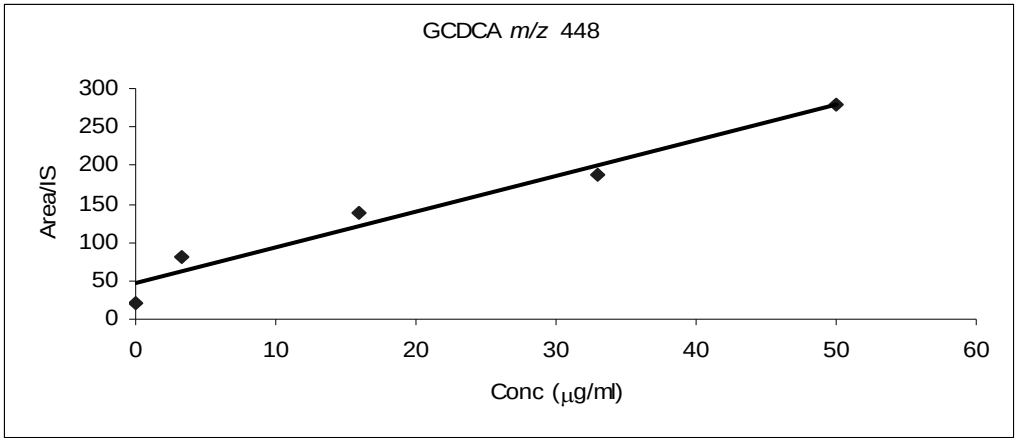
e)


GCA *m/z* 464
Area/IS
600
500
400
300
200
100
0
0
10
20
30
40
50
60
Conc (μg/ml)

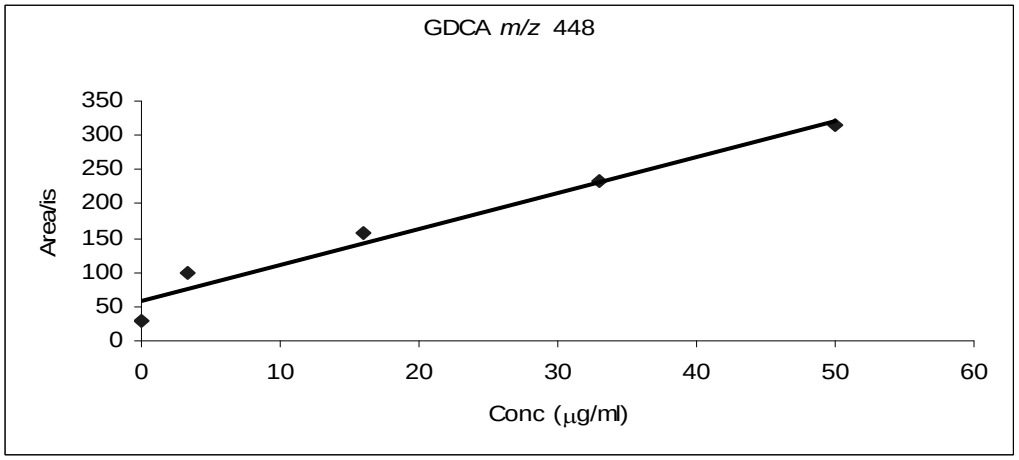
f)


GUDCA *m/z* 448
Area/IS
500
400
300
200
100
0
0
10
20
30
40
50
60
Conc. μg/ml

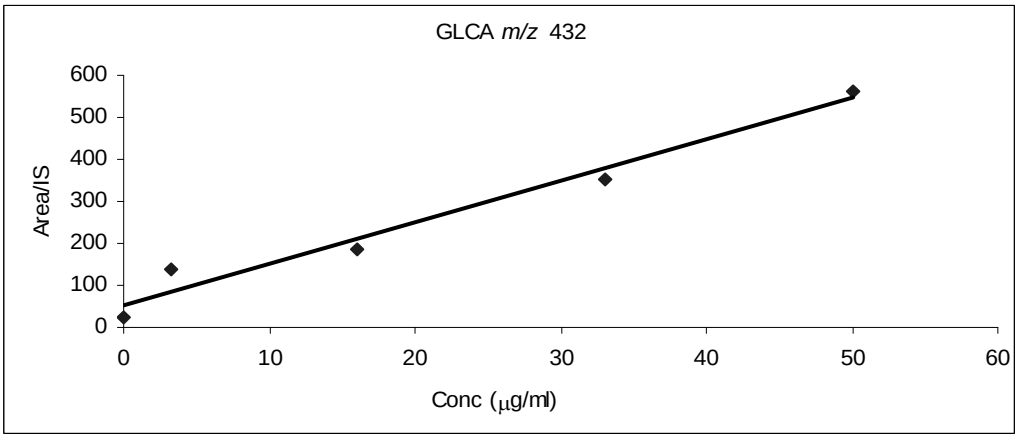
g)


GCDCA *m/z* 448
Area/IS
300
250
200
150
100
50
0
0
10
20
30
40
50
60
Conc (μg/ml)

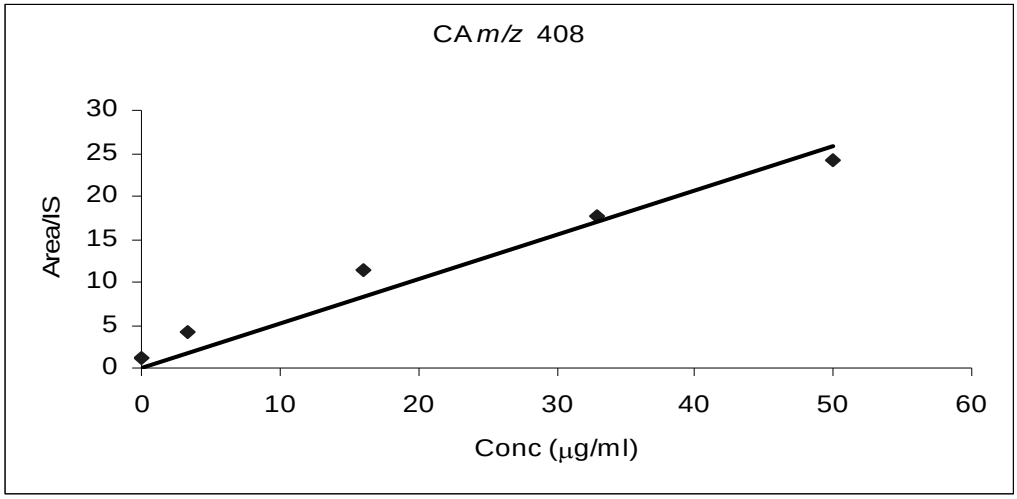
h)


GDCA *m/z* 448
Area/is
350
300
250
200
150
100
50
0
0
10
20
30
40
50
60
Conc (μg/ml)

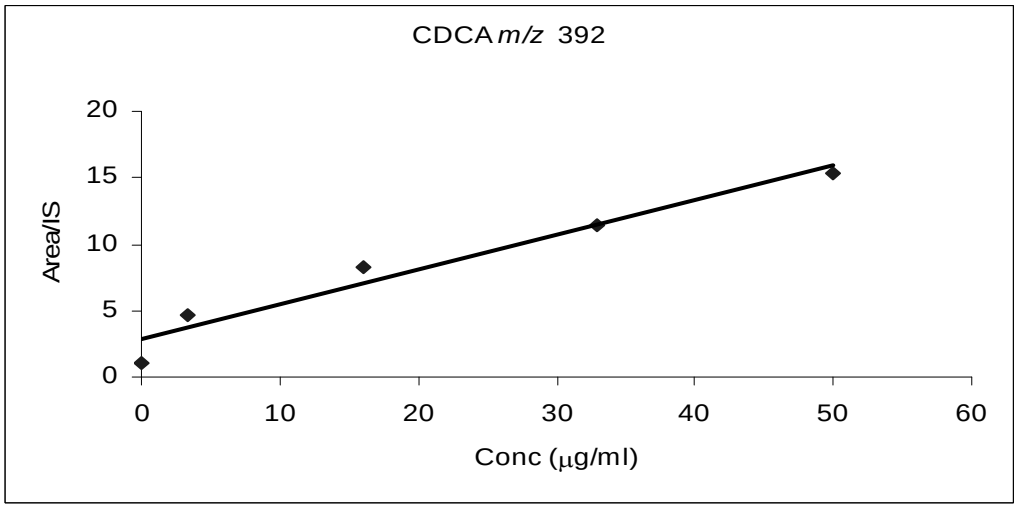
i)


GLCA *m/z* 432
Area/IS
600
500
400
300
200
100
0
0
10
20
30
40
50
60
Conc (μg/ml)

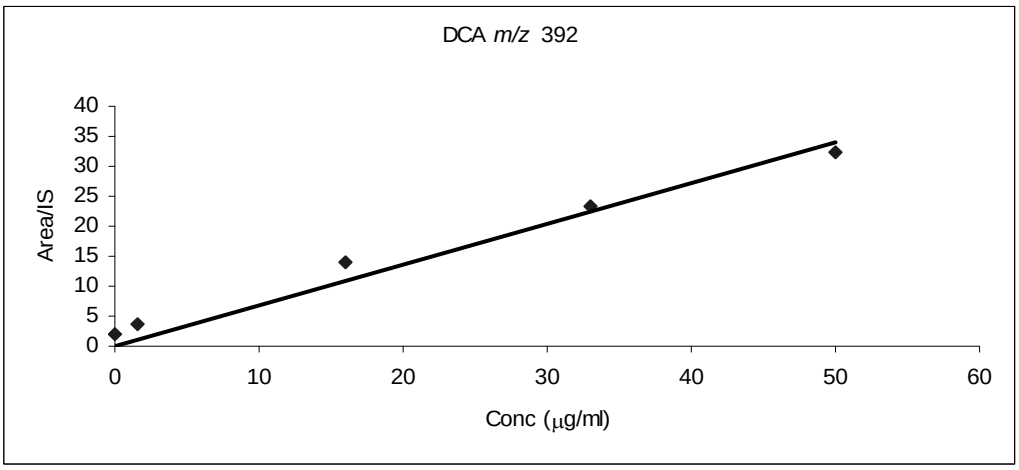
j)


CA *m/z* 408
Area/IS
0
5
10
15
20
25
30
0
10
20
30
40
50
60
Conc (μg/ml)

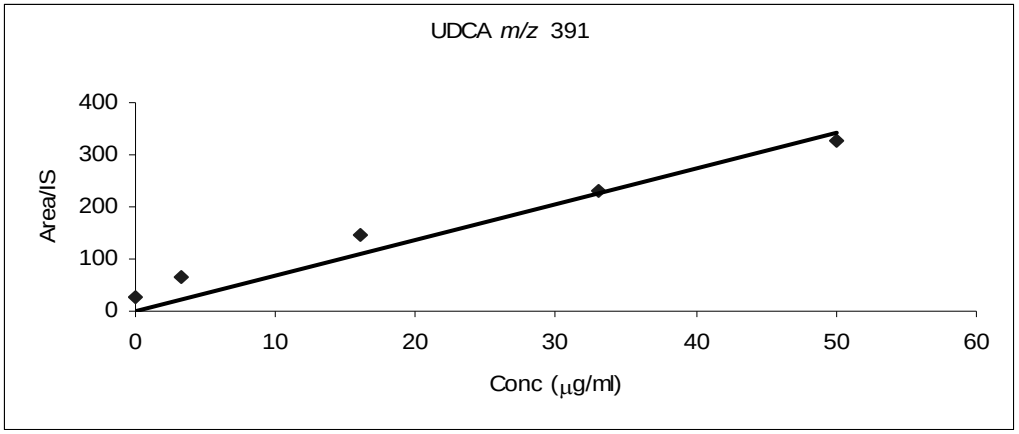
k)


CDCA *m/z* 392
Area/IS
0
5
10
15
20
0
10
20
30
40
50
60
Conc (μg/ml)

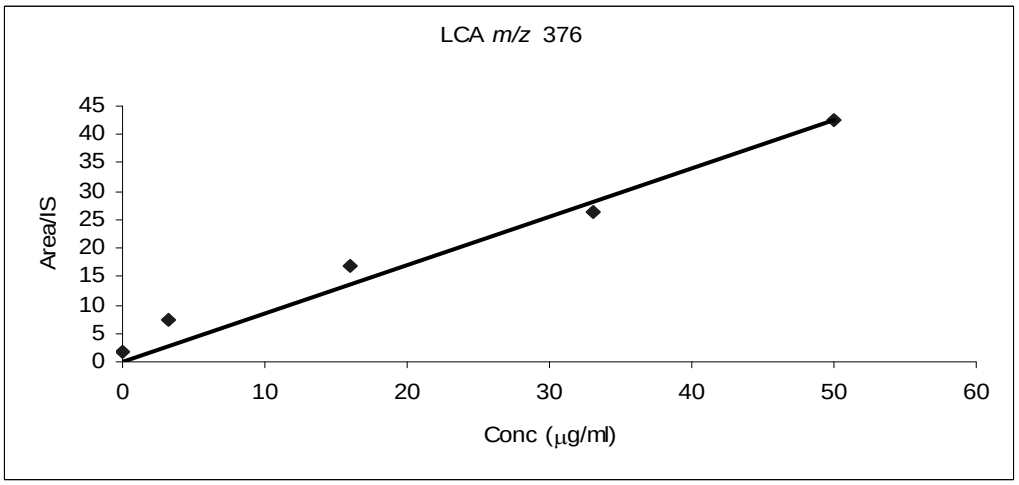
l)


DCA *m/z* 392
Area/IS
0
5
10
15
20
25
30
35
40
0
10
20
30
40
50
60
Conc (μg/ml)

m)



n)



o)

Figure 5.5 a to o. Calibration lines for individual bile salts/ acid.

The calibration lines for the bile acids are in the following order:

a) Taurocholic acid (TCA), b) Tauroursodeoxycholic acid (TUDCA), c). Taurochenodeoxycholic acid (TCDCA), d) Taurodeoxycholic acid (TDCA), e). Taurolithocholic acid (TLCA), f). Glycocholic acid (GCA), g). Glycoursodeoxycholic acid (GUDCA), h). Glycochenodeoxycholic acid (GCDCA), i). Glycodeoxycholic acid (GDCA), j). Glycolithocholic acid (GLCA), k). Cholic acid (CA), l) Chenodeoxycholic acid (CDCA), m) Deoxycholic acid (DCA), n) Ursodeoxycholic acid (UDCA), o) Lithocholic acid (LCA). Calibration line for internal standard is not shown.

Table 5.2. Indicative retention times, linearity, LoD, LoQ of the ESI-LC/MS of bile salts.

Bile acid Standards	Equations	R^2	LoD (ng)	LoQ (ng)	Retention time
TCA	y=5.48x+49.4	0.960	7	25	8.08
TUDCA	y=5.62x+55.4	0.935	5	25	5.81
TCDCA	y=5.186x+51.03	0.951	1	50	11.60
TDCA	y=3.673x+85.36	0.984	8	25	12.57
TLCA	y=8.699x+77.51	0.948	1	50	15.82
GCA	y=7.68x+95.2	0.936	5	25	8.62
GUDCA	y=7.39x+90.1	0.919	7	25	6.17
GCDCA	y=4.63x+46.96	0.962	7	50	12.08
GDCA	y=5.25x+58.95	0.965	3	25	13.20
GLCA	y=9.92x+49.4	0.972	2	50	16.40
CA	y=0.423x+4.05	0.942	9	100	12.31
CDCA	y=0.271x+2.42	0.954	2	100	10.08
DCA	y=0.526x+6.81	0.912	5	100	17.07
UDCA	y=5.45x+57.8	0.969	3	100	10.08
LCA	y=0.658x+7.87	0.916	1	100	20.72

5.3.5. Reproducibility

To ensure method reliability and reproducibility, inter and intra day accuracy and precision were determined and this was determined by using six quality control concentrations distributed throughout the calibration range for each analyte. Each of these six QC points were analysed in replicates for three days to determine the inter-day and between (intra) days variations.

Inter-day accuracy and precision was calculated by dividing the standard deviation of the three replicates by the overall mean area and converting the value to a percentage relative standard deviation (%RSD= SD/mean) respectively. The inter-day precision for all the analytes were less than 15% and is shown in Table 5.3.

A mid-range concentration (30 ng) was selected and injected for a total of five times in succession and the co-efficient of variation was calculated by dividing the standard deviation of the five replicates by the overall mean area and converting the value to a percentage to give the between (inter) day accuracy and precision. The calibrating solutions were freshly prepared from the individual bile acid stock solutions stored at -80ºC. The co-efficient of variation for intra-day precision was less than 5% (except for GDCA). The intra-day precision is summarized in Table 5.4.

5.3.6. Recovery

The average recoveries ranged from 86 to 105 %. The results are summarized in Table 5.4.

	QC1		QC2		QC3		QC4		QC5		QC6	
Conc.	0.16 µg/mL	%RSD	1.6 µg/mL	%RSD	3.3 µg/mL	%RSD	16 µg/mL	%RSD	33 µg/mL	%RSD	50 µg/mL	%RSD
TCA	11	13.4	26	8.1	86	2.9	179	8.6	233	9.1	333	3.8
TCDCA	4.3	13.5	16	12.7	26	17	153	14.3	207	3.6	288	7.4
TDCA	7.7	11.7	17.7	3.3	35.3	13	169	15.9	217	9.8	257	2.5
TUDCA	5.3	9.8	74	6.6	91	5.8	166	6.9	233	8.5	277	7.0
TLCA	8.8	3.8	132	1.6	172	5.8	226	1.3	336	9.0	495	5.2
Nominal Conc.	0.06 µg/mL	%RSD	0.16 µg/mL	%RSD	1.6 µg/mL	%RSD	3.3 µg/mL	%RSD	16 µg/mL	%RSD	33 µg/mL	%RSD
GCA	2.4	8.6	20.7	7.4	125	5.0	141	5.8	261	3.2	367	5.2
GCDCA	1.7	12.4	3.1	3.2	15.2	8.4	86	5.2	133	3.7	179	9.8
GDCA	2.0	1.5	26	13.8	66	6.1	39	11.5	145	8.6	244	8.5
GUDCA	2.0	7.3	34	12.8	144	7.7	144	11.8	249	13.5	332	9.6
GLCA	4.9	10.6	28	9.2	57	10.5	41.3	13.3	174.7	15.7	321	7.4
Nominal Conc.	0.06 µg/mL	%RSD	0.16 µg/mL	%RSD	1.6 µg/mL	%RSD	3.3 µg/mL	%RSD	16 µg/mL	%RSD	33 µg/mL	%RSD
CA	0.15	9.9	1.43	14.5	4.2	8.2	4.5	14	16.6	14	17.3	8.1
CDCA	0.45	9.7	3.1	6.1	3.6	2.6	16.8	7.6	8.0	8.5	11.0	3.5
DCA	0.73	3.6	1.9	9.6	7.1	13.8	13.1	2.1	19.5	11.3	22.9	10.7
UDCA	4.2	4.9	0.0	0.0	18.0	11.0	191	1.7	166	2.4	235	1.6
LCA	0.65	3.2	1.7	4.0	11.6	5.8	14.2	0.55	19.3	9.6	26.7	15.0

Table 5. 3. Inter-day accuracy and precision for the ESI-LC/MS assay of bile salts.

Table 5.4. Intra-day accuracy and precision recovery of bile acids. Mean recovery rate of the extraction procedure presented as of % recovery (n=3, ± SD). The average peak areas of the extracted analyte were compared with the one for the non-extracted standard from which the % recovery was calculated.

Bile acids	Intra-day CV (%)	Recovery (%)
TCA	3.6	90.05±2
TUDCA	5.4	99.10±9
TCDCA	ND	90.21±6
TDCA	3.9	99.6±6
TLCA	5.4	87.77±9
GCA	3.7	104.76±5
GUDCA	4.7	96.24±7
GCDCA	ND	92.70±7
GDCA	6.1	85.52±3
GLCA	2.5	89.47±7
CA	4.1	87.67±12
CDCA	5.0	97.36±1
DCA	2.2	87.39±2
UDCA	4.5	92.06±2
LCA	4.0	83.12±13

ND-Not detected.

5.3.7. Bile salt analysis in clinical samples

The bile salts were extracted from the bile as described in Section 2.7.4. (see methods section). Typical chromatograms for the analysis of clinical samples from patients suffering from cholestatic hepato-biliary diseases are shown in Figure 5.6. The results of the analysis of conjugated and unconjugated acids from patients suffering from cholestatic hepato-biliary disease are shown in Table 5.5.

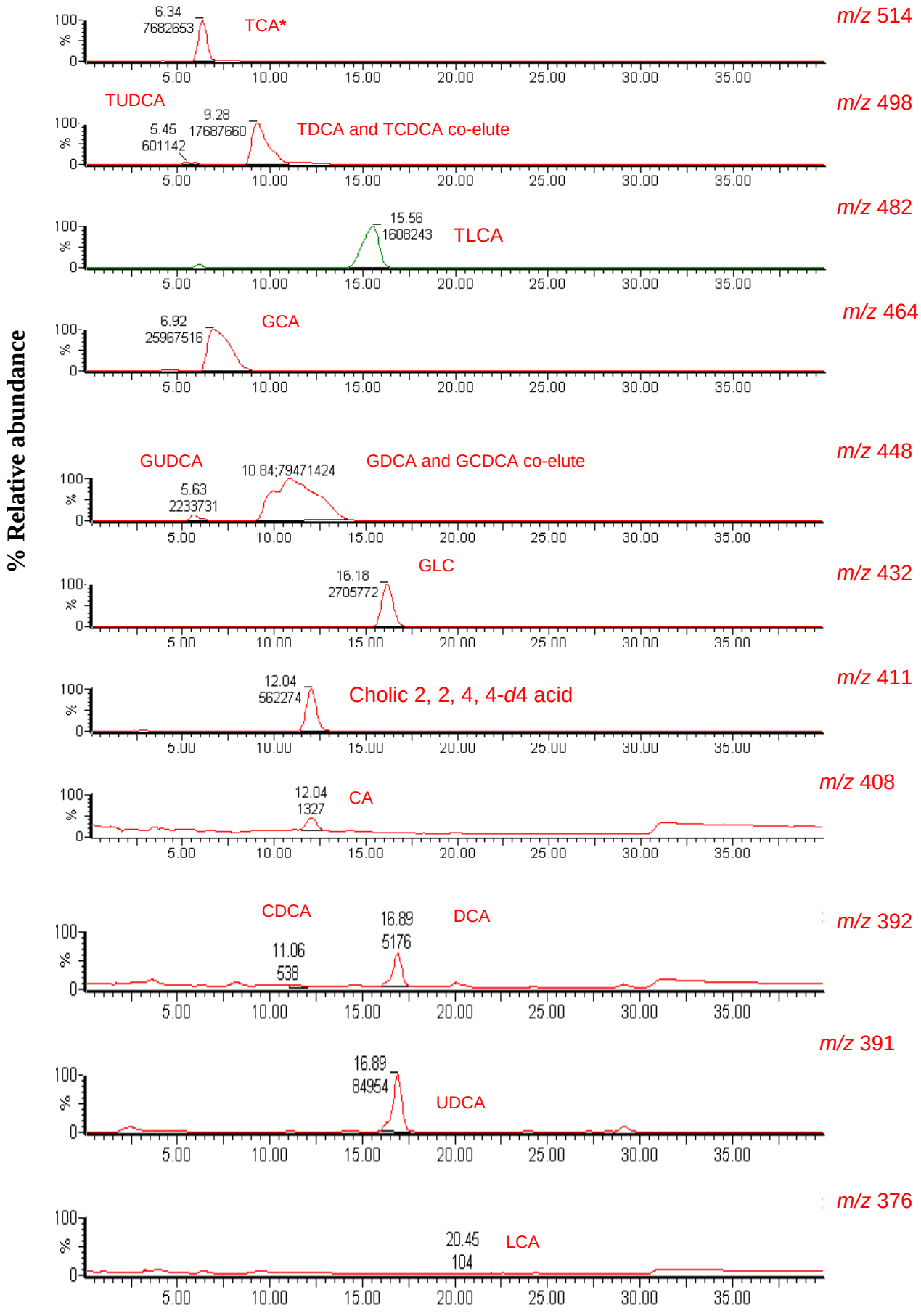


Figure 5.6. Representative mass chromatogram of bile salts from bile sample.

Taurine and glycine conjugates (m/z 498 and m/z 448) co-elute in the bile sample only. The bile acids elute in the following order:

1.Taurocholic acid (TCA), 2. Tauroursodeoxycholic acid (TUDCA), 3. Taurochenodeoxycholic acid (TCDCA), 4. Taurodeoxycholic acid (TDCA), 5. Taurolithocholic acid (TLCA), 6. Glycocholic acid (GCA), 7. Glycoursodeoxycholic acid (GUDCA), 8. Glycochenodeoxycholic acid (GCDCA), 9. Glycodeoxycholic acid (GDCA), 10. Glycolithocholic acid (GLCA), 11. Cholic 2, 2, 4, 4-*d*4 acid (internal standard) 12. Cholic acid (CA), 13. Chenodeoxycholic acid (CDCA), 14. Deoxycholic acid (DCA), 15. Ursodeoxycholic acid (UDCA), 16. Lithocholic acid (LCA).

* TCA eluted 2 minutes early than the expected standard retention time.

Table 5.5. Bile acid levels in bile samples from patients suffering from cholestatic hepato-biliary diseases (mean of three separate analysis). Values shown represent concentrations (µg/mL) analysed from bile collected from six patients with cholestatic hepato-biliary diseases**.** Each value represents the average of three individual determinations run along with the standards in parallel. TCDCA; TDCA and GCDCA; GDCA co-eluted in all the samples. ND, not detected.

Taurine Conjugates	Clinical Condition	TCA	Co-elute		TUDCA	TLCA
			TCDCA	TDCA		
µg/mL		m/z 514	m/z498	m/z 498	m/z 498	m/z 482
Bile Sample 1	Gall Pne	381	3539	0	36	16
Bile Sample 2	Biliary Colic	2182	3753	0	83	60
Bile Sample 3	Sever AP pseudo cyst	463	1269	0	102	2
Bile Sample 4	Mild GS AP	2431	6251	0	423	11
Bile Sample 5	Mild GS AP	3323	6488	0	81	179
Bile Sample 6	Nero endocrine liver	1975	2931	0	126	178
Glycine Conjugates		GCA	Co-elute		GUDCA	GLCA
			GCDCA	GDCA		
µg/mL		m/z 464	m/z448	m/z 448	m/z 448	m/z 432
Bile Sample 1	Gall Pne	970	2202	0	37	43
Bile Sample 2	Biliary Colic	2841	6744	0	128	97
Bile Sample 3	Sever AP pseudo cyst	1862	326	0	488	12
Bile Sample 4	Mild GS AP	5673	18667	0	1919	12
Bile Sample 5	Mild GS AP	773	5970	0	406	404
Bile Sample 6	Nero endocrine liver	5835	8196	0	196	222
Primary Bile Acids		CA	CDCA	DCA	UDCA	LCA
µg/mL		m/z 408	m/z392	m/z 392	m/z 391	m/z 376
Bile Sample 1	Gall Pne	0.35	2.47	1.69	0.10	2.29
Bile Sample 2	Biliary Colic	1.56	ND	0.08	0.10	2.66
Bile Sample 3	Sever AP pseudo cyst	2.41	2.26	1.42	0.16	2.66
Bile Sample 4	Mild GS AP	1.66	4.46	2.56	0.12	4.33
Bile Sample 5	Mild GS AP	1.76	1.00	2.68	0.09	3.79
Bile Sample 6	Nero endocrine liver	0.23	0.01	0.23	0.02	0.36

5.4. Discussion

A resurgence of interest in bile acids over the years as a result of their use in various treatments and their involvement in the aetiology of a number of diseases has led to the development of specific analytical techniques.

The present method has detected and determined the profile of 15 bile acids in a single run. Mass spectrometric conditions have been specifically optimised for each analyte (each bile salt). As acidic function is always present in these compounds, they yielded ions in the negative mode (Warrack and DiDonato, 1993, Evans et al., 1993). Further, the use of ESI has enabled a robust method for bile acid screening in human bile. The elution gradient was optimised for the simultaneous separation of unconjugated and conjugated (taurine and glycine conjugated) bile acids. Furthermore, ammonium acetate was used in the LC separation and was preferred over ammonium formate as it yielded more signal strength and is more volatile, therefore giving better sensitivity (Alnouti et al., 2008, Setchell and Vestal, 1989, Iida and Murata, 1990). Volatile electrolytes like ammonium acetate readily promote ion-molecular reaction in almost neutral pH (Iida and Murata, 1990). Further, using a low concentration of ammonium acetate (5 mM) was useful to minimize the formation of ammonium adducts and the molecular ions remained intact. The signal strength of molecular ions was highest in negative mode. Moreover, 0.012% formic acid, added as a buffer additive (to control ion formation) was suitable for chromatographic behaviour and enhanced for MS sensitivity.

Broad differences in the physiochemical properties of bile acids such as polarity and lipophilicity, required particular care in choosing stationary and mobile phases. A C18 ODS column allows good separation of 15 bile acids using a methanol gradient. To account for any loss of the analyte(s), the quantification of bile acids was based on using deuterium labelled internal standard. The bile samples were semi-purified using

SPE cartridges. The SPE allowed a simple efficient and cost effective of all analytes and internal standard. The deuterated internal standard behaved identically to the analytes (Burkard et al., 2005). All the standard curves were linear in both calibration ranges for all bile acids summarized in Table 5.2. The data obtained from this method in terms of precision and accuracy demonstrated the reproducibility of the assay (See Table 5.4). The mean recovery rates of the extraction procedure were between 86 and 105 %. Glycine conjugates showed better recoveries than the taurine conjugated bile acids. The unconjugated bile acid LCA, in particular showed poor recovery perhaps the lipophilicity could possibly interfere with extraction process. The LoD and LoQ are summarized in Table 5.4.

A crucial parameter in this method development was the chromatographic separation of glycine and taurine conjugates of UDCA, CDCA and DCA as they all possess same parent ion (m/z 498 for taurine conjugates and m/z 448 for glycine conjugates) (see Table 5.1). Hence, it was considered prudent to obtain sufficient peak resolution for chromatographic analyses.

It was also noted that, taurine conjugated bile acids eluted earlier than the glycine conjugates (See Figure 5.4). The glycine conjugates eluted earlier than the primary and secondary bile acids. It was noted from the chromatogram (Figure 5.6) that TCA peak (m/z 514) from the bile sample, eluted two minutes before the expected retention time (as in TCA standard, See Figure 5.4). This probably can be attributed to the detection of a possible isoform of TCA in the bile sample. This need to be further elucidated.

Furthermore, two of the taurine and glycine conjugates co-eluted in all the patients' samples (Figure 5.6) thus making the levels of these bile acids indistinguishable in

patients. The rest of the conjugated and primary bile acids were sufficiently resolved. At this late juncture, it was not possible to fabricate new and better resolving gradient programmes to separate the co-eluting conjugates.

The bile acid levels measured from the patients were quantitated using the standard calibration lines. As evident from Table 5.5, the concentrations of the major endogenous primary bile acids (CA, CDCA, DCA UDCA and LCA) in these patients showed considerable variations. The concentrations of the glycine conjugates were higher compared with taurine conjugates. In addition, very low levels of UDCA were detected in the patients in the present study (Table 5.5). Perhaps low levels of UDCA reflected greater extent of conjugation with glycine rather than with taurine. CA, CDCA and DCA varied in the concentration while LCA remained elevated in all the patients, compared to other primary and secondary bile acids. As it can be seen from the Table 5.5, primary bile acids seemed to be conjugated in most of the patients resulting in elevated levels of taurine and glycine.

An increased proportion in LCA and a reduction in UDCA and CDCA in all the patients was observed. LCA is formed either by 7-β-dehydroxylation of UDCA or by 7-α-dehydroxylation of CDCA. UDCA is poorly absorbed in the GI tract but undergoes significant biotransformation to hydrophobic bile acids CDCA and LCA (Cecilia et al., 1995). This might possibly explain the high concentration of LCA among these patients. It is suggested that LCA is the most toxic bile acid while CDCA is the least toxic bile acid (Staudinger et al., 2001). The levels CA in two of the patients was decreased compared to normal, which perhaps suggest failure of 12α-hydroxylation in the bile synthetic pathway. This decrease in the CA was also noted in cirrhotic patients (McCormick et al., 1973).

Taurine and glycine are the main conjugates of the primary bile acids and their ratio vary with age and in various liver disorders. An accurate measurement of their ratio perhaps is of biological and clinical significance. It is reported that UDCA in its conjugated form could prevent liver cell injury and cholestatic in animal model (Kanai and Kitani, 1983).

In conclusion, this part of the project was a pilot study with a small cohort of patients with varying cholestatic hepato-biliary disease. Nevertheless, the study concluded lower levels of primary bile acids. However, the results suggested that the summation of all the di and tri-hydroxy C_{27} bile acids can possibly be overall indicators of cholestatic hepato-biliary diseases.

CHAPTER 6

General discussion and future work

CHAPTER 6 General discussion and future work

Oxidative stress is a perpetual theme throughout the body of this thesis. Overwhelming free radical production in the presence of limited free radical neutralization leads to accumulated oxidative stress resulting in various injuries in the cell/ tissue/ organ. This has been a constant conjunction in the pathophysiology of AP. One of the aspects the present study was concentrated in evaluating the pathophysiology augmented by oxidative stress in AP in the absence of depleting thiol status.

6.1. Transsulfuration and acute pancreatitis

Ample supplies of data, whether it is pharmacokinetic or biochemical have suggested that oxidative stress was a plausible damaging mechanism in both acute-or-chronic pancreatitis (Braganza, 1990). The role of ROS in the pathogenesis of pancreatic inflammation has been studied almost exclusively in animal models and has received relatively little attention in human patients. This is perhaps due to a high variability in aetiology, severity and clinical course of AP in human patients.

Depletion of thiols in the pancreas was seen as an early feature in experimental AP (Neuschwander-Tetri et al., 1992, Luthen and Niederau, 1995). In an attempt to investigate thiol status, analysis of sulfur amino acids was carried out in the plasma of patients suffering from AP. Clearly, the results suggested that impaired GSH biosynthesis occurs within 24 hrs of onset of abdominal pain associated with AP. Although loss of GSH is perpetual either in acute or chronic pancreatitis, the rationale for this loss is still ambiguous. The following evidences can be germane and possibly explain the reasons for loss of GSH during AP.

Cys is extremely unstable and rapidly auto-oxidises to cystine, generating potentially toxic oxygen radicals that can further augment oxidative stress. Furthermore, Cys is

avidly taken up by pancreas, it is cited that pancreas is relatively low in transsulfuration enzymes (Mudd et al., 1965). Therefore, the acinar cells must depend on GSH for a continuous supply of Cys for synthesis of GSH. From the present data, despite ample Cys supply, GSH synthesis is deficient. This possibly explains GSH being severely compromised with a large load of oxygen radicals generated by Cys auto-oxidation.

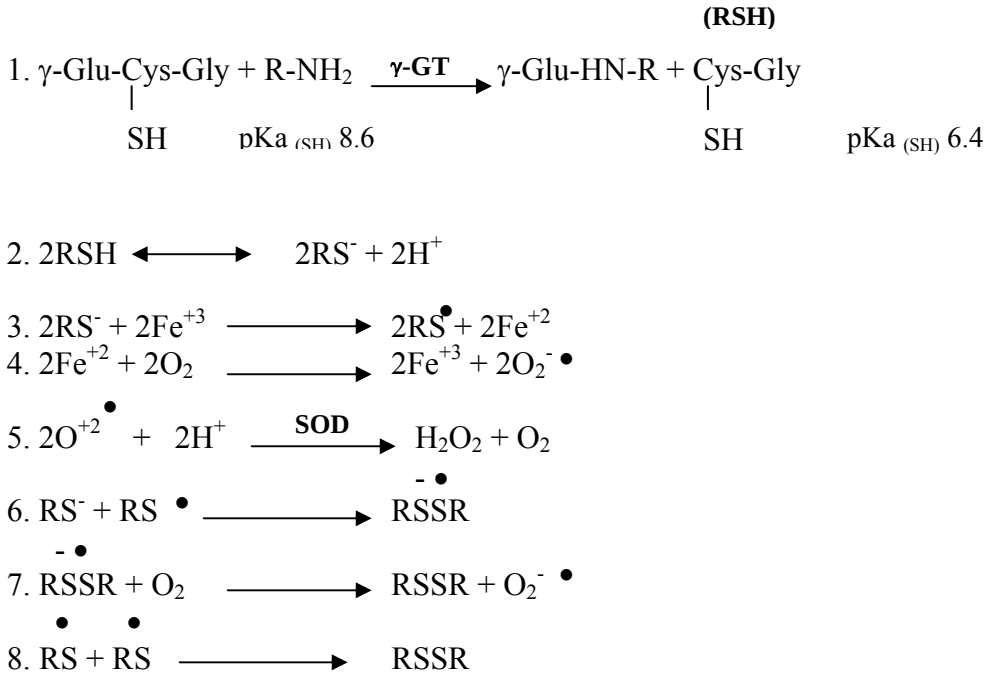
Further, Hcy has also been shown to cause oxidative stress on cells directly by the production of H_2O_2 (Jhee and Kruger, 2005). Severe oxidative stress may overcome the ability of the cell to reduce GSSG, thus deplete cellular GSH (Benedetti et al., 1980, Vander Jagt et al., 1997). Decreased levels of GSH in plasma are reported, but paradoxically an increased GSH levels may also be found as a result of outshoot after enhanced synthesis due to oxidative stress or conjugation of toxic compounds, as has been reported in different disorders (Gut et al., 1995, Plummer et al., 1981, Deneke and Fanburg, 1989).

GSH conjugation with xenobiotic electrophiles is rapidly excreted from the cells, (Orrenius, 1984) although vitally protective, results in permanent loss of GSH. Under heavily oxidative stress conditions, a cell may not be able to replace this disrupted thiol status. This mechanism, depending on the aetiology, can possibly reflect the state of pancreatic acinar cells during a pancreatic attack. Considering all these above-described points, it is perhaps not surprising that abrupt depletion of GSH, itself may seem to initiate changes in AP.

6.1.2. Role of γ-Glutamyl transpeptidase in disease progression

The data originated from the γ-GT expression poses a possible hypothesis for aggravated and/ or progression during pancreatitis. Low GSH levels and γ-GT illustrated a significant correlation at 72 hrs (See section 4.3.5 for results, Table 4.2). As

cited earlier, γ-GT is an abundant ectoenzyme, whose major function is to maintain the catabolism of GSH (Hanigan and Pitot, 1985). An important apparent activity of γ-GT is that γ-GT can act as GSH oxidase (Tate, 1979), giving rise to free radicals (Stark, 1991). Considering the following reactions:



1. Formation of Cys-Gly (CG-SH) from GSH by GGT
2. Facile dissociation of CG-(SH) into thiolate anion due to low pKa (SH)
3. Direct reduction of iron and formation of a thiyl radical
4. Recycling of iron and formation of superoxide
5. Dismutation of superoxide to hydrogen peroxide
6. Formation of a thiolate anion radical
7. Formation of superoxide from thiolate anion radical and molecular oxygen
8. Possible termination: formation of Cys-Gly disulfide.

Scheme 1. Generation of oxygen and thiyl radicals from the oxidation of GSH. (Adopted from (Stark et al., 1993).

During this auto-oxidation of thiols, thiyl radicals can also propagate radical chain reactions by reacting directly with unsaturated bond in lipids and/ or abstract hydrogen from alcohols yielding carbon-centred radicals. These carbon-centred radicals can further react with oxygen to yield oxygen-centred radicals (Ito et al., 1988, Stock et al., 1986, Schoneich et al., 1989). This premise could possibly explain disease progression at 72 hrs following a mild AP attack. To add weight to this argument, non-enzymatic auto-oxidation of Cys-Gly in the presence of iron yields superoxide and H_2O_2 and the auto-oxidation proceeds via, thiolate anion intermediates (Misra, 1974).

As previously explained, oxygen radicals mediate an important step in the initiation and progression of AP, the potential source for enhanced oxygen radicals' production however, remains unexplained. Continuing the hypothesis, auto-oxidation and dissociation of GSH at high pH yields $O2^-$ radical (Richmond and Halliwell, 1982). As explained earlier, γ-GT cleaves the γ-glutamyl bond in GSH releasing Cys-Gly, another abundant thiol, that has received little attention so far.

The conversion of GSH to Cys-Gly results in readily dissociating species, facile auto-oxidation and radical formation at physiological pH (Stark, 1991). An inefficient metabolism of Cys-Gly and/ or the radicals, the action of γ-GT on GSH, may lead to oxidative damage (Stark et al., 1988, Stark, 1991, Stark et al., 1993), which may also explain the aggravated pancreatic attack at 72 hrs. To add to this present conception, free radicals own ample energy and are unstable. Hence, it is implicit that some radicals' have extremely short half-life period, which prevents them from leaving the site of its generation. This could explain depletion of GSH at 72 hrs leading to disease progression.

Oxidative damage does happen in a pancreatic attack but its mode of action is still unclear. Efforts to demonstrate the participation of free radical association during a

pancreatic attack has not been fully successful in human patients despite considerable body of information being available from the animal models. Nevertheless, experimental effectiveness on animal models does not mirror the clinical reality because patients are admitted to the hospital only after the onset of the disease/ attack. This reflects that the disease is fully established and the mechanisms taking place during this time gap has not been identified so far in human patients. It must be stressed, that to-date no direct evidence for these proposed mechanisms is available and it is impossible to prove that GSH depletion, coupled with oxidant stress, underlies the development of AP in humans (Wallig, 1998). From a clinical point of view, the above-explained arguments may seem controversial. However it is, important to realise that these preponderances are an "***association",*** and may/ may not reflect on the theoretical advantage of the present study. Nevertheless, oxidative damage exerted by the GSH and γ-GT system can be considered as a contributing factor to AP attacks.

Of interest AP is an inflammatory disease associated with auto-digestion of the pancreas, intra-hepatic activation and release of digestive enzymes. Current perspectives favour evidence that pancreatic ischemia also plays an important role in AP (Yuzbasioglu et al., 2008). The current consensus is that a disturbance of pancreatic microcirculation leads to the formation of thrombus in the capillaries, following activation of leukocytes, release of proteolytic enzymes and formation of ROS and/ or proinflammatory cytokines. Evidences also suggested that there is a relationship between pancreatitis and homocysteinuria, possibly being secondary to occlusive vascular disease of the pancreas (Makins et al., 2000). Thus, elevated Hcy levels may be a cause of AP with endothelial dysfunction and impaired micro vascular circulation (Yuzbasioglu et al., 2008).

Folate deficiencies depress remethylation of Hcy to Met. Met is an essential amino acid and is avidly taken up by pancreas (Mann et al., 1989) and has a key role in maintaining the integrity and proper functioning of the pancreas (Girish et al., 2010). It was reported that in alcohol-induced relapse of pancreatitis, leukocyte Met levels exceeded those in controls while GSH remained largely oxidized (Martensson and Bolin, 1986). Their study suggested that oxidative stress within the gland interferes with Met metabolism at a point distal to SAM, causing amino acid flux into plasma. Collectively, their results indicated the extension of free radical pathology from the pancreas to the peripheral blood during an attack. Reduced plasma Met levels have been observed in patients with chronic pancreatitis (Girish et al., 2010)

6.2. Redox regulation and glutathione

The concept of redox regulation is suffering much popularity, mainly because of the redox couples functioning to maintain the redox homeostasis and to add to this popularity, redox regulation is an "emerging concept". There will always be a struggle to quantify oxidative stress because the redox couples in the body are not in constant state of equilibrium (Smith, 2005). Furthermore, there are no data (at present), available to show that individuals with increased oxidized (GSSG) redox ratio are at a risk of developing any kind of disease and / or any clinical interventions could possibly normalize this increased oxidized ratio could provide a beneficial effect. However, common quantitative standards would advance the clinical utility of biomarkers of oxidative stress. Based upon available data, the redox state of GSH/GSSG ratio would provide a reasonable place to begin.

A comprehensive compilation of references is available to quantify GSH. Information on GSSG quantification are however scanty. The second aspect of the present study was

to transfer a proficient methodology to determine tGSH and GSSG in whole blood and subtract the two to get the GSH present. The fact that one can assess oxidative stress *in vivo* in plasma by measuring GSH/GSSG in animal (rodent) models was established several years ago (Adams et al., 1983). However, application of the same approach to human studies has been limited due to enhanced oxidation of GSH that allows erroneous errors during its measurements.

The novelty of the present method comes from the use of a mercaptan scavenger M2VP to scavenge GSH and quantify GSSG in whole blood, which no earlier studies have done using M2VP in a HPLC setting. Unfortunately, at this late juncture, this developed method could not be applied on the blood samples from sepsis patients, which may have provided grounds for GSH loss in the pancreatic study. Nevertheless, the method developed as described in detail in chapter 3 allowed suitable venture for GSSG determination.

6.3. Estimation of bile salts in human bile using liquid chromatography-mass spectrometry

There has been steady progress in the bile acid science (cholanology), aimed at elucidating their physiological functions and developing pharmacological applications.

Mass spectrometric methods have become indispensable for the analysis of bile acids to quantify either free or conjugated bile acids. Further, the emergence of soft ionization technique such as ESI has considerably simplified bile analysis. ESI was well suited for polar, non-volatile and thermo liable compounds.

The present study has identified the bile salt levels in a small cohort of patients suffering from various cholestatic-hepatobiliary diseases and in an attempt has tried to explain different responses/ behaviour of bile salts using LC-MS. This method is simple

and provides high sensitivity and specificity to perform quantitative-profiling of individual bile salts. Negative ion mass spectra were characterized by intense $[M-H]^-$ ions and product ions due to consecutive loss of Na^+/ water corresponding to number of hydroxyl groups present. Quantifying bile acids in these patients with the use isotypically labelled internal standard is an improvement and novel in this method. Deuterated bile acid analogs behave identically to their analytes and differ in their mass-to-ratio (m/z) (Burkard et al., 2005).

A number of hepato-biliary diseases have been identified which are caused by defects in bile acid biosynthesis. Current perspectives upon a comprehensive evaluations of six patients in this study favour evidence for the existence of high levels of hydrophobic bile salts in conjugation with deoxycholic acid (tauro and glyco conjugates) in all the patients. It is suggested that retention of hydrophobic bile like taurine and glycine conjugates of the secondary bile salt deoxycholate is the main cause of hepatotoxicity (Grattagliano et al., 2005). This is often associated with impaired mitochondrial metabolism, heightened oxygen radical production and oxidative damage (Krahenbuhl et al., 1994, Sokol et al., 1994).

The current study is far too small to provide a meaningful interpretation for hepatobiliary diseases. However, the small cohorts of six patients have demonstrated valuable observation in terms of their levels in diseased state. The use of LC-MS has resulted in a resurgence of interest in the determination of bile acids, accurate identification and quantification of bile acids in hepatobiliary diseases.

Finally, a comprehensive exploration of bile acid in patients with various cholestatic-hepatobiliary diseases in large numbers will probably be a clinical intervention with new therapies in future. It is claimed that UDCA is extensively used in therapy for

biliary diseases (Combes et al., 1999). However, relatively little information is available on the effect of UDCA in compensating for low primary bile acids levels.

6.4. Future work

The present work has focussed on oxidant stress leading to depleted thiol status, focussing on GSH status, in an auxiliary inflammatory condition called AP. Although various theories have been pin-pointed for the depleted GSH status, it still remains blurred, whether the current data, pose that oxidative stress and GSH depletion play a significant role in the development of severe pancreatitis and whether the maintenance of intracellular GSH concentration has beneficial therapeutic implication.

The corollary that ancillary antioxidant systems (vitamin C, vitamin E or GPx), exert a GSH-sparing facility has been exploited by way of supplementing antioxidants (Uden et al., 1990). However, the question whether antioxidant therapy is beneficial or functionally redundant remains unanswered to date. Although data produced by the use of GSH analogues in sepsis remains controversial due to conflicting results (Spies et al., 1994, Peake et al., 1996), their use in AP cannot be advocated without a better understanding of GSH metabolism in this disease. If GSH depletion is the principal mediator of disease progression, a better understanding of its kinetics in clinical pancreatitis needs to be ascertained to reduce the chances of treatment failure.

In addition, future work needs to address cellular loss of GSH and the biochemical processes that separate mild from severe attacks of pancreatitis. This may fall into three broad categories: reduced synthesis (either due to inadequate amino acid availability or decreased activity of key regulatory enzymes), reduced recycling of GSSG (disulfide

pool), or increased cellular GSH loss (through oxidative stress, reduced recycling, or xenobiotic conjugation).

It may be inferred that a fall in GSH reflects a build up of GSSG (GSSG may accumulate in the face of oxidative stress), and a number of experimental studies supports this notion. Hence, measuring GSSG along with sulfur amino acids would provide useful information about the GSH/GSSG ratio in patients suffering from AP. Plasma Met, SAM, folate, vitamin B12 levels determination upon admitting AP patients would provide useful information about transsulfuration intermediates. In addition, conjugation of GSH through mercapturic acid pathway would possible estimate loss of GSH. In addition, urinary excretion of oxoproline can suggest the loss of GSH. Hence, an estimation of this loss would be valuable indicator of redox balance.

Pancreas is low in transsulfuration enzymes (Wallig, 1998) in normal physiological conditions, an inflammation in the organ, can explain if transsulfuration enzymes are expressed during AP. Cogent with these statements, the catabolism of intracellular GSH by other mechanism cannot be ruled out. Although, GSH is considered resistant to normal pancreatic intracellular enzymes, an inappropriate release (characteristic of pancreatic attacks) of digestive enzymes that could yet play an anonymous role in GSH loss. This can potentially provide subject of further studies.

The γ-GT expression during inflammatory conditions are tantalising but unproven during AP progression. Only a well-designed clinical study will provide a meaningful interpretation of γ-GT expression during AP. As stated elsewhere, GCL is a heterodimer and takes part in the synthesis of GSH. The activity of two subunits can be compromised during an oxidative stress. The stress response model as described by

(Yang et al., 2002), can possible be correlated to humans and during a stress response like in AP. The cellular pathway approaches towards oxidative stress are significantly complex than originally envisioned. Hence, any pharmacological interventions will only or probably be promising when they target the regulatory pathways linked to these diseases. This perhaps, can potentially ameliorate pancreatic injury caused by free radicals.

The present study also reported bile acid levels in cohort of patients suffering from various degrees of cholestatic hepato-biliary disorders. The data reported increased levels of LCA compared to other primary and secondary bile acids. Increased levels of LCA are toxic in normal physiology. Further studies can explain the toxicity of LCA in cholestatic hepato-biliary diseases. In addition, further studies can be focussed on increasing the resolution of taurine conjugates and perhaps identify a probable isoform of TCA eluting 2 min earlier than the normal standard retention time (see Figure 5.6). In the present study, poor peak shape resulted in varied limits of LoD and chromatograms. Future studies can perhaps enhance these limitations.

References

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APPENDICES

Table 3.1. Calibration line for GSH

Conc. of GSH mM	GSH (µL)	MPA (µL)	Water (µL)	Volume Taken(µL)	OPA (µL)	phosphate (µL)
10	100	400	500	100	100	800
8	100	400	500	100	100	800
6	100	400	500	100	100	800
4	100	400	500	100	100	800
2	100	400	500	100	100	800
1	100	400	500	100	100	800

Table 3.2. Choosing appropriate concentration for DTT reduction

Conc. of DTT (mM)	GSSG (µL) 0.5 mM	GEE (µL)	Water (µL)	DTT (µL)	TRIS (µL)	MPA (µL)	Volume taken (µL)	OPA (µL)	Phosphate (µL)
50	100	50	300	100	50	400	100	100	800
35	100	50	300	100	50	400	100	100	800
30	100	50	300	100	50	400	100	100	800
25	100	50	300	100	50	400	100	100	800
20	100	50	300	100	50	400	100	100	800
10	100	50	300	100	50	400	100	100	800
50	0	50	550	0	0	400	100	100	800
35	0	50	550	0	0	400	100	100	800
30	0	50	550	0	0	400	100	100	800
25	0	50	550	0	0	400	100	100	800
20	0	50	550	0	0	400	100	100	800
10	0	50	550	0	0	400	100	100	800
Blank	100	50	300	100	50	400	100	100	800
Blank	0	0	450	100	50	400	100	100	800

Table 3.3. Calibration line for GSSG with and without DTT

Conc. of GSSG (μM)	GSSG (μL)	GEE (μL)	Water (μL)	DTT (μL)	TRIS (μL)	MPA (μL)	Volume Taken (μL)	OPA (μL)	Phosphate (μL)
1000	100	50	300	100	50	400	100	100	800
500	100	50	300	100	50	400	100	100	800
300	100	50	300	100	50	400	100	100	800
100	100	50	300	100	50	400	100	100	800
50	100	50	300	100	50	400	100	100	800
10	100	50	300	100	50	400	100	100	800
5	100	50	300	100	50	400	100	100	800
1000	100	50	450	0		400	100	100	800
500	100	50	450	0		400	100	100	800
300	100	50	450	0		400	100	100	800
100	100	50	450	0		400	100	100	800
50	100	50	450	0		400	100	100	800
10	100	50	450	0		400	100	100	800
50	100	50	450	0		400	100	100	800
Blank	0	50	400	100	50	400	100	100	800
Blank	0	0	600	0	0	400	100	100	800

Table 3.4. Calibration line for tGSH

Initial conc. standard mixture GSH+GSSG	Final Conc. (mM)	Mix (μL)	GEE (μL)	Water (μL)	DTT (μL)	TRIS (μL)	MPA (μL)	Volume Taken (μL)	OPA (μL)	Phosphate (μL)
1	0.5	100	50	300	100	50	400	100	100	800
2	1	100	50	300	100	50	400	100	100	800
4	2	100	50	300	100	50	400	100	100	800
5	2.5	100	50	300	100	50	400	100	100	800
1	0.5	0	50	400	100	50	400	100	100	800
2	1	0	50	400	100	50	400	100	100	800
4	2	0	50	400	100	50	400	100	100	80
5	2.5	0	50	400	100	50	400	100	100	800
Blank	0	0	50	400	100	50	400	100	100	800

Table 3.5. Choosing a suitable M2VP and NEM concentration for scavenging

Conc. of scavenger (mM)	M2VP (µL)	NEM (µL)	GSH (µL)	GEE (µL)	Water (µL)	DTT (µL)	TRIS (µL)	MPA (µL)	Volume Taken µL)	OPA (µL)	Phosphate (µL)
Blank	0	10	0	50	290	100	50	400	100	100	800
15	10	0	100	50	290	100	50	400	100	100	800
10	10	0	100	50	290	100	50	400	100	100	800
8	10	0	100	50	290	100	50	400	100	100	800
5	10	0	100	50	290	100	50	400	100	100	800
3	10	0	100	50	290	100	50	400	100	100	800
1	10	0	100	50	290	100	50	400	100	100	800
0	0	0	100	50	300	100	50	400	100	100	800
15	0	10	100	50	290	100	50	400	100	100	800
10	0	10	100	50	290	100	50	400	100	100	800
8	0	10	100	50	290	100	50	400	100	100	800
5	0	10	100	50	290	100	50	400	100	100	800
3	0	10	100	50	290	100	50	400	100	100	800
1	0	10	100	50	290	100	50	400	100	100	800
0	0	0	100	50	300	100	50	400	100	100	800
Blank	0	10	0	0	290	100	50	400	100	100	800

3.6. Comparison of scavenging capabilities of M2VP and NEM.

Initial Conc. (μM)	Actual Conc. (μM)	M2VP Or NEM	MIX (μL)	GEE (μL)	Water (μL)	DTT (μL)	TRIS (μL)	MPA (μL)	Volume taken (μL)	OPA (μL)	Phosphate (μL)
tGSH											
500	250	0	100	50	300	100	50	400	100	100	800
300	150	0	100	50	300	100	50	400	100	100	800
100	50	0	100	50	300	100	50	400	100	100	800
50	25	0	100	50	300	100	50	400	100	100	800
10	5	0	100	50	300	100	50	400	100	100	800
0	0	0	0	0	450	100	50	400	100	100	800
GSSG		**M2VP**									
500	250	10	100	50	300	100	50	400	100	100	800
300	150	10	100	50	300	100	50	400	100	100	800
100	50	10	100	50	300	100	50	400	100	100	800
50	25	10	100	50	300	100	50	400	100	100	800
10	5	10	100	50	300	100	50	400	100	100	800
0	0	0	100	50	300	100	50	400	100	100	800
Blank	0	0	0	0	450	100	50	400	100	100	800
		NEM									
500	250	10	100	50	300	100	50	400		100	800
300	150	10	100	50	300	100	50	400	100	100	800
100	50	10	100	50	300	100	50	400	100	100	800
50	25	10	100	50	300	100	50	400	100	100	800
10	5	10	100	50	300	100	50	400	100	100	800
0	0	0	100	50	300	100	50	400	100	100	800
Blank	0	0	0	0	450	100	50	400	100	100	800
GSSG											
250	250	0	100	50	300	100	50	400	100	100	800

Table 3.7. Comparison of scavenging capabilities of M2VP and NEM with increasing concentration of GSH

Conc. Of GSH (μM)	M2VP 15 mM (μL)	NEM 15 mM (μL	GSH (μL)	GSSG (μL)	GEE (μL)	Water (μL)r	DTT (μL)	TRIS (μL)	MPA (μL)	Volume Taken (μL)	OPA (μL)	Phosphate (μL
2000	10	0	100	100	50	190	100	50	400	100	100	800
1000	10	0	100	100	50	190	100	50	400	100	100	800
450	10	0	100	100	50	190	100	50	400	100	100	800
100	10	0	100	100	50	190	100	50	400	100	100	800
50	10	0	100	100	50	190	100	50	400	100	100	800
30	10	0	100	100	50	190	100	50	400	100	100	800
25	10	0	100	100	50	190	100	50	400	100	100	800
20	10	0	100	100	50	190	100	50	400	100	100	800
10	10	0	100	100	50	190	100	50	400	100	100	800
5	10	0	100	100	50	190	100	50	400	100	100	800
2000	0	10	100	100	50	190	100	50	400	100	100	800
1000	0	10	100	100	50	190	100	50	400	100	100	800
450	0	10	100	100	50	190	100	50	400	100	100	800
100	0	10	100	100	50	190	100	50	400	100	100	800
50	0	10	100	100	50	190	100	50	400	100	100	800
30	0	10	100	100	50	190	100	50	400	100	100	800
25	0	10	100	100	50	190	100	50	400	100	100	800
20	0	10	100	100	50	190	100	50	400	100	100	800
10	0	10	100	100	50	190	100	50	400	100	100	800
5	0	10	100	100	50	190	100	50	400	100	100	800
Blank	0	0	0	100	50	300	100	50	400	100	100	800