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Pharmacokinetic Evaluation of a Novel Compound, Sn79, a Putative Sigma-2 Receptor Antagonist, By Intravenous and Oral Administration in Rats

Harsha Vinnakota

Pharmacokinetic Evaluation of a Novel Compound, SN79, a Putative Sigma-2 Receptor Antagonist, by Intravenous and Oral Administration in Rats

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Harsha Vinnakota

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ABSTRACT

Considering the alarming rates at which substance drug abuse, especially cocaine, is increasing in today's society, there is a lot of impetus on the development of medications that can effectively help alleviate its toxicity and addiction. The affinity of cocaine to sigma receptors (sigma-1 and sigma-2) rendered the hypothesis that blocking sigma receptors could be a possible mechanism to attenuate cocaine-induced toxicity and addiction. In this view, **SN79**, a synthetic compound with selectivity to both sigma-1 and sigma-2 receptors garnered our attraction for its use as an antagonist. This dissertation encompasses detailed investigation of SN79 from drug discovery and development perspective. Development and validation of a bio-analytical method using ultra performance liquid chromatography-mass spectrophotometry to selectively separate and identify SN79 in biological matrix was a crucial part of this project. Determination of various physicochemical parameters including aqueous solubility, chemical stability, Log P and pK_a etc are presented. A number of *in vitro* tests necessary for predicting the compound's profile in the body were also performed. Single dose pharmacokinetic studies were conducted in fasted and fed state rats that help determine the disposition of SN79 *in vivo*.

DEDICATION

This doctoral dissertation is dedicated to my loving parents, Narasimha Rao Vinnakota and Dr. Revathy Vinnakota who taught me the value of education, my husband, Dr. Venkat Tumuluri and to the fulfillment of our dreams, and to the memory of my grandparents.

SYMBOLS AND ABBREVIATIONS

5-HT – 5-hydroxytryptamine

AIDS – Acquired immune deficiency syndrome

ADME - Absorption, distribution, metabolism and excretion

AUC – Area under the curve

BBB – Blood brain barrier

CNS - Central nervous system

ºC – degree Celsius

DA – Dopamine

DAT - Dopamine reuptake transporter

DAWN - Drug Abuse Warning Network

DXM – Dextromethorphan

ER – endoplasmic reticulum

FDA – Food and Drug Administration

F_u – Fraction unbound

GABA - γ-aminobutyric acid

GC – Gas Chromatography

GI – Gastro intestinal

GIT – Gastro intestinal tract

HCl – Hydrochloride

HIV – Human immune deficiency virus

HPLC – High pressure liquid chromatography

I.P. – Intraperitoneal

IV – Intravenous

IACUC - Institutional Animal Care and Use Committee

ICSS - Intracranial Self Stimulation

IS – internal standard

LC – Liquid Chromatography

LLOQ - Lower limit of quantification

Log P - Octanol/buffer partition coefficient

MAM - mitochondrion-associated ER membrane

ME – Matrix Effect

MMC - Migrating motility complex

NaDPH – Nicotinamide adenine dinucleotide phosphate

NCE – New chemical entity

NET - Norepinephrine transporters

NIDA - National Institute on Drug Abuse

NMDA - N-methyl-D-aspartic acid

NMR – Nuclear magnetic resonance

NSDUH - National Survey on Drug Use and Health

ON – Oligonucleotides

p.o dosing – Oral dosing

PCP – Phencyclidine

QC – Quality Control

RD – Relative Difference

RDS – Rate determining step

RE – Recovery

RSD – Relative Standard Deviation

RT –Retention time

SAMHSA - Substance Abuse and Mental health Services Administration

SD - Standard Deviation

SERT - Serotonin transporters

SGF – Simulated Gastric Fluid

SIF – Simulated Intestinal Fluid

SIR – Single ion reaction

T1/2 – half life

UPLC/MS – Ultra performance liquid chromatography-mass spectrometry

USP – United States Pharmacopeia

VTA - Ventral Tegmental Area

σ-1 – Sigma-1

σ-2 – Sigma-2

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Harsha Vinnakota

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I: INTRODUCTION

1.1 Substance Drug Abuse and Addiction

Substance abuse and addiction is a major concern in today's society. It is vital to address drug addiction considering the alarming rates at which people, especially teenagers, are drawing themselves towards drug abuse. There are several categories of illicit drugs: hallucinogens, inhalants, tranquilizers, sedatives, prescription type pain relievers like hydrocodone and stimulants such as cocaine. The 2009 annual survey from National Survey on Drug Use and Health (NSDUH) in collaboration with Substance Abuse and Mental health Services Administration (SAMHSA) reported that an estimated 21.8 million Americans aged 12 or above were illicit drug users. This represents 8.7 percent of the population aged 12 or older are on any of the drug categories listed above [3]. In addition to the obvious health risks associated with some drugs, abuse can lead to higher risk behavior or amplify the course of existing problems. Some of the more common medical problems associated with drug abuse include cardiovascular disease, stroke, cancer, HIV/AIDS, hepatitis, and lung disease.

Treatment statistics reflect the rising use of illicit drugs and prescription psychotherapeutics. According to a survey conducted by NSDUH in 2009, 23.5 million Americans (ages 12 and older) needed treatment for drug addiction of which only 2.6 million (1.0% of the population) received treatment [3]. Treatment for individual disorders was as follows: alcohol (2.9 million), marijuana (1.2 million), cocaine (787, 000), and heroin (507, 000 [3].

Cocaine abuse and addiction continues to plague the society. About one in six Americans has tried cocaine by the age 30, and 7% of the population have tried it by their senior year of high school [4]. Cocaine is associated with the maximum number of emergency visits caused by illicit drugs [5]. The use of cocaine is correlated with medical and psychiatric morbidity. According to the Drug Abuse Warning Network (DAWN) system in 2002, approximately 200,000 people specified cocaine use in case of emergency medical or psychiatric treatment in emergency departments [6]. Most of the cases are cocaine coupled with ethanol or an opiate. Cocaine abuse can be treated with behavioral therapies. According to Carroll et al. [7], some patients respond well to behavioral treatments, while others continue to abuse cocaine with reduced frequency and still others drop out of treatment [8].

Current break-through discoveries regarding brain anatomy and other genetic studies provide unprecedented openings in combating this public health issue. In view of cocaine's immense abuse potential, it is crucial to develop medications that can effectively help alleviate its toxicity and addiction.

1.2 Cocaine

1.2.1 History and Uses

Cocaine, originally extracted from the *Erythroxylon coca* bush, was primarily found native in Peru and Bolivia [9]. It is well known that cocaine was used for panacea and local anesthetic effect in the Incan Empire of Peru. The leaves were first consumed by Aymara Indians of the Andes Mountains [9]. They chewed the leaves to help elevate mood, decrease hunger and increase their stamina for work [9]. In 1884, Karl Koller in Europe demonstrated its therapeutic potential [10]. He used its tissue-numbing capability to anaesthetize cornea of the eye [10]. The

next twenty years saw a steep rise in the use of cocaine to treat pain (physical and mental) in patients with terminal diseases. In 1879; it began to be used for the treatment of morphine addiction [11]. By 1885, cocaine was being marketed by the U.S. manufacturer Parke Davis in the form of cigarettes, powder and as a mixture to be administered intravenously [12]. By the turn of the twentieth century, the addictive potential of cocaine became clear capturing public attention. On December 17, 1914, the Harrison Narcotics Tax Act was approved as a United States Federal law to illegalize the sale and distribution of cocaine in the United States [5]. Cocaine was not considered a controlled substance until the United States included it in the Controlled Substances Act in 1970 [13]. Today, cocaine possesses the status of a Schedule II drug under the Controlled Substances Act of 1970. This status categorizes it as a drug of high abuse potential but can be used legitimately in medical treatments, such as local anesthesia for some eye, ear, and throat surgeries [13].

Two chemical forms of cocaine are commonly available: the water-soluble hydrochloride salt and the water-insoluble cocaine base (or freebase). The principal routes of administration are: oral, intranasal, intravenous and inhalation. The drug can also be rubbed on mucous tissues. Any route of administration can lead to absorption of toxic amounts of cocaine, possible acute cardiovascular or cerebrovascular emergencies, and seizures—all of which can result in sudden death [4].

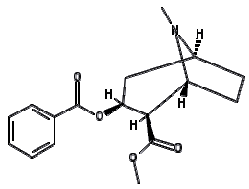

Figure 1.1 Cocaine

1.2.2 Mechanism of Action

Cocaine is believed to cause euphoric effects by acting on the reward circuitry in the brain. One possible explanation is that cocaine exerts major effects on parasympathetic,as well as sympathetic, function [9]. Through extensive research of the neuronal circuitry and neurochemistry, scientists have identified various regions in the brain stimulated by reinforcing stimuli such as food, sex and illicit drugs. One such neural system that is affected by cocaine is called Ventral Tegmental Area (VTA) that originates in a region of the midbrain. This region extends into one of the brain's key areas involved in reward called nucleus accumbens [14]. The mesolimbic and mesocortical dopamine systems projecting from the VTA to the nucleus accumbens and frontal cortex have been found to have psychomotor stimulant reward function [15] (Figure 1.2).

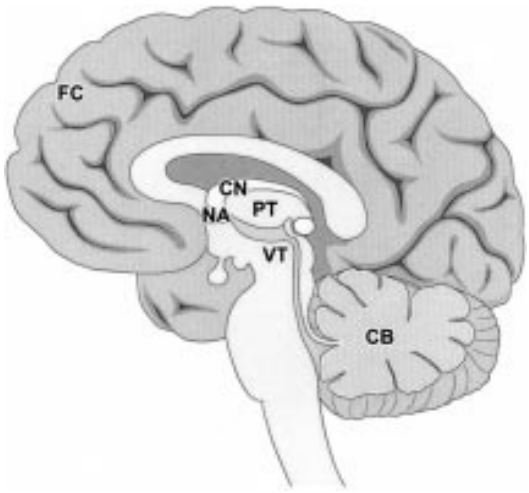

Figure 1.2 Certain regions of the brain are known to be involved in reward/reinforcement due to drugs. These include the nucleus accumbens (NA), ventral tegmental (VT) and frontal cortex (FC). Cerebellar (CB), putamen (PT) and caudate nucleus (CN) [1].

Rewards are responsible for enhancing brain levels of dopamine, thereby increasing neural activity in the nucleus accumbens. When dopamine is released by a neuron into the synapse, it binds to specialized proteins (dopamine receptors) on the neighboring neuron thereby sending a signal to that neuron. Subsequently, dopamine is removed by dopamine reuptake transporter (DAT) from the synapse to be recycled for further use [16-17]. The rationale for selecting dopaminergic drugs to treat cocaine abuse and dependence is based on the dopamine hypothesis. According to the "Dopamine Hypothesis", cocaine attaches to the dopamine reuptake transporters (DAT) thereby blocking the normal communication process [16]. This results in an accumulation of dopamine neurotransmitter in the synapse, which sends an amplified signal to the receiving neurons. This mechanism is what causes the "euphoria" associated with drugs of abuse [16, 18]. The increase in dopamine levels in the brain due to cocaine's interaction with DAT is a critical element in cocaine's reinforcing properties [1].

1.2.3 Scope of Pharmacotherapy

The National Institute on Drug Abuse (NIDA) funds a broad variety of research focused on drugs of abuse and the causes and treatment of addictive disorders. Researchers have tried their best to develop anti-cocaine agents by blocking its primary site of action, DAT (Figure 1.3). In developing cocaine antagonists, several compounds were screened but most of them exhibited cocaine-like effects [1, 19]. In an experiment conducted on DAT knock-out mice, it was observed that the subjects had conditioned place preference to cocaine suggesting that cocaine had affinity to other sites [20]. It was established that cocaine also interacts with other neurotransmitter systems such as norepinephrine transporters (NET), serotonin transporters (SERT) and sodium ion channels [21-25].

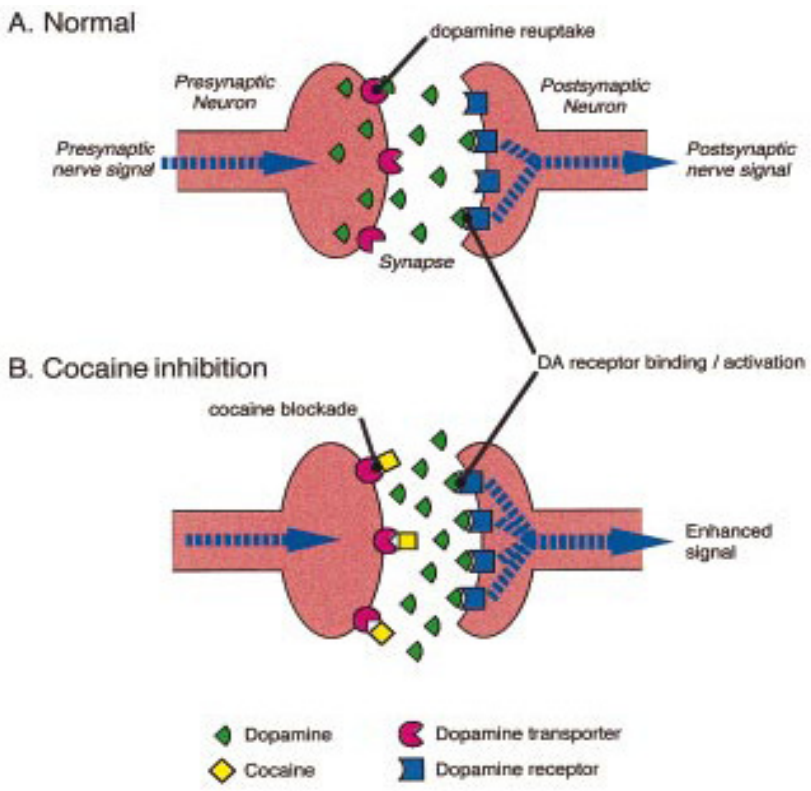

Figure 1.3 Schematic representation of the dopamine hypothesis of cocaine's reinforcing effects. (A) Stimulation of neuron releases DA from the presynaptic terminal into the synapse where it may bind to DA receptors on the postsynaptic terminal, resulting in transmission of signal; reuptake of DA via the transporter teriminates the action. (B) Cocaine blocks this reuptake of DA, flooding the synapse with excess DA thereby causing an enhanced signal. A direct competitive antagonist of cocaine would block cocaine binding but would not block dopamine [1].

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Efforts to develop therapeutic agents by blocking these previously identified sites did not yield satisfactory results characterized by low efficacy and undesirable side effects [1].

To develop a compound into a medication, the compound should possess certain properties. On careful perusal, the following characteristics have been listed as desirable in potential candidate compounds for cocaine abuse:

- Must be potent and selective with minimal side effects [1].
- Must remain active following the desired route of administration [1].
- Must possess suitably long duration of action so as to allow for reasonable dosing schedules [26].
- Must enter the brain slowly owing to the fact that compounds entering the brain rapidly are associated with high abuse liability [27-29].
- Possess low potential for self-administration in animal models [1].

Table 1.1 lists the various animal behavioral models that are used in the development of medications for cocaine abuse [1]. Reproduced with kind permission from American Chemical Society Rightslink (License number: 2640561044896)

Table 1.1. Animal Behavioral Models for Drug Screening
Drug Self Administration
Drug Discrimination
Schedule-Controlled Responding
Intracranial Self Stimulation (ICSS)
Conditioned Place Preference
Locomotor Activity

Similarly, table 1.2 lists several neurochemical assays that help identify potential compounds in the drug development process [1]. Reproduced with kind permission from American Chemical Society Rightslink (License number: 2640561044896)

Table 1.2. Some Neurochemical/Neurophysiologic/Neuroanatomic Assays [30-35]
Receptor binding
In situ hybridization
Receptor autoradiography
Immunocytochemistry
Microdialysis
Voltametry
Gene Cloning
Antisense technology

In 1996, Mello and Negus [36]recommended that an ideal medication for cocaine abuse should be capable of depressing the dose-response curve for cocaine self administration. This curve, typically in the form of an inverted "U" is presented in figure 1.4. A medication that depresses this "U" downward will be effective in decreasing cocaine-intake behavior over a broad range of doses.

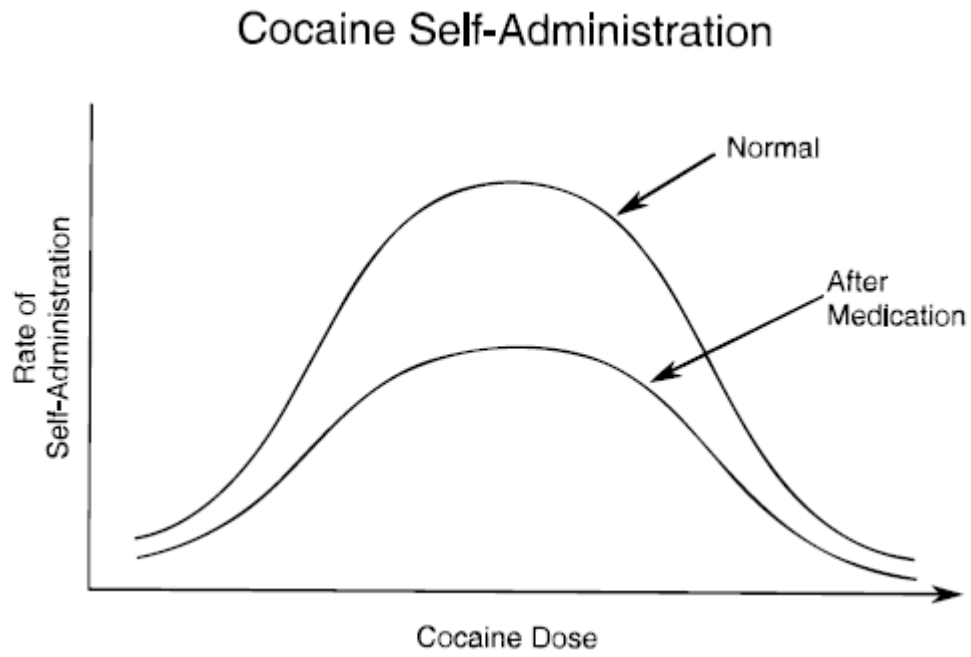


Figure 1.4 Effect of medication in an ideal dose-response curve for cocaine in self-administration studies. The typical dose-response is an inverted "U" [1, 36]. Reproduced with kind permission from American Chemical Society Rightslink (License number: 2640561044896)

In 1989, as per directives from the U.S. Congress, the NIDA Medications Development Program (MDP) initiated the drug development process to evaluate and develop medications for cocaine dependence [6]. To achieve this goal, the MDP worked independently as well as in conjunction with the pharmaceutical industry. The MDP used a top-down as well as bottom-up approach in the development of medications [6]. Some of these medications underwent clinical trials with some success noted but also associated with efficacy problems.

1.3 Dopamine System

The drug screening sequence most commonly used in developing medications for cocaine related toxicity and addiction is receptor binding. Based on the strong recommendations received for the "dopamine hypothesis" [16, 37-39], research was largely focused on targeting cocaine's access to dopamine transporters, its primary site of action. Various laboratory studies [40-43] showed that dopamine uptake inhibitors and direct-acting dopamine agonists could maintain drug self-administration in laboratory animals. Some drug-discrimination studies suggested that certain dopamine uptake inhibitors substitute completely for cocaine [44-45] while the direct-acting dopamine agonists substitute partially for cocaine [46-47]. On the other hand, dopamine antagonists can attenuate specific behavioral effects of cocaine including its reinforcing effects [48-49], locomotor activity effects [50], discriminative stimulus effects [51-53] and rare-altering effects on schedule-controlled behavior [41, 54-56]. There are some promising results with a number of these dopamine agents but side effects such as abuse potential associated with these drugs limit their acceptability in drug abusers [1].

1.4 Serotonergic Drugs

Owing to cocaine's complex pharmacology, it has been found to interact with more than one neurotransmitter system [1]. Based on these findings, a broad range of drug classes have been studied in pre-clinical and clinical areas for their effectiveness in treatment of cocaine abuse. Wolf and Kuhn [57]reported that cocaine binds the serotonin (5HT) receptor with greater affinity than the dopamine receptor. Consistent with these results, administration of the 5HT uptake inhibitor, fluoxetine or the 5HT precursor, L-tryptophan decreases IV self-administration of cocaine in rats [58-60]. Conversely, several $5HT_3$-selective antagonists such as MDL 72222 and

ondansetron failed to block the reinforcing [61-62] or discriminative-stimulus effects [61, 63] of cocaine in laboratory animals.

1.5 Excitatory and Inhibitory Amino Acids

Reports also suggest that excitatory and inhibitory amino acid pathways may provide useful targets in the treatment of substance abuse [64-67]. Several N-methyl-D-aspartic acid (NMDA) noncompetitive antagonists, MK801 and SGB-017 and the competitive antagonist NPC 12626 were examined in behavioral studies [68-71].

In addition, inhibitory amino acid systems such as γ-aminobutyric acid (GABA) are known to be widely distributed in the central nervous system (CNS) [72] and are also involved in the reinforcing effects of cocaine [64-67]. Baclofen, a $GABA_B$ receptor agonist that inhibits the release of dopamine, norepinephrine, serotonin and glutamate, was shown to suppress cocaine self-administration in rats [73]. Another candidate, vigabatrin, a GABA-transaminase (GABA-T) inhibitor also blocked cocaine self-administration [65, 74]. Vigabatrin is currently in human trials to examine its effectiveness in the treatment of cocaine abuse. Vigabatrin is on market in Europe for cocaine abuse and in the United States for partial seizures [75].

1.6 Opioid Receptor System

Another neurotransmitter system linked to the pharmacology of cocaine is the endogenous opioid system. Naltrexone, an opioid antagonist showed no alteration to cocaine self-adminstration in laboratory animals [36]. Some δ-opioid receptor selective antagonists such as naltrindole and naltriben also showed inconsistent results in rats and rhesus monkeys [73, 76].

The κ-selective agonists 50, 488, spiradoline [77], enadoline and bremazocine [78], κ-opioid receptor antagonist norbinaltorphimine [79] – all showed varied results in altering cocaine self-administration in laboratory animals.

1.7 Sigma Ligands

In 1988, studies conducted by Sharkey et al. established the micromolar affinity of cocaine towards sigma receptors [80]. These receptors were initially viewed to be a part of opioid receptor family. They derive the name "sigma" from the first letter of the compound SKF-10047 (N-allylnormetazocine) that was considered to be an archetype sigma ligand[81]. Owing to the irreversible effects of (+)-SKF-10047 by classic opioid antagonists like naloxone, sigma receptors were eliminated from the opioid class [82]. Further studies indicated that (-)-SKF-10047 had an affinity for mu (μ) and kappa (κ) receptors. (+)-SKF-10047 exhibited affinity towards a different site from opioid receptors [83-85]. Investigations also revealed interactions of (+)-SKF-10047 with PCP sites affiliated to NMDA receptors [86-88]. Eventually, in 1988, Wong et al. discovered that (+)-SKF-10047 could not be displaced by very sensitive PCP/NMDA ligands [88]. Thus, this receptor site discrete from opioid and NMDA receptors was labeled as the sigma receptor.

1.7.1 Sigma-1 and Sigma-2

There are two subtypes of sigma receptors, namely sigma-1 and sigma-2 respectively [89]. Both of these subtypes are distinct in terms of molecular weight, distribution in the body and ligand binding profile [89-90]. Sigma-1 receptors have a molecular weight of 25-29 kDa and composed of 223 amino acids [91-94]. Sigma-2 receptors have a molecular weight of 18-22 kDa[95].

Researchers have been able to clone sigma-1 receptors from mouse, rat, guinea pig and human tissues but no sigma-2 receptors have been cloned as of yet [79, 93, 96].

Sigma-1 and Sigma-2 receptors are extensively distributed in the body. They can be found in the brain, liver, gastrointestinal tract and kidney [95, 97]. Highest densities of sigma receptors are found in brainstem motor nuclei, cerebellum, red nucleus and in cranial nerves such as facial, hypoglossal and trigeminal nerves [98]. In the heart, approximately 80% of the sigma receptors are of the sigma-1 subtype and linked to many aspects of cardiovascular function [99].

In comparison to the research conducted on sigma-1 subtype antagonists, work indicating the involvement of sigma-2 receptors in cocaine-induced effects is nominal. This is attributed to the fact that sigma-2 receptors have not yet been cloned and there are no antisense oligonucleotides (ON) to reduce their number. Therefore, it is important to study the pharmacological and toxicological profile of compounds specific to sigma-1 and sigma-2 subtypes to aid in identifying the most effective compounds for treatment of drug abuse.

1.8 Sigma Receptors

Ever since Michael Kuhar et al. [16] discovered the micromolar affinity of cocaine for sigma receptors (σ), much attention has been given for further investigation of this new site. Sigma receptors have been implicated in many neuropsychiatric disorders as well as schizophrenia, seizures and anxiety [80]. Contrary to initial beliefs, this class of receptors was declared distinctly different from the opioid and NMDA receptors [81-88]. Sigma receptors are distributed extensively in the brain and peripheral organs [95, 97-98]. They are also expressed throughout the central nervous system.

1.8.1 Subtypes of Sigma Receptors

Sigma receptors have been classified into two main subtypes, sigma-1 (σ-1) and sigma-2 (σ-2) [89]. These subtypes were differentiated based on their molar mass, expression throughout various tissues, different drug selectivity patterns and ligand binding profile [89-90] (Table 1.3).

Table 1.3 Subtypes of Sigma Receptors [2]

Characteristics	Sigma-1 Receptor	Sigma-2 Receptor
Molecular Weight	25-29 kDa	18-22 kDa
Tissue Distribution	Brain, heart, liver, spleen, gastrointestinal tract	Brian, liver, gastrointestinal tract
Relative affinity of benzomorphans	(+)-isomer	(-)-isomer

1.8.2 Sigma-1 (σ-1) Receptor Subtype

The sigma-1 receptors found to be discrete from the opioid receptors [83-88] reside specifically at the endoplasmic reticulum (ER)-mitochondrion interface called the MAM (mitochondrion-associated ER membrane) [100]. Sigma-1 receptors have been cloned and well characterized due to the availability of selective sigma-1 ligands. The sigma-1 receptor cDNA has been cloned from guinea-pig liver, human placental cell line, T leukemia Ichikawa cell line and human brain, mouse kidney and brain, and rat brain [101]. It consists of 223 amino acids and the sequences of the purified proteins have been found to be highly similar. The protein sequence also showed similarity, a 33% identity and 66% homology, with a yeast sterol C_8-C_7isomerase [79]. However, no similarity has been observed with any mammalian enzyme or any other mammalian protein,

indicating that it is a unique receptor from any other existing receptors [101]. Their anatomical distribution has been well established based on initial auto-radiographic studies in rodent brain and more recently using in situ hybridization or immuno-histochemical techniques. They are understood to be concentrated in specific areas throughout limbic systems and brainstem motor structures [101]. Its presence is dense in the granular layer of different structures such as olfactory bulb, hypothalamic nuclei and hippocampus [102-103]. This receptor is particularly concentrated with post-synaptic thickenings, although can be observed at the pre-synaptic level. It contains an endoplasmic reticulum sequence, which upon activation translocates the sigma-1 receptor from the endoplasmic reticulum to the plasma or nuclear membrane [104-105]. The sigma-1 receptor is also widely expressed throughout the peripheral organs such as the digestive tract [106-107], vas deferens [108-109], liver [95, 108, 110], kidney [111], heart [112], adrenal medulla [113-114], pituitary testis and ovaries [113-115] and blood mononuclear cells [114]. The subcellular distribution profile of [^{3}H](+)-SKF-10,047 of the radioligand binding to sigma-1 sites coincided with that of NaDPHcytochrome c reductase, indicating the presence of sigma-1 receptor on the endoplasmic reticulum [101]. Sigma-1 receptors have been shown to play an important part in regulation of cardiac function by exerting a complex effect on the amplitude and frequencies of contraction, Ca^{2+} fluxes and intracellular Ca^{2+} concentration transients [112]. In particular, sigma-1 receptor agonists such as DTG, (-)-3-PPP, (+)-pentazocine, or BD737 increase the electrically-evoked amplitude of the contraction and Ca^{2+} transients [99, 112]. DuP 734, a sigma-1 receptor antagonist reversed the decrease in ventricular fibrillation threshold induced in conditions like post-infarction cardiosclerosis or immobilization stress [99].

Sigma-1 receptors are found to be highly mobile at the ER membrane suggesting a chaperone action [100, 116]. The mechanism of translocation is however unknown.

- Translocation of sigma-1 receptors has been observed from the ER to the neurite process and the nucleus when NG108 cells were treated with cocaine [117-118].
- Another example includes translocation of sigma-1 receptors from the MAM following loss of glucose or deficiency of ER Ca^{2+} by thapsigargin [100].
- Presence of a sigma-1 receptor agonist, (+) pentazocine caused the sigma-1 receptors to translocate from lipid raft fractions to non-raft fractions [117, 119].

It is perceived that ligand or stress induced translocation of sigma-1 receptors might change their target proteins from those at MAM to others at the plasmalemma or nuclear envelopes [120]. In mouse, these receptors were found to regulate voltage gated potassium channels in posterior pituitary nerve terminals [91]. In rats, sigma-1 receptors modulate various Ca^{2+} channels in sympathetic and parasympathetic neurons [104, 121]. This ability of sigma-1 receptors to act as an inter-organelle signaling modulator can be potentially used to develop medications for peripheral diseases as well as those related to central nervous system [120].

Considering the concentration of sigma-1 receptors in peripheral organs mediating physiological and behavioral effects at pharmacologically relevant doses, it is of immense interest in cocaine addiction. Cocaine is known to act not only on the brain, but also in tissues such as heart, lung, digestive tract and kidney. In cases of overdose or toxicity, peripheral sigma-1 receptors may influence the effects of cocaine.

1.8.3 Sigma-2 (σ-2) Receptor Subtype

Sigma-2 receptors have a molecular weight of 18-22 kDa, which is less than that of sigma-1 receptors [95]. Unlike sigma-1 receptors, these receptors have not yet been cloned. These receptors are found to be densely located in parts of the brain associated with motor activity like

the substantia-nigra pars reticulate, central gray matter, occulomotor nuclei, cerebellum and motor cortex [98]. In the peripheral organs, their presence is less dominant compared to that of sigma-1 receptors in heart and spleen [99]. The literature is abundant in reports on sigma-2 ligands[122-130]. Most of these reports are based on the reasonable affinity to sigma-2 receptor relative to sigma-1, with little emphasis on selectivity as compared to non-sigma binding sites. Functional studies conducted by Bowen et al and other researchers have shown that few sigma-2 compounds that were tested behaved like agonists [123-124, 131-132]. On the other hand, (±)-SM 21 (3α-tropanyl-2-(4-chlorophenoxy) butyrate), a sigma-2 preferring compound has been shown to exhibit antagonist activity against dystonic head movements and cocaine-induced behaviors in rodents [133-134]. Sigma-2 receptors have also been implicated in tumor cell proliferation [135-137]. It has been suggested that sigma-2 receptors induce apoptosis by causing depletion of cytoplasmic Ca^{2+} stores resulting in subsequent cell death [138]. Sigma-2 receptors have also been linked to potassium channels and intracellular calcium release in NCB-20 cells [79]. Selective sigma-2 ligands such as CB64D, CB184 and ibogaine and non-selective sigma-2 ligands such as haloperidol have been shown to affect growth and cause cell death in various tumor cell lines [138-140].

One of the hindrances in evaluating the profile of sigma-2 receptors is the lack of selective pharmacological sigma-2 antagonists and an amino acid sequence from which antisense oligonucleotides with sigma-2 selectivity can be developed. Of the two characterized subtypes, there is sufficient evidence reflecting the role of sigma-1 receptors in attenuating cocaine-related effects but the contributions of sigma-2 receptors are less clear.

1.9 Drug development targeting Sigma ligands

The discovery of cocaine's interaction with sigma-receptors opened new possibilities for research involving the development of suitable medications for cocaine-induced toxicity and addiction [80]. Three different mechanisms were proposed to understand the influence of sigma receptors on the effects of cocaine – 1) direct binding to the receptors, 2) modulation of other neurotransmitter systems, and 3) alterations in gene expression [21, 141-144]. It was hypothesized that blocking these sigma receptors could help attenuate cocaine related effects.

Certain non-selective sigma receptor antagonists such as BD1008 (N-[2-(3, 4-dichlorophenyl) ethyl]-N-methyl-2-(1-pyrrolidinyl) ethylamine) and its analogs successfully mitigated cocaine-induced toxic effects like convulsions and lethality in some cases [145-148].

Rimcazole (9-[3-(cis 3, 5-dimethyl-1-piperazinyl) propyl] carbazole), known as an "atypical antipsychotic" was found to lessen convulsions, locomotor stimulatory activity and sensitization in mice treated with cocaine [149-151]. Some synthetic analogs of rimcazole were also reported to have the same effect as rimcazole on cocaine-administered mice [149, 152-153]. Despite the dual affinity of rimcazole and its analogs towards both sigma-receptors and dopamine transporters (DAT), it was believed that the mitigation of cocaine induced effects correspond to their affinity for the sigma receptor [149].

In vitro studies conducted on AC927 (phenethylpiperidine), a compound with mixed $sigma_{1/2}$ affinity, were found to have antagonist actions through sigma-2 receptors [131]. Among its analogs, UMB24 (1-(2-phenethyl)-4-(2-pyridyl)-piperazine) was identified as a lead compound for development of selective sigma-2 receptor agents [127]. Behavioral studies in mice showed

that pretreatment with UMB24 resulted in attenuation of cocaine-induced convulsions and locomotor activity but not lethality [54, 154].

Recently, CM156 (3-(4-(4-cyclohexylpiperazin-1-yl) butyl) benzo[*d*]thiazole-2[*3H*] thione) (Figure 1.5), a highly selective sigma receptor antagonist, was able to vitiate cocaine-related toxic effects such as convulsions, locomotor stimulatory activity and expression of sensitization [54, 154].

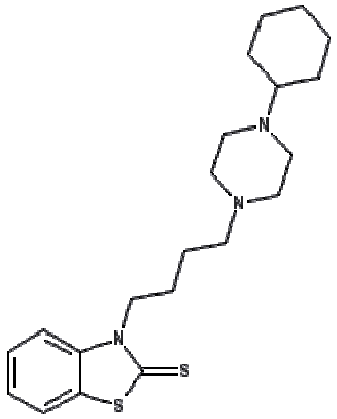


Figure 1.5 CM156

The literature also shows compounds that have agonist actions at sigma receptors. These compounds such as BD1031 and DTG were successful in aggravating the acute locomotor stimulant effects of cocaine in mice [145-146, 148, 155].

1.9.1 Targeting Sigma-1

Cocaine has been shown to have 10-fold higher affinity to sigma-1 receptors as sigma-2 (Table 1.4) [149]. Because of this, the sigma-1 receptor has been extensively examined as a target for mitigating cocaine effects. This was also made possible due to the cloning of the sigma-1

receptor [79, 93, 96]. Using this data, synthesis of antisense ON targeting the mRNA of sigma-1 receptors was carried out. Subsequently, these ON led to a 40% reduction in the number of sigma-1 receptors in the brain by interfering with their synthesis [145]. Subsequently, it was observed that this decrease in brain sigma-1 receptors caused significant attenuation in cocaine-induced convulsions, locomotor stimulant activity and conditioned place preference [145, 147, 156]. This data was substantiated by conducting the same study using sense and mismatch ON as additional controls which resulted in no effect on cocaine-induced toxic effects thus concluding the reduction in these effects as a direct consequence of decrease in brain sigma-1 receptors [145, 147]. One major drawback of this study was the intracerebroventricular route of administration that had to be used because of the poor permeation of ON across the blood brain barrier (BBB). Despite ON showing potential in attenuating the cocaine-induced toxic effects, therapeutically, this was not a feasible option.

Table 1.4 Affinity of cocaine for sigma receptors [2]

Drug	**Radioligand**	**Tissue**	**Sigma subtype**	**Affinity K_i, nM**
Cocaine	[^{3}H](+)-pentazocine	Rat brain without cerebellum	σ-1	2909 [145]
Cocaine	[^{3}H]DTG + (+)-pentazocine	Rat brain without cerebellum	σ-2	29,175[145]

Improvising on what ON had to offer, candidates capable of crossing the BBB were examined using selective sigma-1 ligands. A behavioral study using BMY14802 [150] and rimcazole were compared against effects of a non-sigma agent, clozapine and a classical sigma receptor antagonist haloperidol and (+)-3-PPP [157].

All these historic ligands exhibited the ability to reduce cocaine-induced convulsions but it wasn't clear if this was a result of their binding to sigma receptors since they exhibit an affinity to other sitesas well [146]. BMY14802 had a significant affinity for 5-HT1A and it was unclear if this affinity played a role in preventing the development of the cocaine-induced sensitization.

Dextromethorphan (DXM) was among other candidates that were examined as a target for sigma-1. Despite its moderate sigma-1 receptor affinity (K_i = 419 nM), it was reported to prevent cocaine-induced behavioral effects [68-69, 158-159]. Dextromethorphan was found to have higher affinity for the NMDA receptors. These receptors are considered to play a role in the attenuation of cocaine-related effects. Analogs of DXM were synthesized with enhanced selectivity for sigma-1 receptors over phencyclidine (PCP) binding sites. None of these DXM analogs possessed good affinity for binding to sigma-2 receptors. Although these compounds could protect against cocaine-induced convulsions at higher doses, the lack of data correlating their anti-convulsant potencies to their affinities for sigma-1 receptors gave rise to speculations regarding involvement of other receptor mechanisms or an associated pharmacokinetic issue [160-162].

Aside from the antagonist activity associated with antisense ON and sigma-1 antagonists, certain sigma-1 receptor agonists exhibited potential in enhancing cocaine-induced convulsions,

locomotor stimulant activity and other effects thus establishing the role of sigma-1 receptors in cocaine related toxic effects and addiction [145-146].

1.9.2 Targeting Sigma-2

Cocaine has been known to have affinity not just for sigma-1 but also sigma-2 receptors. It is believed to cause the inhibition of NMDA-stimulated dopamine release from key areas of the brain. Research related to the discovery of antagonists for sigma-2 receptors is not as extensive as that for sigma-1. The literature does show some examples of sigma-2 antagonists with the ability to attenuate cocaine-related effects [163-164]. Ibogaine, an indole alkaloid obtained from the root of the African shrub *Tabernantheiboga* and its analogs displayed preferential sigma-2 receptor affinity over sigma-1 subtype [124]. However, it was observed that ibogaine and its analogs have interactions with other receptors as well, hence was considered unsuitable for *in vivo* studies [124, 165]. Lu28-179 or Siramesine (1'-[4-[1-(4-fluorophenyl) – 1*H*-indol-3-yl] butan-1-yl] spiro [isobenzofuran-1(3*H*), 4'-piperidine], a piperidine analog was identified as a sigma-2 antagonist with preferential affinity for sigma-2 receptor (K_i = 0.12nM) [166-167]. It was believed to have a 140-fold affinity for sigma-2 over the sigma-1 subtype. Additionally, Siramesine also exhibited low affinities for dopaminergic D_2, α-adrenergic, 5-HT_{1A} and 5-HT_{2A} receptors [167]. CB-184, a benzylidenephenylmorphan was one of the first sigma-2 ligands to be tested for its selectivity for the sigma class. This sigma-2 agonist had a 555 fold affinity for sigma-2 over sigma-1 receptors [123] but also had agonist activity with mu (μ)-opioid receptors [131-132, 138]. A series of (E)-8-benzylidene derivatives of 2-methyl-5-(3-hydroxyphenyl) morphans were synthesized which had preferential sigma-2 receptor affinity over sigma-1 in the range of 32-238 fold. These compounds also retained their potent *in vivo* affinity for μ-opioid receptors [122].

A series of 1, 4-dibenzylpiperazine compounds seemed to have anticonvulsant effects in mice administered with cocaine at 60mg/kg intraperitoneally[168]. Their *in vivo* potency had no correlation with the sigma-1 receptor affinity and selectivity. These findings further corroborated the role of sigma-2 receptors in attenuation of cocaine-induced convulsions.

S14905, a selective sigma-2 compound was found to attenuate cocaine-induced locomotor stimulant activity in mice but had an affinity for other receptors as well [169]. In 2007, Matsumoto et al. identified two compounds, UMB24 and (±)-SM 21 with a high preferential affinity for sigma-2 receptors over the sigma-1 subtype [154].Behavioral studies conducted using these two compounds in mice resulted in significant attenuation of cocaine-induced convulsions and locomotor activity but had no influence on the lethality. This inability on the part of these two compounds may be due to fewer of sigma-2 receptors compared to sigma-1 in peripheral organs such as heart. It has been hypothesized that antagonists with mixed preferential affinity for sigma-1 and sigma-2 receptors may have better potential in mitigating cocaine-related convulsions than highly selective antagonists [154]. Also, the high dopamine (D_2) affinity of UMB24 has been perceived to be the cause for locomotor depressant action in mice.

Recently, Dr. Christopher R. McCurdy's group from the Department of Medicinal Chemistry at the University of Mississippi synthesized a compound that is a derivative of 2(3H)-benzothiazolone with 11 fold preferential subnanomolar affinity for sigma-2 receptors over sigma-1 [170]. However, it still has affinity for sigma-1 receptors.

These compounds have established the critical role of sigma receptors in alleviating cocaine-induced convulsions and locomotor stimulant activity; however more understanding is required to develop a medication for cocaine abuse and toxicity. Because of the lack of data regarding

structural information, amino acid sequence, the pharmacological characterization of sigma-2 subtype and the absence of a truly selective sigma-2 ligands, the scope of research done in this area so far is very limited. Pharmacological studies show that sigma-2 receptors may be lipid raft proteins that affect calcium signaling via sphingolipid products [89, 131, 171]. It is difficult to ignore sigma-2 receptors because evidence suggests their involvement in the attenuation of cocaine-induced convulsions although their role in doing so is unclear [154]. The above-mentioned compounds exhibit some ability in mitigating cocaine-related toxic effects in spite of their mixed receptor activity. Since cocaine has affinity to more than one target site, interpretation of any behavioral data can become challenging. Therefore, it is only reasonable to develop a pharmacological antagonist that is capable of crossing the blood brain barrier while having great preference for sigma-2 over sigma-1 receptors in addition to having high selectivity over other receptors and transporter systems in the brain. This will help delineate the exact role of sigma-2 receptors in cocaine abuse. A truly selective sigma-2 ligand will also aid in unmasking the protein sequence of the sigma-2 receptor thereby shedding light on its mechanism of action and biochemical role in various physiological effects.

1.10 SN79

In search of a selective sigma-2 ligand, Dr. Christopher R. McCurdy's group from the Department of Medicinal Chemistry at the University of Mississippi synthesized a series of 2(3*H*)-Benzoxazolone and 2(3*H*)-Benzothiazolone derivatives by varying the linker length from two to six carbons [170]. This was achieved by simple *N*-alkylation of 2(3*H*)-Benzoxazolone and 2(3*H*)-Benzothiazolone with various dibromoalkanes and 4-cyclohexylpiperazine to produce the final analogs. These analogs were then converted to hydrochloride salts and tested in *in vitro* competition binding assays for sigma-1 and sigma-2 receptors from rat brain homogenates.

[^{3}H](+)-Pentazocine was used to label sigma-1 receptors. [^{3}H]DTG was used for sigma-2 receptors in the presence of a blocking concentration of (+)-pentazocine. Non-specific binding was determined using haloperidol. It was found that these scaffolds had high affinity in the range of 2-10 nM for both the subtypes [170].

It was noted that compound CM121 with a four-carbon linker had a six-fold preference for the sigma-2 subtype over the sigma-1 subtype. Compounds with 2, 3, 5 and 6 carbon linkers exhibited similar affinities for both sigma-1 and sigma-2 receptors [2].

Similarly, among the 2(3*H*)-Benzothiazolone derivatives synthesized, the four-methylene linker compound, CM145, exhibited an 11-fold selectivity with a subnanomolar affinity for sigma-2 receptors. Upon conducting further studies, it was hypothesized that presence of a four-carbon linker, 6-keto functionality in 2(3*H*)-benzoxazoloneheterocycle may be significant for sigma-2 receptor affinity and selectivity [2]. From a set of compounds previously synthesized by Dr. Christopher R. McCurdy's group from the Department of Medicinal Chemistry at the University of Mississippi, compound CM170 had 11-fold preference or sigma-2 receptor while compound CM142 had 7-fold selectivity for sigma-2 receptors.

The presence of a 1-(4-fluorophenyl) piperazine moiety in CM170 and a 6-acetyl group in CM142 was considered significant for enhancement of sigma-2 receptor affinity and selectivity.

Based on these observations, the following features were deemed salient to design SN79 (Figure 1.6) a putative selective sigma-2 receptor ligand:

- Presence of a four methylene linker between two hydrophobic regions
- Presence of a 6-acetyl group in the 2(3H)-benzoxazoloneheterocycle
- Presence of a 1-(4-fluorophenyl) group at the piperazine ring

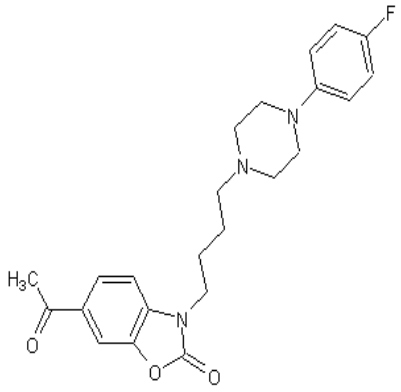


Figure 1.6 Structure of SN79

Based on radio-ligand binding assays, compound SN79 exhibited high affinity for sigma-2 receptors (K_i = 6.89±0.09 nM) with partial affinity for sigma-1 receptors (K_i = 28.03±3.39 nM) [172].

1.10.1 Pharmacological evaluation of SN79 in rodents

Researchers in Dr. Rae R. Matsumoto's lab from the Department of Basic Pharmaceutical Sciences at West Virginia University studied the antagonist effect of SN79 on cocaine actions in male Swiss Webster mice [172]. When pre-treated with SN79 (0.1-10 mg/kg) i.p. followed by convulsive dose of cocaine administered after 15 min, percentage of mice with convulsions decreased in a dose dependent manner [172]. According to previously conducted studies, sigma

receptor agonist as well as antagonists and 5-HT_2 antagonists tend to have anticonvulsant effects thereby mitigating cocaine-induced convulsions [173-174]. Pre-treatment with SN79 given orally followed by a convulsive dose of cocaine (80 mg/kg) also had a significant effect on percentage of mice exhibiting convulsions, causing a decrease from 78 to 20% [172]. SN79 was reported to have caused a dose dependent decrease in cocaine-induced hyperactivity having some sedative effect of its own [172]. Apart from the above mentioned observations, SN79 also inhibited development of cocaine-related sensitization [172], an attribute previously associated with 5-HT_2 antagonists [175]. Studies conducted by Dr. Matsumoto's researchers reinforce the role of sigma receptors in mitigating cocaine-induced effects [172].

SN79 caused no inhibition of some major cytochrome P450 enzymes such as CYP1A2, CYP2A6, CYP2C19, CYP2C9*1, CYP2D6 and CYP34A [172] thereby eliminating the possibility of any drug-drug interactions *in vivo* [172].

Based on these results, SN79 can be categorized as a potential pharmacotherapy to mitigate acute as well as subchronic effects of cocaine thereby making it necessary to study critical ADME properties of this compound.

CHAPTER II: IMPORTANCE OF PHARMACOKINETICS IN DRUG DISCOVERY AND DEVELOPMENT

2.1 Role of Pharmacokinetics and Metabolism in Drug Discovery and Development

Drug discovery and development encompass the functional process of searching for new drugs. The discovery stage is characterized by identifying the target enzyme/receptor for a particular disease establishing surrogate markers (or suitable models) for biological activities, and testing the new drug candidates for in vitro and/or in vivo biological activities [176]. The development stage consists of evaluation of the toxicity and efficacy of the new drug molecules. In the United States, the average time taken for a new chemical entity to enter the market involves 10-15 years of research at a cost of almost a billion dollars [176]. Compounds that fail in preclinical or clinical studies largely affect the drug discovery process and also gives rise to obvious time and cost penalties.

Lead compound optimization is achieved through the screening of a multitude of compounds based on desired target affinity. These results aid medicinal chemists in modifying the structure of the compound based on a variety of empirical and semi-empirical structure activity relationships to amplify its *in vitro* activity. Along with *in vitro* studies, supporting *in vivo* data is required to understand whether or not the compound of interest has good bioavailability and duration of action. When a new chemical entity (NCE) is discovered, it is important to understand its therapeutic activity in the body and achieving ways to optimize its potency.

Information pertaining to its biological, physical and physicochemical properties will help design a drug delivery system that enhances *in vivo* targeting efficacy. It is not easy to fully determine the pharmacokinetics of a drug *in vitro*, therefore early preclinical pharmacokinetics is a rational approach to address this issue. In this stage, certain characteristics are measured that help decide whether or not the NCE is a potential drug candidate. This data guides us to better understand if the compound of interest after administration, acts and remains at the targeted site producing a concentration-time profile to elicit the desired response. These studies help reduce attrition rate at the *in vivo* and clinical phases. For this reason, pharmacokinetics and metabolism play instrumental roles not just in drug development phase but should also be employed in discovery stage as determinants of *in vivo* drug action.

The experimental design of studies and selection of animal species play a vital role in providing crucial data that help predict drug absorption and elimination in humans. Any errors in the consideration of these two parameters can cost the researchers loss of a good compound. Regulatory agencies require a thorough understanding of the pharmacokinetics and metabolic processes of a new drug. Many psychotropic drugs have shown tendency to form metabolites with their own biological activity [177]. This kind of behavior offers an explanation in cases of toxicity for some drugs. Before studying metabolism in humans, *in vitro* studies are done to establish the compound's metabolic half-life. During the drug development stage, it is desirable to identify the enzymes responsible for drug metabolism in humans. This data can prove useful in designing clinical studies, specifically those involving drug-drug interactions. Comparison of metabolism in humans and animals is important in the early stage of drug development to aid in selection of appropriate animal species for toxicity studies. Latest technologies such as LC-MS,

high-field NMR and LC-NMR techniques aid in obtaining early information on the metabolic pathways of new drug candidates in the drug development phase.

The literature contains references to many compounds discovered serendipitously – the antituberculosis agent, isoniazid [178-179], anxiolytics such as diazepam and chlordiazepoxide [177, 180-181]. Through a better understanding of disease pathways and recent advances in molecular biology and protein chemistry, mechanism-based drug design became feasible. This helps interrupt specific biochemical processes by targeting certain types of enzymes and/or receptors. Computer-aided graphics has also introduced structure-based design. These approaches have been successfully employed to maximize drug activity within certain structural limits. However, good *in vitro* activity does not necessarily warrant good *in vivo* activity in case of specific drugs. This may be a consequence of undesirable pharmacokinetic parameters, and toxicity resulting from metabolites with biological activity of their own. Pharmacologically, the metabolite may behave similarly as the parent compound, via different mechanisms or even antagonism. Therefore, incorporating pharmacokinetic and metabolism data with biochemical and pharmacological data characterize an optimal drug design.

2.1.1 Pharmacokinetics and Drug Design

Many drug candidates are discarded because of their weak pharmacokinetic properties, such as too long or too short half-life, undesirable absorption, and extensive hepatic first-pass metabolism. The high rate of attrition of new drug candidates as a result of to their serious pharmacokinetic problems indicate that the significance of pharmacokinetics in the drug discovery and development process. Two important parameters that define good pharmacokinetics properties are good bioavailability and a desirable half-life. Therefore, through

suitable chemical modifications to the drug design, it is possible to attain drug candidates with optimum pharmacokinetics. The following factors help define drug absorption and disposition.

2.1.1.1 Absorption

Absorption of a drug is largely dependent on physicochemical and biological factors. Two important physicochemical properties influencing drug absorption are lipophilicity and solubility [182] (Figure 2.1). The oral bioavailability of a drug is characterized by the amount of oral drug dose that reaches systemic circulation.

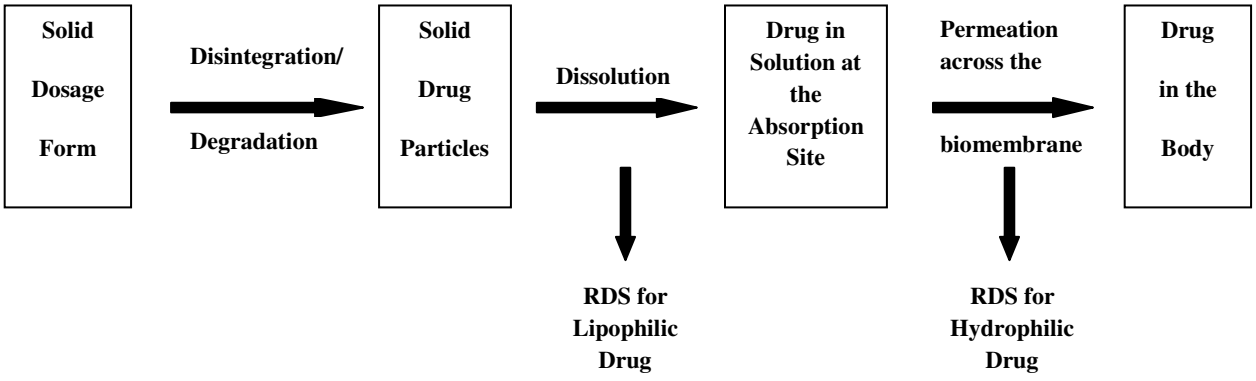


Figure 2.1 The two important rate determining steps (RDS) for absorption of drugs from oral administration [183].

Drugs entering the body via oral route encounter the membrane of gastrointestinal tract made of phospholipids interspersed with proteins. The rate and extent of transcellular passage of drugs across this lipid bilayer is determined by the lipophilicity of the drugs. In case of barbiturates, it has been observed that increasing lipophilicity causes increased membrane permeability thereby exemplifying the correlation between lipophilicity and increased permeability [184]. Drug absorption from the gastrointestinal tract is also defined by the susceptibility of the drug to

metabolize in the liver. Before the drug reaches systemic circulation, blood supply of the upper gastrointestinal tract passes through the liver thereby exposing the drug to first-pass effect. A drug with high metabolic clearance is extensively metabolized by the liver and gut wall during first passage of absorption, hence resulting in low bioavailability. From studies conducted by Seydel et al., it has been understood that the lipophilicity of a drug is directly proportional to membrane permeability and first–pass metabolism [185-186]. Therefore, optimization of lipophilicity is crucial in maximizing membrane permeability and minimizing first-pass metabolism to enhance bioavailability.

Solubility also plays a significant role in ascertaining drug absorption into systemic circulation. Drugs with poor aqueous solubility pose a hindrance to absorption. By applying modifications to drug design, solubility can be altered to make the drugs suitable for oral delivery. One such approach is using the salt form of the drug instead of the free base. Merck research laboratories designed a HIV protease inhibitor called indinavir sulfate [187-188]. It was found to be a better oral dosing agent as against its free base indinavir. The free base suffered from high lipophilicity and low aqueous solubility (0.07 mg/mL at pH-7.4), thereby limiting its absorption. The salt form had enhanced solubility (60 mg/mL at pH-3.5) adverse to the indinavir free base, facilitating its aqueous solubility, subsequently enhancing drug absorption in acidic pH of the gastrointestinal tract [187-188]. Apart from structural modifications, formulation optimization also helps augment drug absorption [189].

2.1.1.2 Distribution

Distribution of the drug in the body plays a vital role as one of the determinants of bioavailability. Distribution is also characterized by the lipophilicity of the drug. Highly

lipophilic drugs exhibit high protein binding, thereby displaying a high distribution level [185-186]. A study conducted on 15 basic drugs in dogs exemplified the effect of increased lipophilicity on enhancing volume of distribution [190]. Aside from lipophilicity, the extent of the drug binding to plasma and tissue proteins also governs drug absorption in adipose tissues [191]. Delivery to brain has several limiting factors in comparison to other organs in the body. The presence of a highly lipophilic barrier, known as blood-brain-barrier (BBB), necessitates perfusion of drugs into the brain via simple diffusion. For drugs whose activity is warranted in the central nervous system (CNS), it is important to understand the functioning of the BBB in order to gain access to the site of action. Most drugs crossing the BBB do so by passive diffusion, thereby establishing the significant role of lipophilicity in brain penetration. Recent studies indicate that an octanol/buffer partition coefficient (log *P*) value between 1 and 2 is deemed optimum for CNS drugs [192](Figure 2.2).

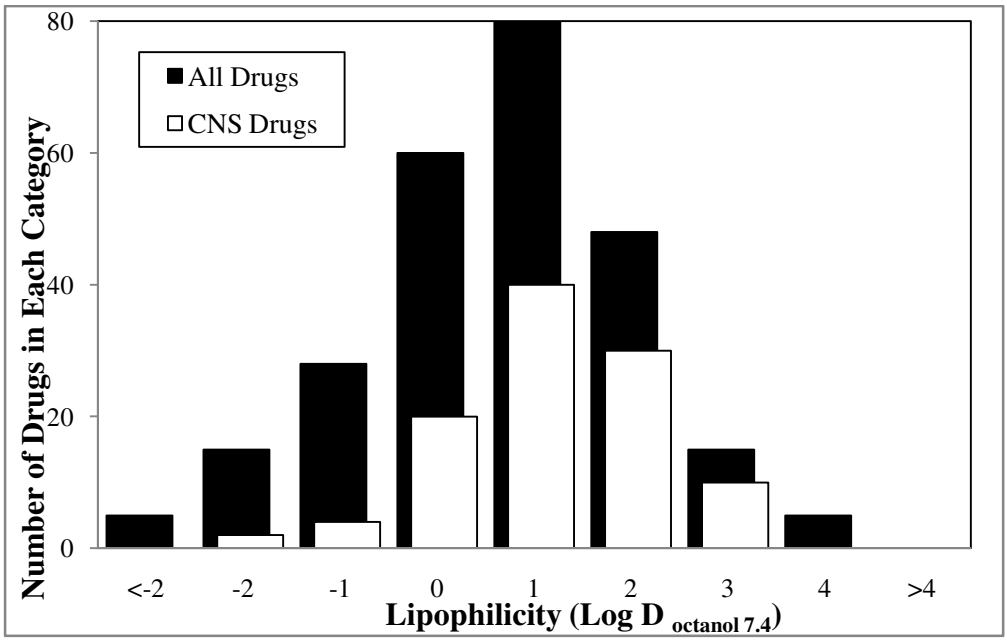


Figure 2.2 Graph showing correlation between distribution and lipophilicity of 257 marketed drugs [192].

Contrary to popular beliefs that increase in lipophilicity causes enhanced brain permeation, it has been reported that a linear relationship between lipophilicity and brain permeation occurs only within a certain range. Inhibition of P-glycoprotein has also shown enhancement in BBB permeation of some highly lipophilic drugs [193-194].

2.1.1.3 Drug Half-life

The biological half-life of a drug is a crucial determinant of the dosage regimen. *It is defined as the time taken for the amount of drug in the body as well as plasma concentration to decline by one-half or 50% its initial value.* The half-life of a drug reflects the duration of drug action. In order to obtain therapeutically significant drug levels in plasma, fixed dosing is given at regular time periods. A drug with shorter half-life postulates frequent dosing thereby affecting patient compliance. Today, half-life is considered a secondary parameter that is defined by primary parameters - clearance and apparent volume of distribution. In order to attain desired pharmacokinetics, a drug with prolonged half-life is reckoned as ideal. Two approaches to prolong drug half-life and reduce dosing frequency are by increasing the volume of distribution or decreasing elimination clearance. Chemical modifications made to the structure seem to offer the requisite plasma half-life in certain drugs. An example of this is nifedipine, a calcium channel blocker used to treat hypertension. With its limitations such as short plasma half-life (~2 hours), high dosing frequency and substantial first-pass effect [195], investigators were tasked with developing a drug with improved bioavailability and prolonged half-life. Structural modifications led to the development of amlodipine, which is also a 1, 4-dihydropyridine derivative, similar to nifedipine that exhibits antihypertensive activity. This compound when examined in clinical studies displayed good oral bioavailability (50-60%) and increased plasma half-life (~30 hours) [196-197]. Another strategy to prolong half-life is formulation modification.

A sustained-release drug delivery system or co-administration of inhibitors of drug-metabolizing enzymes has been shown to have an escalating effect on plasma half-life [198].

2.1.1.4 Stereoisomers

Many marketed pharmaceutical drugs contain racemic mixtures without much knowledge about the pharmacokinetic data for each individual stereoisomer. It is imperative to understand the role of stereochemistry in drug development because stereosiomers of a chiral drug are known to exhibit marked differences in their pharmacokinetic and pharmacodynamic behavior [176]. It is common for one stereoisomer to exhibit desirable bioavailability and plasma half-life over the other stereoisomer of a chiral drug. Careful perusal of the similarities/dissimilarities between the activities of (+) and (-) isomers is necessary to identify the most optimal enantiomer for drug development. For specific drugs, the use of a racemic mixture resulted in better therapeutic profile than with a single enantiomer. Indacrinone, a drug used for its uricosuric and diuretic activities, is marketed as a 9:1 racemic mixture of the (+) - and (-)-isomers [199-200]. Specificity of receptors is also a determinant in selection of isomers. β_1-adrenergic receptors have better affinity than β_2 subtype to timolol, a nonselective β-adrenergic antagonist due to the presence of a chiral center in the amino-hydroxypropoxy side-chain [201].

The examples mentioned in this topic exemplify the role of pharmacokinetics in guiding drug disovery and development.

2.1.2 Metabolism and Drug Toxicity

Regulatory agencies warrant extensive toxicity studies to be conducted in laboratory animals before proceeding to clinical studies. Despite exploring safety in animals, extrapolating toxicity assessments from animals to humans can be challenging. Drug-induced toxicity is species-

dependent due to the tendency of certain laboratory animals to exhibit typical mechanisms of developing toxicity that extrapolation of such data to humans would be incorrect [202-205]. In order to accurately interpret this behavior, it is necessary to understand the various metabolic pathways and other mechanisms influencing species variation in metabolism and pharmacokinetics.

2.1.2.1 Species Induced Differences in Metabolism

Most of the mammals, despite descending from a common ancestor have evolved into different beings by adapting to their environment. There are many variations with respect to amino acid sequences in species. Considering one of the most instructive cytochromes, P-450, biochemists have been able to isolate at least 14 P-450 families to date [206]. Though P-450 was perceived to have evolved from a single ancestral gene, there are significant differences in the amino acid sequences across species [207]. Changes in amino acid sequence invariably influence substrate specificity [208]. This has a profound effect on both rate of drug metabolism and the metabolite pattern between animal species. Species differences have been observed by both phase I and phase II reactions. In phase I reactions, both quantitative and qualitative differences in the enzyme and its activity have been reported. On the other hand, phase II reactions only illustrated qualitative differences. Drugs undergo metabolism in a number of pathways depending on the species. The metabolism of Indinavir, a potent HIV protease inhibitor, yielded different values of intrinsic clearance in animals and humans [209]. In humans, indinavir was metabolized through a number of pathways – (a) glucoronidation at the pyridine nitrogen yielding a quarternized ammonium conjugate, (b) pyridine *N*-oxidation, (c) para-hydroxylation of the phenylmethyl group, (d) 3'-hydroxylation of the indan, and (e) *N*-depyridomethylation[209]. It was observed that indinavir produced oxidative metabolites in humans and animals, while N-glucoronide was

formed only in human and monkey urine [210]. The intrinsic clearance (cL_{int})(V_{max}/K_m) values of indinavir was in the following order: rat (157 mL/min/kg) ≈ monkey (162 mL/min/kg) > dog (29 mL/min/kg) > human (17 mL/min/kg) [210]. These values exemplify the quantitative and qualitative variations in metabolism of indinavir across different species. Induction is another mechanism that influences metabolism. In a study conducted by Conney et al [211], animals treated with 3-methyl-cholanthrene displayed enhanced ability in metabolizing methylatedaminoazo dyes. Omeprazole, a gastric-acid suppressant, acts as a CYP1A2 inducer in humans but not in mice or rabbits [212-213]. Induction of drug-metabolizing enzymes may or may not increase toxicity depending on the balance between detoxification and metabolite activation. Aside from species variation, difference in sex is also an important determinant of toxicity. According to recent studies, sexual dimorphism in laboratory animals is largely attributed to the differential expression of sex-dependent cytochrome P-450s [214-216]. Hepatic cytochrome P-450 is responsible for metabolic inactivation and/or activation of a drug. Any differences in the levels of P-450 expression due to sex affect the toxicological response as it affects the susceptibility of the tissue to drug-exposure. Thus, regulatory agencies warrant safety assessment data in equal number of males and females of each species.

The onset of toxicity is not always an attribute of the parent drug, but can also arise due to the formation of reactive toxic metabolites for certain drugs. Studies indicated that acetaminophen, commonly used for its antipyretic and analgesic activities, tends to induce hepatotoxicity in mice at doses ~ 200-300 mg/kg while in rats (doses >1500mg/kg), barely detectable hepatotoxicity was observed. These results suggest that the amount of reactive metabolite formed varies from species to species; sometimes completely absent in one particular species. Thus, monitoring the parent drug alone in vivo has very little relevance.

2.1.2.2 Stereoselectivity and Toxicity

Safety assessments also extend to studying stereochemistry of parent compound. Pharmacokinetic and pharmacodynamic differences of enantiomers are a consequence of their varied interactions with biological macromolecules [217-220]. Steroselective plasma protein binding is a species-dependent phenomenon. Also, stereoselective metabolism has been documented in case of certain drugs. *In vitro* metabolism of Mephenytoinenantiomers was studied in different species and humans [221]. *R*-mephenytoin underwent microsomal 4'-hydroxylation 2 to 6 times higher than*S*-mephenytoin in rabbits, dogs and rats whereas the rates were 5 to 15 times higher with *S*-enantiomer than *R*-mephenytoin in monkey and humans. These results manifest the significance of species selection and help decide whether to develop a racemate mixture or individual enantiomer while conducting safety assessments in laboratory animals before the data are extrapolated to humans.

2.2 Kinetics of Cocaine distribution and elimination

The measurement of cocaine and its metabolites has considerable relevance to cocaine research, both clinical and basic, and to that controversial and fuzzy interface between medicine and social policy defined as drug abuse screening [222]. Cocaine hydrochloride, a crystalline salt, is commonly inhaled or injected. Cocaine is a tertiary amine with pK_a of 8.6. It is a weak base and crosses cell membranes quickly and efficiently [223]. At higher pH values, the un-ionized or freebase form predominates while at a more acidic pH, cocaine exists in the salt form. Traditionally, the free base was prepared by alkalinizing an aqueous solution of the salt form and extracting the freebase using an organic solvent such as ether that was then evaporated. The route of administration largely influences the rate and bioavailability of cocaine entering systemic.

Figure 2.3 illustrates the differences in peak plasma levels of cocaine when approximately equipotent doses were administered to the same ten subjects via various routes [223]. When cocaine was administered intranasally in surgical parents for vasoconstriction and local anesthesia prior to intubation, peak plasma levels were detected in blood one hour after administration [224]. Vasoconstriction limits the rate of absorption and thus variable amounts are swallowed. When inhaled, absorption from nasal mucosa was found to be similar to absorption from mouth and the gastrointestinal tract after oral administration and much slower than after smoking or after intravenous administration [225-226]. After inhalation or oral administration, peak plasma levels were found after 60 minutes although great variability between individuals exists ranging between 30 to 120 minutes [223]. Oral and nasal bioavailability has been found to be 30 to 40 percent with the remainder eliminated by first-pass metabolism [223]. Depending on the route of administration, Cocaine doses commonly range from 0.2 to 3 or 4 mg/kg. The rate of injection during intravenous administration is an important determinant of peak cocaine levels in the systemic circulation. Peak plasma levels can range from 50 to 2000 ng/mL depending on route and rate of injection.

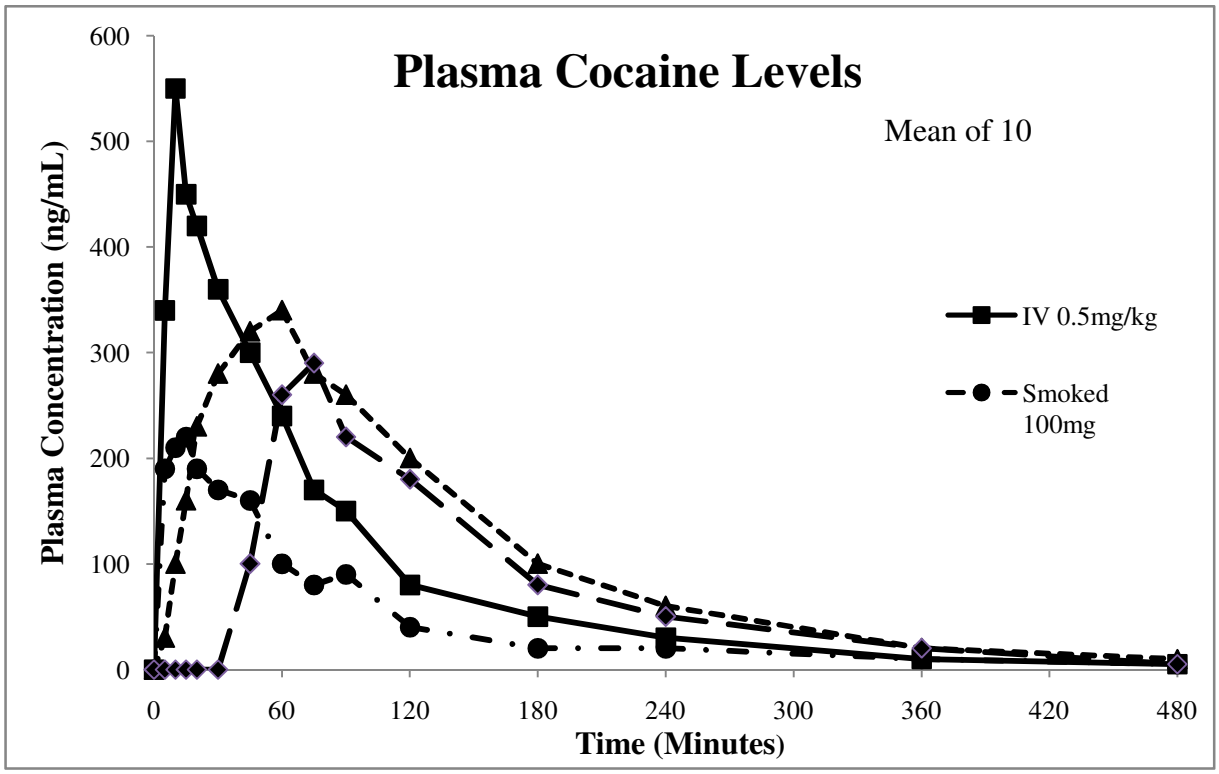


Figure 2.3 Plasma levels of cocaine when dosed via various routes. [223].

Cocaine, once administered, is found to be widely distributed through body tissues with a volume of distribution ranging from 1.5-2 L/kg [225, 227]. Its volume of distribution is small compared to some psychotropic drugs such as methadone (3.6-6.7 L/kg) [228-229], desipramine (20 L/kg) [230] and naltrexone (15 L/kg) [231]. Cocaine is found in low concentrations in body fluids and is known to exhibit very rapid metabolic disposition. Also, cocaine is susceptible to enzymatic and spontaneous hydrolysis both in vitro and in vivo; for the afore-mentioned reasons, accurate measurement of cocaine represents an interesting challenge to researchers [222]. When cocaine incubated with human plasma or with liver homogenates, it rapidly hydrolyzes to form an ecgonine methyl ester, which is one of cocaine's two major metabolites [232-235]. About 1 to 5 percent of an administered dose of cocaine is excreted unchanged in urine [223]. About 75 to 90 percent is eliminated as inactive metabolites such as the ecgonine methyl ester and

benzoylecgonine[224, 236-238]. An N-demethylation of cocaine in hepatic microsomes produces norcocaine, the only pharmacologically active metabolite of cocaine [239]. Cocaine is rapidly eliminated from the body with an elimination half-life of about 1 hour and total body clearance of about two liters per minute [240-242]. The elimination half lives of cocaine's metabolites, benzoylecgonine and ecgonine methyl ester are 6-8 hours and 3-8 hours respectively [223].

There is continuing controversy over using a one-compartment or two-compartment system for studying the distribution of intravenous cocaine. In 1977, Kogan et al. [243] reported for the first time that cocaine plasma concentrations declined multi-exponentially when dosed at 100 mg intravenously with half-lives of 20-40 min for a distribution phase and 2.8 h for the terminal phase. This data was found to be best fit by a two compartment model [242]. On the contrary, Javaid et al. [244] found the distribution of intravenous cocaine to decline mono-exponentially at a dose of 32 mg. Barnett et al. [240] studied the distribution of cocaine in two subjects using a 200 mg dose and found that the plasma levels were best described by a bi-exponential function in one subject and by a mono-exponential function in the other. Chow et al. studied the pharmacokinetics of cocaine in five healthy subjects at a low dose of 32 mg [241]. The concentration data was modeled using a two compartment system and the authors speculate that the very rapid distribution phase of cocaine was not taken in account in the earlier studies because sampling was not done early enough after drug administration [241]. Cocaine dosed at 100 mg to three patients resulted in concentration data following mono-exponential function. This prompted Barnett et al. and other investigators to conclude that the disposition of cocaine in the human body has pronounced dose dependence over the dose range studied [244]. Many pharmacokinetic parameters are dose-defined over the range of 1.1 – 2.9 mg/kg. The resolution

of the dose-dependent disposition of cocaine relies on further investigations such as measurements of both drug and metabolites in plasma, urine and consideration of an extended dose range.

2.3 Pharmacokinetics of Sigma Receptor Antagonists

Early reports on opioid-induced behavioral and psychotomimetic effects were originally thought to be produced by sigma receptors [81]. Many typical and atypical antipsychotic drugs, including haloperidol, bind with high affinity to the sigma binding site leading to the hypothesis that a sigma ligand may be an effective therapeutic solution for a new class of antipsychotic drugs without the serious motor side effects of traditional neuroleptic agents. This approach has led to the identification of several new antipsychotic agents in animal behavioral studies with binding affinity to sigma receptors [157]. The preclinical behavioral effects of DuP 734 indicate its potential as an antipsychotic agent without the extrapyramidal symptoms of neuroleptics[245]. DuP 734 exhibits high affinity for both sigma (K_i= 10 nM) and 5-HT_2 receptors (K_i= 15 nM), weak affinity at D_2 and PCP receptors (K_i> 1.0 μM) and no appreciable affinity for any other receptor, ion channel or second messenger system *in vitro*[246]. Kapil et al. investigated the pharmacokinetics of DuP 734 [247] in mice, rats, beagle dogs and cynomolgus monkeys at different intravenous and oral doses. Mice were given an IV bolus dose of 3.1 or 8.0 mg/kg and p.o. dose of 3.1, 11.2 or 31.1 mg/kg. Rats were given an IV bolus injection of 3.7 mg/kg and p.o. dose of 12.5, 25 and 50 mg/kg. Beagle dogs were given a single IV bolus dose of 1 or 3 mg/kg and oral dose of 1, 3 and5 mg/kg. Cynomolgus monkeys received single IV bolus injection of 0.77 mg/kg and p.o. dose of 9.3 mg/kg respectively [247]. The distribution of DuP 734 in plasma was characterized by high total body systemic clearance ranging between 46 to 87 mL/min/kg and large steady state volume of distribution (3.6 to 6.8 L/kg). The terminal elimination half-life

was in the range of 50 to 83 min. DuP 734 was found to have extensive distribution in all species, a desirable pharmacokinetic property for a compound that acts on the central nervous system. This compound exhibited rapid gastrointestinal absorption from an aqueous solution in mice and rats with peak plasma concentrations achieved within 5 and 20 min following administration. The peak plasma concentrations were attained within 45 and 130 min in dogs and monkeys. DuP 734 was also found to have a high systemic clearance, indicating that it will be susceptible to hepatic first pass elimination upon oral dosing. This assumption was based on the understanding that the blood to plasma distribution ratio of DuP 734 approximates unity. Studies conducted by infusing DuP 734 directly into the portal vein of rats [248] reported that approximately 94.5% of drug delivered to the liver via portal route was eliminated and only 6.5% reached the systemic circulation. This may be the underlying cause for low oral bioavailability of DuP 734 in all the species. Low bioavailability at lower doses in mice, rat, dog and monkeys was observed which could be attributed to one of more processes including incomplete absorption from gastrointestinal tract, chemical degradation in the gastrointestinal tract, metabolism by the intestinal flora and/or gut wall and/or hepatic first-pass metabolism [247].

Dextromethorphan (DXM), a sigma receptor agonist and an NMDA receptor antagonist, has long been examined for its ability to attenuate acute opiate and ethanol withdrawal [68-69, 158-159]. The interest in DXM relates to its CNS pharmacological properties including the binding to high and low affinity binding sites in the brain [160, 249-252]. There isn't enough information on the pharmacological mechanism of DXM abuse in humans. However; several studies reported that its metabolite, dextrorphan, is responsible for its phencyclidine (PCP)-like behavioral effects [253-254]. When injected to rats at a dose of 30 mg/kg intraperitoneally (i.p.), the bioavailability

of DXM was 1.3 fold lower and the formation of dextrorphan and other metabolites was 3 fold greater as compared to the same dose given subcutaneously [255]. Wills et al. reported that in comparison to the IV route, the absolute bioavailability of DXM in the rat was 25% after i.p administration and 10% after p.o. administration [256]. These findings clearly indicate that DXM undergoes extensive hepatic first pass metabolism. In considering pharmacokinetic studies, it is therefore prudent to understand the effect of route of administration on disposition of the drug and its metabolites in addition to many other factors such as dosage and time of testing.

2.4 Relevance of Pharmacokinetics and Metabolism in furthering of SN79

In 1988, Sharkey et al. reported cocaine's affinity to sigma receptors [80]. Based on the hypothesis that blocking sigma receptors can help attenuate cocaine related toxicity and addiction, researchers have focused their attention in developing highly selective sigma-1 and sigma-2 ligands. In this view, as mentioned in Section 1.10, SN79, preferring sigma-2 receptor antagonist was synthesized by Dr. Christopher R. McCurdy's group from the Department of Medicinal Chemistry at the University of Mississippi. In comparison to the research conducted on sigma-1 subtype antagonists, work indicating involvement of sigma-2 receptors in cocaine-induced effects are nominal. This is attributed to the fact that sigma-2 receptors have not yet been cloned and there are no antisense oligonucleotides to reduce their number. For reasons mentioned in Section 2.5, extensive pharmacokinetic and pharmcodynamic evaluation of SN79 in laboratory animals will help define accurately the biological role of sigma-2 receptors in attenuating cocaine-induced toxicity and addiction before furthering into clinical studies.

CHAPTER III: BIOANALYTICAL METHOD DEVELOPMENT AND VALIDATION USING ULTRA PERFORMANCE LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY

3.1 Introduction

There is an emerging opportunity to surpass the rate of sample generation with the rate of sample analysis, considering the high number of compounds generated during the drug discovery stage. In order to narrow the timeframe for analysis, traditional analytical approaches need to be adjusted keeping in mind the advances in sample generation. Rapid, high throughput, sensitive and selective methods are warranted for pharmaceutical analysis [257]. Breakthrough advances have been made in analytical methods used to expedite the drug development process allowing for the detection of trace elements/metabolites at the nanogram and pictogram per liter range. A significant number of analytical methods based on liquid chromatography tandem mass spectrometry (UPLC^{TM}) enable the detection of pharmaceutical compounds in different biological matrices such as plasma, serum, urine and feces [258]. LC/MS techniques are applicable to a wide range of pharmaceutical compounds and are characterized by powerful analytical figures of merit such as selectivity, sensitivity, speed of analysis and cost effectiveness. The idea that UPLC/MS is now frequently used in every stage of drug development exemplifies its acceptance. In view of the widened scope of applications that UPLC/MS technique offers in addition to the afore-mentioned figures of merit, we employed this tool for most of the analytical evaluations mentioned in this dissertation.

3.2 Bio-analytical Method Development and Validation

The Food and Drug Administration (FDA) provides validation guidelines [259] to be followed for bio-analytical procedures such as gas chromatography (GC), high-pressure liquid chromatography (LC) and other hyphenated LC mass spectrometric procedures. Based on these recommendations, we developed a rapid, sensitive and selective UPLC/MS method for quantization of SN79 in rat plasma.

3.3 Chemicals and reagents

HPLC grade acetonitrile, methanol, chloroform, and water were from Fisher (Fair lawn, NJ, USA). Reagent grade trifluoroacetic acid was purchased from Sigma-Aldrich (95%, St. Louis, MO, USA). Drug-free rat plasma was bought from Innovative Research Inc. (Southfield, MI, USA). Rat liver microsomes were purchased from CellzDirect Inc. (Durham, NC, USA). Sodium chloride (0.9% w/v) was purchased from Abbott Laboratories (North Chicago, IL, USA). SN79 and CM156 were supplied by Dr. Christopher R. McCurdy's group from the Department of Medicinal Chemistry at the University of Mississippi. The purity of the compounds was proved to be over 98% by H^1-NMR and C^{13}-NMR.

3.4 Liquid chromatography and Mass spectrometry

3.4.1 *SN79*

A Waters Acquity UPLC system was used (Milford, MA, USA). Chromatographic separations were performed on a Waters Acquity UPLC™ BEH C_{18} column (1.7 µm, 2.1 × 50 mm). Collision gas used in mass spectrophotometer was argon while nitrogen was used as desolvation and cone gas for mass spectrophotometer. The mobile phase consisted of acetonitrile: methanol

(95:5) and 0.3% trifluoroacetic acid (50:50, v/v). The flow rate was held at 0.2 mL/min, the sample injection volume was 10 µL, and the duration of the run was 2 min.

The mass spectrometry equipment consisted of a Waters Micromass Quattro Micro™ triple-quadrupole system (Manchester, UK). The mass spec system was controlled by version 4.0 Mass Lynx software. Ionization was performed in the positive electro spray mode. Mass spec conditions were the following: capillary voltage 4.69 kV, cone voltage 51V, extractor voltage 4V, RF lens voltage 0.3V. The source and desolvation temperatures were maintained at 120°C and 450 °C, and the desolvation and cone gas flows were held at 380.2 and 95.8 L/hr, respectively. The selected mass-to-charge (m/z) ratio transition of SN79 $[M+H]^+$ used in the single ion reaction (SIR) was m/z: 412.3, respectively. The dwell time was set at 500 ms. The mass scan of SN79 is shown in Figure 3.1.

Figure 3.1 Mass scan of SN79

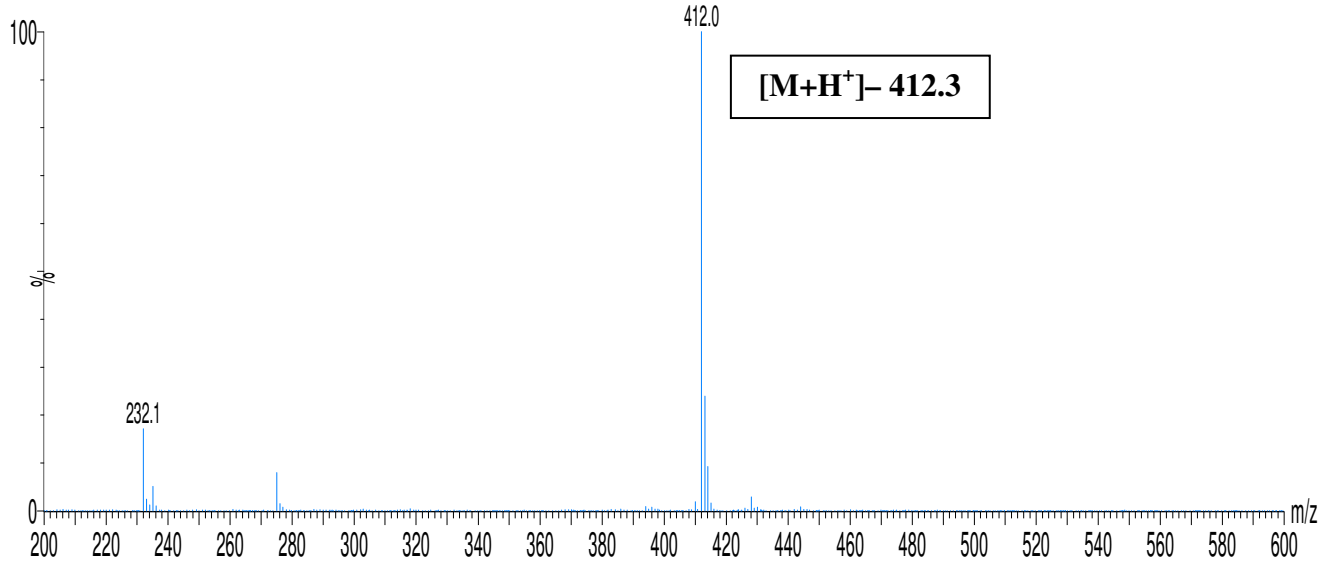


Figure 3.2 Test for specificity

Chromatogram of blank rat plasma

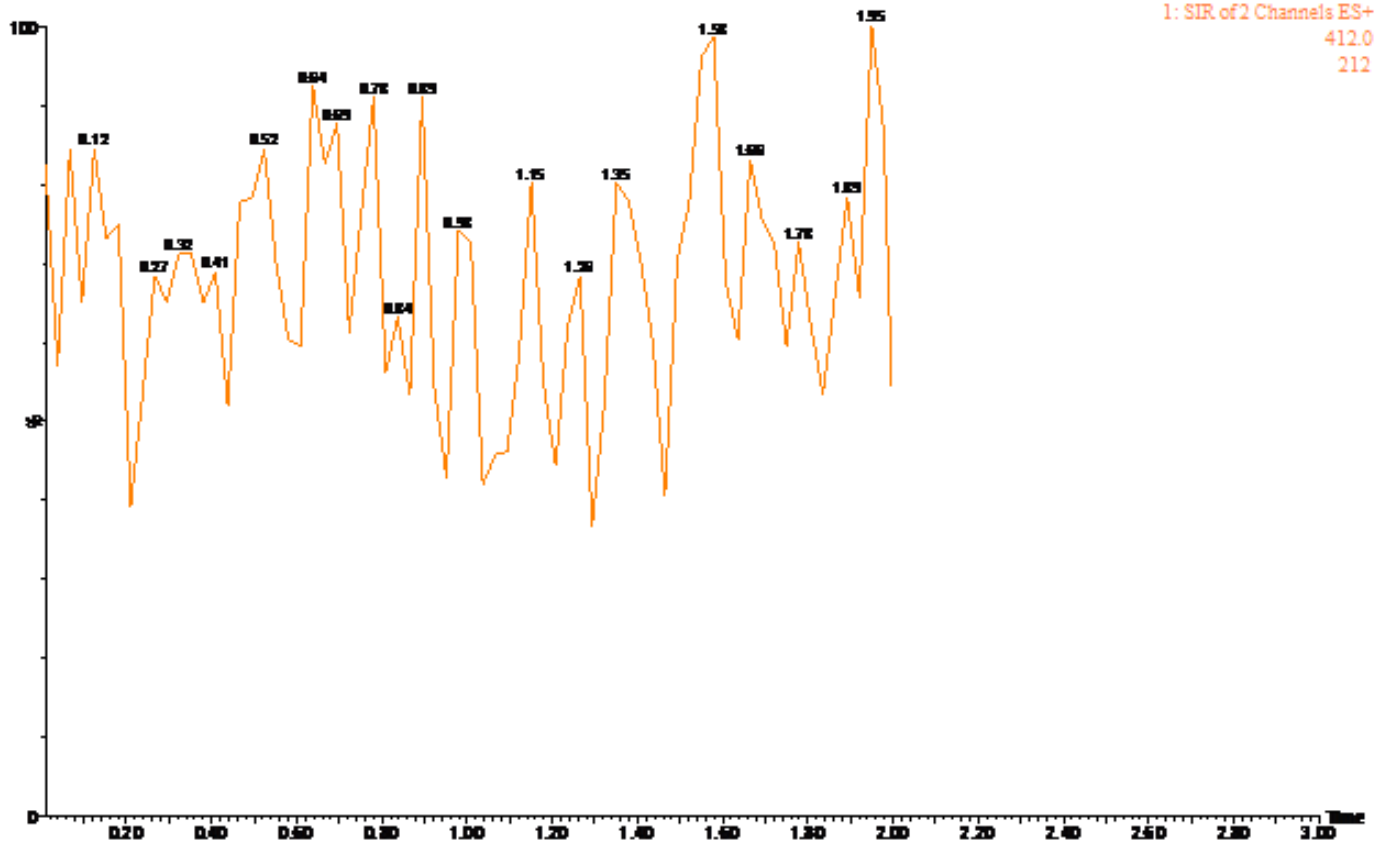
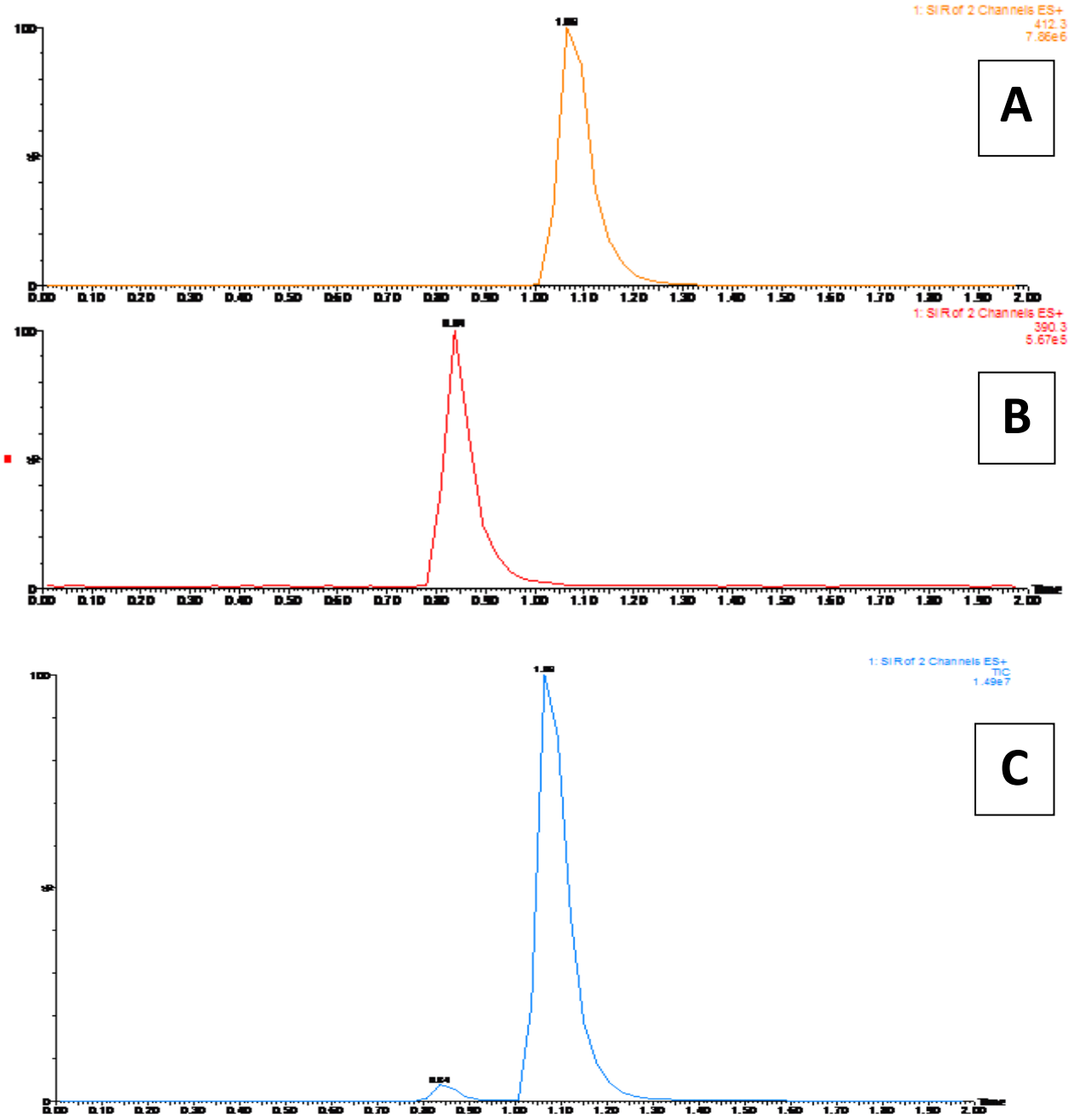


Figure 3.3 Chromatograms of SN79 and rat plasma spiked with SN79



A- Blank plasma spiked with SN79.

B- Blank plasma spiked with CM156 (internal standard).

C- Plasma sample collected from rat 3 hours after oral administration of SN79 spiked with CM156.

3.4.2 Internal Standard

An internal standard in analytical chemistry **is** a chemical substance of known amount, different from the analyte, which is added to the unknown sample. The internal standard must provide a signal that is similar to the analyte signal but sufficiently different so that the two signals are readily distinguishable by the instrument. Peak area of the analyte is compared to peak area of the internal standard to calculate the amount of analyte as a function of the analyte standard concentration. This ratio is used to obtain analyte concentrations from a calibration curve. provides numerous advantages in liquid chromatography/tandem mass spectrometric bioanalytical techniques such as reduction of the analysis run time, improvement in the intra-injection reproducibility, impact reduction of matrix and ionization effects [260]. Internal standards are particularly useful when you have considerable "sample clean-up" to do. Use of an IS will prevent sample to sample fluctuations in extraction efficiency from ruining your precision and accuracy. The internal standard acts as a carrier for low concentrations of the analyte such that it can determine the lower limit of detection for every sample. The addition of IS corrects for analyte losses during sample preparation [261].

An external standard is similar to the internal standard (known behavior), but is not added to the unknown sample. It is run alone, as a sample, and usually at different concentrations, to generate a standard curve. Again, the peak areas are related to the known amounts of external standard run. Unlike internal standards, external standards do not correct for losses that may occur during preparation of the sample, such as extraction, centrifugation, evaporation, etc.

3.5 Method validation in Rat Plasma

Bio-analytical method validation must be performed to ensure that the analytical procedures developed generate reproducible and reliable data in order to correctly interpret preclinical data such as bioavailability and bioequivalence from pharmacokinetic, pharmacology and toxicology studies. The validation process establishes the robustness of analytical methods when transferred between labs and/or analysts. Full validation is necessary for all new chemical entities. The validation of a UPLC/MS method includes linearity, selectivity, inter- and intra-assay precision and accuracy, stability, recovery and ion suppression studies.

3.5.1 Standards preparation

SN79-*di*-hydrochloride was used to prepare the standard solutions. To prepare a stock solution with a concentration of 1 mg/ml of SN79, 1.18 mg of SN79-*di*-hydrochloride was dissolved in 1 mL of HPLC grade water. Further dilutions were made using HPLC grade water to get final concentrations of 50, 100, 150, 500, 1,000, 2,000, 5,000, 10,000, 30,000 and 50,000 ng/ml. CM156 (internal standard) was prepared at a concentration of 10 µg/ml in HPLC grade water. To prepare the standards, 80 µL of blank rat plasma was taken, to which 10 µL each of standard stock solution was added to get final concentrations of 5, 10, 15, 50, 100, 200, 500, 1,000, 3,000 and 5000 ng/ml. To these standard solutions, 10 µL of the internal standard (10µg/ml) was added to get a final volume of 100 µL. Chloroform was used as the extraction solvent. Five hundred micro-liter of chloroform was added to these samples and the micro-centrifuge tubes were vortexed for 10 minutes on VWR Signature® pulsing vortex mixer (VWR Scientific Inc., U.S.A). The samples were centrifuged at 3000 rpm for 20 minutes using an Accuspin Micro 17R centrifuge (Fisher Scientific, U.S.A). The organic phase (400 µL) was transferred into 1.5 mL

eppendorf tubes (Fisher Scientific, U.S.A) and dried in a vacuum oven (Precision, Jouan. Inc, USA) at 25 ^{o}C. To the dried samples, 100 µL of mobile phase was added and vortex mixed for 2 minutes. The mobile phase consisted of acetonitrile: methanol (95:5) and 0.3% trifluoroacetic acid (50:50, v/v). All the samples were filtered with a 0.2 µm syringe filter (Waters 13 mm GHP 0.2 µm) and analyzed using the UPLC/MS.

Quality Control (QC) samples were prepared separately at concentrations of 5, 15, 200 and 3000 ng/ml to assess accuracy and precision of the analytical method. All calibration and QC samples were extracted with the method described in the subsequent section and stored at -20 °C (Kenmore, USA) until they were analyzed.

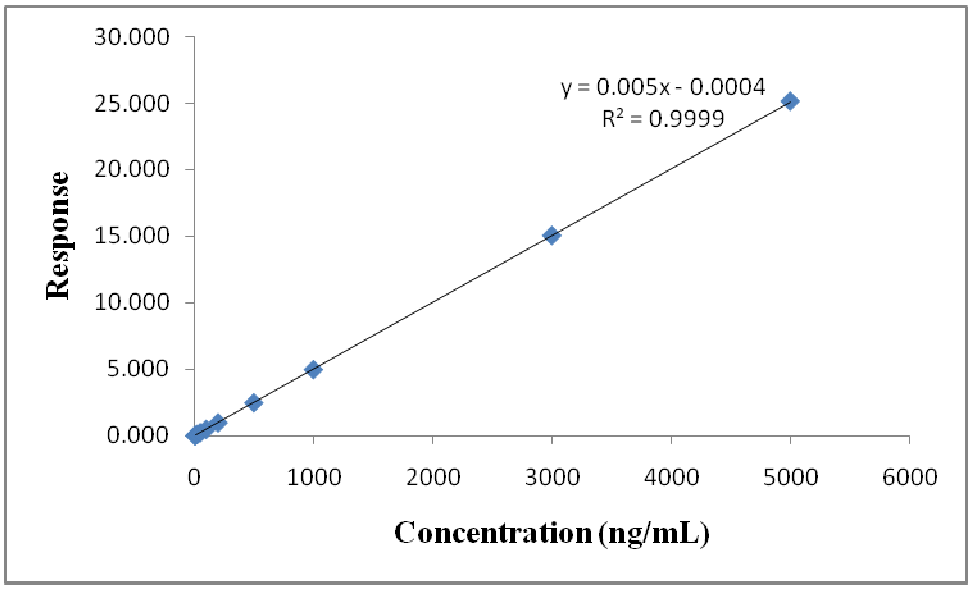


Figure 3.4 Calibration curve of SN79 extracted from rat plasma

3.5.2 Selectivity

It is important to show that a chromatographic procedure is selective for the analyte. It should be capable of differentiating and quantifying the analyte in presence of potential matrix components from plasma proteins, urine etc. Each blank sample should be checked for interference and selectivity must be established at the lower limit of quantification (LLOQ).

The selectivity of the method was evaluated by comparing the chromatograms of a pool of six different batches of blank rat plasma samples with the corresponding spiked plasma samples to make sure there were no significant interfering peaks at the retention times of SN79 and IS at lower limit of quantification.

3.5.3 Linearity and lower limit of quantification (LLOQ)

Calibration curves were calculated based on the relationship between the ratio of the peak area of SN79 to that of the internal standard and the theoretical concentration of analyte. The calibration data was processed with MassLynx 4.0 QuanLynx software. The lower limit of quantification was defined as the concentration of SN79 at which the response of SN79wass ten times the response compared to the blank response.

3.5.4 Accuracy and precision

For all assays, accuracy of the results is vital [262]. Accuracy may be improved by use of replicate sample analysis. Accuracy is defined as closeness of the mean test results to the true concentration of the analyte. The precision of an analytical method is a measure of closeness of individual test results of an analyte.

Intra-day accuracy and precision were evaluated by analysis of the four QC samples with six determinations per concentration in the same day. The inter-day accuracy and precision was measured over three days. Precision was measured by inter- and intra-assay % R.S.D.

Recovery, % RE = (Area_of_pre_extraction_spike / Area_of_post_extraction_spike)*100 [2]

The accuracy was evaluated by the deviation or bias (%) of the observed concentration from the actual concentration.

3.5.5 Recovery and matrix effects

Recovery experiments were conducted as described by Z. Li. Et al. [263]. Rat plasma samples were spiked with SN79 at three final concentration levels (3,000, 200, and 15 ng/mL) and extracted in the same manner as the plasma study samples (n = 5) in Section 3.5.1. Then 10 µL (10µg/mL) of I.S. was added to the extracts and the sample was injected into the UPLC–MS system. The peak-area ratio of SN79 to I.S. was calculated. Standard mixtures of SN79 and I.S. were made in mobile phase at concentrations same as those in the rat plasma. These standards were directly injected into the UPLC–MS system and the peak-area ratio of SN79 to the I.S. was calculated. The recovery of SN79 was evaluated by comparing the peak-area ratios of SN79: I.S. in the rat plasma extracts to that of the standard mixtures. The matrix effect on the ionization of the analytes was evaluated by comparing the peak areas of the analytes dissolved in the blank sample (the final solution of blank plasma after extraction and dissolution) with that dissolved in the mobile phase. Three different concentration levels of SN79 (3,000, 200, and 15 ng/mL) and the I.S. were evaluated by analyzing six samples at each level. The blank plasmas used in this

study were six different batches of blank rat plasmas. If the ratio is <85% or >115%, an exogenous matrix effect is implied. [50]. Matrix effect was calculated as follows:

Matrix effect, %ME = (Area_of_post_extraction_sample/Area_of_standard)*100 [2]

3.5.6 Stability

Anticipating the actual storage conditions the samples might encounter, the stability of the analyte in the matrix should be evaluated after long term (frozen at intended storage temperature) and short-term storage and upon subjecting to freeze thaw cycles. The QC samples (n=6) at four final concentrations (3,000, 200, 15, and 5 ng/mL) and a volume of 1 mL were stored in micro-centrifuge tubes and used to study stability.

For freeze-thaw stability, unprocessed QC samples were subjected to three freeze-thaw cycles. Each cycle lasts for 24 h. At the end of each cycle, the samples were processed, analyzed and compared with the freshly prepared QC samples. When completely thawed, the samples were refrozen for 12–24 h under the same conditions. After three cycles, the percent loss of the analyte was determined by comparing the concentrations with those obtained before freezing.

For the short-term stability test, the QC samples at the same four concentrations were thawed at room temperature and kept at this temperature from 4 to 24 h which exceeds the routine preparation time of the samples. At different time points, the samples were processed, analyzed and compared with the freshly prepared QC samples.

Long term stability is studied to determine how long samples can be stored at −20 °C (Kenmore, USA) before they undergo degradation. This data is helpful while storing pharmacokinetic study samples before they are analyzed. The duration of this study was set as 30 days to reflect the possible time it might take before analyzing samples collected during pharmacokinetic studies. QC samples were stored at −20 °C (Kenmore, USA) for 30 days. The samples were then processed, analyzed and compared with the freshly prepared QC samples.

3.6 Results

3.6.1 Method Validation in Rat Plasma

3.6.1.1 Selectivity

UPLC/MS analysis of the plasma samples showed no endogenous peak interference with the quantification of SN79 and the internal standard. Representative chromatograms of blank rat plasma spiked with SN79 and internal standard, blank plasma and extracted plasma samples are shown in Figure 3.2 and 3.3.**3.6.1.2 Linearity and lower limit of quantification**

A calibration curve was constructed based on the working standard SN79 solutions at 10 concentrations in the range of 5-5000 ng/mL (Table 3.1) The ratio of peak areas of SN79 to that of the IS was used for the quantification of SN79. Linear regression was applied to the calibration curve to determine the correlation coefficient, r^2. The calibration curve (Figure 2.4) was determined to be linear over the concentration range from 5 to 5000 ng/mLwith a correlation coefficient of 0.9999, indicating good linearity. The calibration/ linear equation was $Y = 0.005X - 0.00041$. Each standard point was back calculated with the calibration. The non-zero standards showed less than 20% deviation at the LLOQ and less than 15% deviation at all other

concentration levels. The calibration range was selected according to the concentrations anticipated in the samples to be determined. The final calibration range was between 5–5,000 ng/mL. The lower limit of quantification was 5 ng /mL. Calibration curve and data are presented in Figure 3.4 and Table 3.1.

3.6.1.3 Accuracy and precision

The results of the accuracy and precision tests at the four concentrations are presented in Table 3.2 and Table 3.3 respectively. The QC test concentrations measured after analysis using UPLC/MS were compared against true concentrations using a calibration curve constructed on the same day. For inter-day accuracy and precision, the same procedure as above was repeated for three consecutive days. Accuracy was measured in terms of % recovery and should not deviate from the mean value by more than 20% at LLOQ and 15% at all other concentrations. Similarly, precision values measured as % relative standard deviation (RSD) should not exceed 20% for LLOQ and 15% at all other concentrations. Intraday precision values for LLOQ, low QC, mid QC and high QC were found to be 8.71%, 5.44 %, 0.42% and 3.61%. These values are well within the above-mentioned acceptance criteria. Intra-day accuracy values were in the range of 93.7 – 101.82%. For inter-day, precision values for LLOQ, low QC, mid QC and high QC were found to be 2.32%, 1.86%, 1.23% and 1.53%. These results met the acceptance criteria satisfactorily. Inter-day accuracy values ranged between 97.05-101.25%.

3.6.1.4 Recovery and matrix effect

The mean recoveries of this method were 96.18 ± 2.38% at 3,000ng/ml, 94.41 ± 2.28% at 200ng/ml and 95.22 ± 7.94% at 15ng/ml. ANOVA was used as a statistical tool to compare the recovery values among three different concentrations. The results indicated that there were no significant differences ($p<0.05$) in the extraction recoveries at different concentration levels. Recovery values of SN79 from rat plasma were listed in Table-3.4.

A matrix effect is defined as the direct or indirect alteration or interference in response due to the presence of unintended analytes or other interfering substances in the sample. It could be a severe problem when developing LC/MS assays. In our experiment, the matrix effect of the studied plasma was calculated using ratio of the response of spiked samples after extraction over the response of the corresponding mobile phase samples. The matrix effect was found to be -3.82± 2.38% at 3,000 ng/ml, -5.59 ± 2.28% at 200ng/ml and -4.78 ± 7.94% at 15ng/ml. A matrix effect less than 15% is acceptable [50, 52]. Therefore, the matrix effects from endogenous plasma components on the ionization of the analytes were negligible. The chromatographic parameters were optimized in such a way that the analyte and the internal standard were eluted at different times. The absolute peak area of CM156 was used as a marker to monitor possible suppression effects with study samples.

3.6.1.5 Stability

The results of freeze-thaw, short-term and long term stabilities are presented in Table 3.5, Table 3.6 and Table 3.7. SN79 was found to be stable after three freeze-thaw circles at four different concentrations. SN79 in rat plasma at four QC concentrations stored at room temperature was found to be stable for at least 8 h. The mean recoveries from the nominal concentrations were between 70 and 100% at three different concentrations.

FIGURES AND TABLES

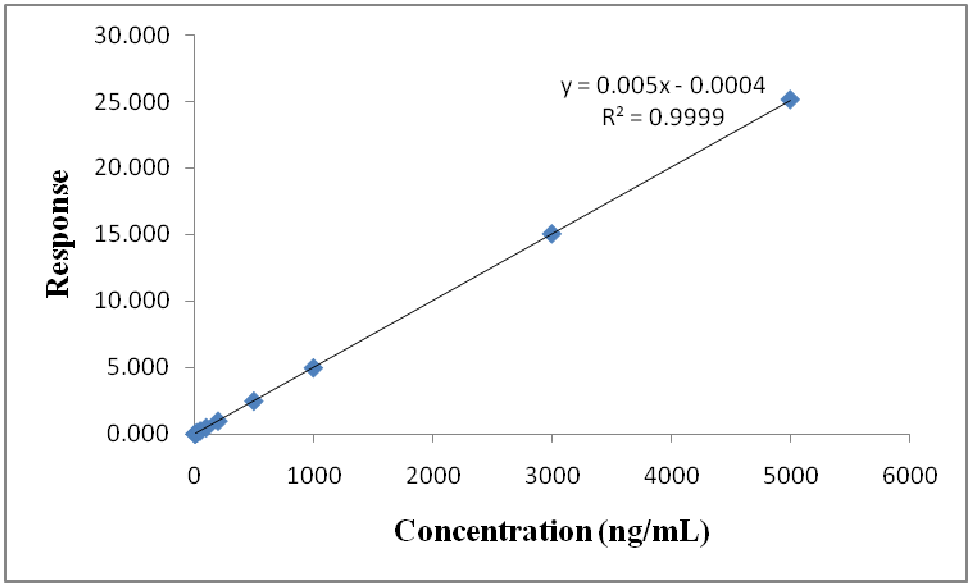


Figure 3.4 Calibration curve of SN79 extracted from rat plasma

Table 3.1 Calibration curve data for SN79 extracted from rat plasma

#	Conc (ng/mL)	RT (min)	Area	Response	Measured Conc (ng/mL)
1	**5**	**1.06**	1404	0.024	4.91
2	**10**	**1.06**	3146	0.050	10.03
3	**25**	**1.06**	8850	0.124	24.96
4	**50**	**1.06**	14790	0.249	49.94
5	**100**	**1.06**	29759	0.499	99.92
6	**200**	**1.06**	57431	0.996	199.27
7	**500**	**1.06**	137681	2.491	498.26
8	**1000**	**1.06**	307714	4.983	996.77
9	**3000**	**1.06**	1023848	15.044	3008.85
10	**5000**	**1.06**	1696067	25.111	5022.24

Table 3.2 Intra-day precision and accuracy of SN79 in rat plasma

Time (hr)	Concentration (ng/mL)			
	3000	**200**	**15**	**5**
0	3098.94	200.05	15.07	4.94
4	2852.13	199.09	15.34	4.80
6	3041.68	200.06	14.34	4.08
8	3049.09	201.15	16.34	4.92
Mean	3010.46	200.09	15.27	4.69
S.D.	1.08	0.84	0.83	0.41
% RSD	3.61	0.42	5.44	8.71
Recovery	100.35	100.04	101.82	93.7

RSD – standard deviation/mean;

Recovery = 1-(nominal concentration-measured concentration)/nominal

Table 3.3 Inter-day precision and accuracy of SN79 in rat plasma

DAY	Concentration (ng/mL)			
	3000	**200**	**15**	**5**
1	3007.23	204.00	14.04	4.95
2	3083.10	201.26	14.42	5.18
3	2998.94	199.05	15.07	5.05
Mean	3029.76	201.44	14.51	5.06
S.D.	4.64	2.48	0.27	0.12
% RSD	1.53	1.23	1.86	2.32
Recovery	100.99	100.63	97.05	101.25

RSD – standard deviation/mean;

Recovery = 1-(nominal concentration-measured concentration)/nominal

Table 3.4 Recovery values of SN79 from rat plasma (n=3)

Actual Concentration (ng/mL)	Measured Concentration (ng/mL)	Mean	S.D.	% RSD	Recovery
3000	2912	2885	2.38	2.48	96.18
	2804				
	2940				
200	193	189	2.28	2.41	94.41
	189				
	184				
15	14	14	7.94	8.34	95.22
	14				
	16				

SD – standard deviation; RSD – standard deviation/mean;

Table 3.5 Stability of SN79 in rat plasma after three freeze thaw cycles

Concentration (ng/mL)	Recovery (%) ± S.D.	R.S.D. (%)
3000	94.45±2.73	2.89
200	98.28±8.36	8.50
15	103.16±1.28	1.24
5	102.33±1.27	1.25

SD – standard deviation; RSD – standard deviation/mean;

Table 3.6 Short term stability of SN79 in rat plasma

Time (hr)	Concentration (ng/mL)							
	3000		200		15		5	
	Recovery (%) ± S.D.	R.S.D. (%)	Recovery (%) ± S.D.	R.S.D. (%)	Recovery (%) ± S.D.	R.S.D. (%)	Recovery (%) ± S.D.	R.S.D. (%)
4	101.10 ± 8.05	7.96	96.29 ± 8.53	8.86	92.82 ± 8.61	9.27	90.06 ± 3.09	3.43
6	80.46 ± 3.20	3.98	83.87 ± 2.66	3.17	88.88 ± 3.38	3.80	86.38 ± 1.23	1.42
8	80.75 ± 8.03	9.94	83.42 ± 3.70	4.44	83.93 ± 1.06	1.27	82.56 ± 6.17	1.34

SD – standard deviation; RSD – standard deviation/mean;

Table 3.7 Long term stability of SN79 in rat plasma after 30 days

Concentration (ng/mL)	Recovery (%) ± S.D.	R.S.D. (%)
3000	88.36 ± 0.83	0.94
200	92.78 ± 2.57	2.77
15	91.60 ± 2.98	3.26
5	92.53 ± 1.87	1.73

SD – standard deviation; RSD – standard deviation/mean;

CHAPTER IV: PHARMACOKINETICS OF SN79 IN RATS

4.1 Experimental

4.1.1 Materials

HPLC grade acetonitrile, methanol, chloroform, water, monobasic potassium phosphate, potassium chloride, sodium hydroxide, hydrochloric acid were purchased from Fisher (Fair lawn, NJ, USA). Reagent grade trifluoroacetic acid, pepsin (derived from porcine stomach mucosa, 800-2500 units/mg protein), pancreatin, potassium biphthalate, boric acid, ammonium acetate, nicotinamide adenine dinucleotide phosphate, glucose-6-phosphate dehydrogenase, glucose-6-phosphate and magnesium chloride were purchased from Sigma-Aldrich (95%, St. Louis, MO, USA). Drug-free rat plasma was bought from Innovative Research Inc. (Southfield, MI, USA). Rat liver microsomes were purchased from CellzDirect Inc. (Durham, NC, USA). Sodium chloride (0.9% w/v) was purchased from Abbott Laboratories (North Chicago, IL, USA). SN79 and CM156 were supplied by Dr. Christopher R. McCurdy's group from the Department of Medicinal Chemistry at the University of Mississippi. The purity of the compounds was proved to be over 98% by H^1-NMR and C^{13}-NMR.

Collision gas used in mass spectrophotometer was argon supplied by nexAir (Memphis, TN, USA). Nitrogen was used as desolvation and cone gas for mass spectrophotometer. Nitrogen generator used in our laboratory is from Peak Scientific (Billerica, MA, USA).

Other equipment used for sample processing includes Accuspin Micro 17R centrifuge (Fisher Scientific, U.S.A), VWR Signature® pulsing vortex mixer (VWR Scientific Inc., U.S.A), vacuum oven (Precision, Jouan. Inc, USA) and Kenmore (USA) refrigerator.

4.2 Liquid chromatography and Mass spectrometry

SN79

A Waters Acquity UPLC system was used (Milford, MA, USA). Chromatographic separations were performed on a Waters Acquity UPLC™ BEH C_{18} column (1.7 μm, 2.1 × 50 mm). The mobile phase consisted of acetonitrile: methanol (95:5) and 0.3% trifluoroacetic acid (50:50, v/v). The flow rate was 0.2 mL/min, the sample injection volume was 10 μL, and the duration of the run was 2 min.

The mass spectrometry equipment consisted of a Waters Micromass Quattro Micro™ triple-quadrupole system (Manchester, UK). The mass spec system was controlled by version 4.0 of Mass Lynx software. Ionization was performed in the positive electro spray mode. Mass spec conditions were the following: capillary voltage 4.69 kV, cone voltage 51V, extractor voltage 4V, RF lens voltage 0.3V. The source and desolvation temperatures were maintained at 120°C and 450 °C, respectively, and the desolvation and cone gas (nitrogen) flows were held at 380.2 and 95.8 L/hr, respectively. The selected mass-to-charge (m/z) ratio transition of SN79 $[M+H]^+$ used in the single ion reaction (SIR) was m/z: 412.3, respectively. The dwell time was set at 500 ms.

4.3 Physicochemical parameters

As mentioned in Section 2.3 of Chapter 2, close scrutiny of the physicochemical parameters may help predict oral bioavailability of a new chemical entity prior to the commencement of

laboratory experiments. Efficient oral absorption is governed by the ability of the drug to dissolve completely and become available to the intestinal mucosal surface to traverse the epithelium. Dissolution is determined by the highly interdependent influences of aqueous solubility, ionizability (pKa), and lipophilicity (octanol/water log P or log $D_{7.4}$). In order for a drug to be distributed to its site of action, it must cross at least one or more membranes. Owing to the largely lipophilic nature of the membrane, most of the drugs cross the membrane by virtue of diffusion, thereby making lipophilicity of the drug molecule a primary determinant of the rate of absorption. To enhance the ability of the drug to traverse both aqueous and lipid environments of the membrane, an understanding of the relative affinity of the drug for both phases is useful in predicting whether or not the drug will be absorbed across the blood–brain barrier. For this purpose, the partitioning of a drug between water and octanol at a constant temperature is studied to give the partition coefficient (P) or log P. Likewise, log P is a crucial determinant of passive membrane partitioning, influencing permeability opposite to its effect on solubility (i.e. higher log P value increases permeability while reducing solubility). In light of this proportionality, a lop P value in the range of 0.5 to 2.0 is recommended for oral absorption [259].

Certain other characteristic properties of the drug such as degree of ionization also play a crucial role in determining its ability to penetrate membranes. Ionized molecules possess an electrostatic charge, thereby dissolve preferentially in water because they tend to associate with a similarly charged environment [264]. Alternately, un-ionized molecules have no net charge or polarity and thus are lipophilic. Also, the pH of the environment is instrumental in determining the ability of a drug to diffuse into and across lipid membranes. The effect of pKa in oral absorption is correlated to the pH dependence of drug permeability across membrane barriers and that of drug solubility in luminal fluid.

4.3.1 cLog P and pKa determination

The cLog P value was calculated with ACD labs software. The pKa and cp$K_a$values of the compound were determined by potentiometric titration method and ACD labs software. The conventional titration technique was used to determine the pKa value of SN79. A solution of sodium hydroxide at a concentration of 0.01M was prepared and pH measured using a Mettler Toledo SevenEasy^{TM}pH meter S20 as 11.81. Similarly, 0.01M hydrochloric acid was prepared and the pH measured as 2.13. A solution of SN79 was prepared by adding 0.31 mg of the compound to 50 mL of water. The pH of the drug solution was measured at a value of 5.62 at room temperature (28.9 ^{0}C). The solution was ultra-sonicated for 10 minutes .To the SN79 drug solution, 0.1mL portions of sodium hydroxide were added and pH recorded (Mettler Toledo SevenEasy^{TM}pH meter S20) until it became relatively constant. Subsequently, 0.1 mL portions of hydrochloric acid were added and pH recorded till it became constant. A titration curve was plotted as pH versus volume of base/acid added. The intersection point of these two curves is noted as the pKa value of the compound.

4.3.2 Aqueous and pH dependent Solubility

Solubility of SN79 was calculated in water (pH-6.98) and in buffers at pH 1.2, 3.0, 5.0, 7.4 and 9.0 using shaking flask method [265] and ACD labs software. Excess compound was added to the 0.5 mL of solvent and placed in glass vials and capped. Presence of excess compound was established by visual examination. The samples were placed in a shaking water bath (Fisher Scientific, USA) at 100 rpm (at 25ºC). Samples were analyzed after 24 and 48 hr. Calibration curve was run using fresh standard solutions of SN79. The calibration/ linear equation was used

to measure the amount of SN79 present in the solubility samples to calculate solubility. Buffers were prepared according to protocol mentioned in the US Pharmacopeia.

- Buffer of pH-1.2 was prepared by taking 12.5 mL of 0.2M potassium chloride solution and 21.25 mL of 0.2M hydrochloride solution in a 50 mL volumetric flask. Water was added to bring up the volume to 50 mL.
- Buffer of pH-3.0 was prepared by taking 12.5 mL of 0.2M potassium biphthalate solution and 5.575 mL of 0.2M hydrochloride solution in a 50 mL volumetric flask. Water was added to bring up the volume to 50 mL.
- Buffer of pH-5.0 was prepared by taking 12.5 mL of 0.2M potassium biphthalate solution and 5.65 mL of 0.2M sodium hydroxide solution in a 50 mL volumetric flask. Water was added to bring up the volume to 50 mL.
- Buffer of pH-7.4 was prepared by taking 12.5 mL of 0.2M potassium phosphate, monobasic solution and 9.775 mL of 0.2M sodium hydroxide solution in a 50 mL volumetric flask. Water was added to bring up the volume to 50 mL.
- Buffer of pH-9.0 was prepared by taking 12.5 mL of 0.2M boric acid and potassium chloride solution and 5.20 mL of 0.2M sodium hydroxide solution in a 50mL volumetric flask. Water was added to bring up the volume to 50 mL.

4.4 pH dependent Stability

The stabiliy of SN79 was estimated in various buffers in the pH range of 1.2- 9.0 at -20ºC (Kenmore Refrigerator, USA), 25ºC and 37ºC (Fisher Scientific, USA). The compound was dissolved in HPLC water to obtain a known concentration of 1mg/mL. From this solution, 100ug/mL and 10ug/mL solutions were made in water. A hundred microliter sample of 10ug/mL solution was added to 0.9mL of the buffers in triplicate and placed at -20ºC, 25ºC and 37ºC. The

stability samples were analyzed on zero day and after 24 hr, 48 hr, 7 days, 15 days, 30 days and 45 days. Calibration curve was run using fresh standard solutions of SN79 on each day of analysis. The calibration/ linear equation was used to measure the amount of SN79 present in the stability samples to calculate percent remaining of the compound. Buffers were prepared according as mentioned in Section 4.3.2.

4.5 Stability in Stimulated Gastric and Intestinal Fluids

The stability of the compound in simulated gastric and intestinal fluids helps us better understand drug plasma levels in the gastrointestinal tract and the role of hepatic metabolism. The stability of SN79 was evaluated in stimulated gastric (SGF) and intestinal fluids (SIF). The compound was dissolved in water to obtain a known concentration of 1mg/mL. From this solution, 100ug/mL and 10ug/mL solutions were made in water. A five hundred microliter of 10ug/mL solution was added to 0.5mL of SIF and SGF in capped glass vials and placed at 37ºC in a water bath (Fisher Scientific, USA). Simulated gastric fluid samples were collected at zero min and 60 min. Simulated intestinal fluid samples were collected at zero min, 60 min, 120 min and 180 min. The samples were analyzed on UPLC/MS for SN79 content. The stability of the compound in simulated gastric and intestinal fluids was calculated. Simulated gastric and intestinal fluids were prepared in accordance with the US Pharmacopeia.

- Simulated gastric fluid is prepared by dissolving 0.1g of sodium chloride and 0.16g of purified pepsin (derived from porcine stomach mucosa, 800-2500 units/mg protein) in 0.35mL of hydrochloric acid. The resulting solution is diluted with water to 50mL with a final pH of 1.2.

- Simulated intestinal fluid is prepared by dissolving 0.34g of potassium phosphate, monobasic in 12.5mL of water. To this, 3.85mL of 0.2N sodium hydroxide, 25mL of water and 0.5g of pancreatin are added and pH of the resulting solution is adjusted to 6.8±0.1 using either 0.2N sodium hydroxide or 0.2N hydrochloride solution. The final volume is made up to 50mL using water with a pH of 6.8.

The relative difference (RD) between the amount added and the amount after incubation was calculated as follows [266-267]:

$$RD = [(C_i - C_f) / C_i] \times 100\%$$

Where C_i is the amount of drug at zero time and C_f is the amount after incubation.

Significant difference (>5%) of a drug assessed in this manner could suggest potential instability in the GIT [266].

4.6 *In vitro* Plasma Protein Binding Studies

The plasma protein binding capability of SN79 was studied *in vitro* at specific concentrations in the range of 5-5000 ng/ mL. The desired concentrations were obtained by diluting with rat plasma. The compound was dissolved in water to obtain a known concentration of 1 mg/mL. From this solution, 0.1mL was taken and diluted with 0.9 mL of water to obtain a final concentration of 100 ug/mL solution. This solution was further diluted with rat plasma to obtain solutions with final concentrations of 0.5ug/mL and 1 ug/mL respectively. These plasma samples were placed in Centrifree® devices (Millipore 30,000 NMWL) and incubated at 37ºC for 30 min. The samples were then centrifuged for 10 min at 1000 g and ultrafiltrates were collected. To 100µL of ultrafiltrate, 100 µL of acetonitrile was added along with 10 µL of internal standard

solution (10 µg/mL of CM156). The samples were vortex mixed for 2 min and centrifuged (Accuspin Micro 17R centrifuge, Fisher Scientific, U.S.A) for 10 min. The supernatant collected was filtered using a 0.45 µM filter (Waters 13 mm GHP 0.45 µm) and analyzed using UPLC/MS. Nonspecific binding was determined and incorporated in the calculation of plasma protein binding values.

Percent of free drug concentration was calculated as follows. Percent unbound drug concentration f_u is given by [268]:

$$f_u = [C_f / C_i - C_f] \times 100$$

Where C_i = intial concentration of drug, C_f = final concentration of free drug, f_u = percent unbound drug concentration.

4.7 *In vitro* GIT Permeation Studies

The absorption pattern of SN79 across various segments of GIT was studied *in vitro*. Animals were anesthetized using ketamine and the duodenum, jejunum and ileum were harvested from male Sprague dawley rats and stored in saline. Permeability study was performed using PermeGear diffusion apparatus (Bethlehem, PA). The tissues were cut along the mesenteric line and mounted between standard 3mm side-by-side diffusion cells. Absorption was studied from mucosal to serosal side as well as serosal to mucosal side at 37ºC. The donor and receptor cells were filled with ammonium acetate buffer (pH adjusted to 7.4) while stirring throughout the study with magnetic stir bar. The compound was dissolved in ammonium acetate and added to the donor cell. Permeability was studied at two different concentrations (0.25mg/mL and 0.5mg/mL) in the range of 5-5000ng/ mL. Aliquots of 0.2mL were withdrawn at pre-determined

time points and immediately replaced with equal amount of blank buffer. Samples were analyzed using UPLC/MS.

4.8 *In vitro* Metabolic Stability

Phase-I metabolism stability of SN79 was studied in rat, mouse and human liver microsomes. The total incubation volume was 200 µL. Incubation mixture consisted of liver microsomal protein (1 mg/mL), SN79 (10 µM), 100 mM ammonium acetate buffer (pH adjusted to 7.4) and NaDPH regenerating system. The NaDPH regenerating system was prepared by taking nicotinamide adenine dinucleotide phosphate (1mM, pH-7.4), glucose-6-phosphate (10mM), magnesium chloride (10mM) and glucose-6-phosphate dehydrogenase (2U/mL). The NaDPH reagents were dissolved in 1 mL of 100 mM ammonium acetate buffer in an eppendorf tube. Ammonium acetate buffer was prepared by addition of 385 mg of ammonium acetate to 50 mL of water to attain a final concentration of 100 mM. In clearly marked 20mL wide mouthed vials (Fisher Scientific, FairLawn, NJ, USA), 150µL of buffer, 20µL of drug solution (1µg/mL) and 10µL of liver microsomes were added and equilibrated for 5 min in a water bath (Fisher Scientific, USA) at 37ºC. The addition of 20µL of the NaDPH regenerating system triggered the reaction. Reaction was then terminated at 0, 5, 10, 15, 20, 30, 45, 60 and 90 min with 0.2 mL of ice cold ACN and the samples were then held on the bench until further processing. The samples were centrifuged (Accuspin Micro 17R centrifuge, Fisher Scientific, U.S.A) at 4°C for 15 minutes at 12000 rpm and the supernatant analyzed using UPLC/MS for residual SN79 content. The solvent controls were also processed similarly.

The elimination half-life ($T_{1/2}$) was calculated as follows:

$$t_{1/2} = -0.693/k$$

Where k is the slope of the line obtained by plotting natural logarithmic percentage (Ln %) of SN79 remaining in the reaction mixture versus incubation time (minutes).

Intrinsic clearance (CL'_{int}, mL/min/kg) was calculated from the following equation [269]:

$$CL'_{int} = \frac{0.693}{\text{In vitro } t_{1/2}} \times \frac{\text{mL incubation}}{\text{mg microsomes}} \times \frac{\text{45mg microsomes}}{\text{g liver}} \times \frac{\text{45g liver}}{\text{kg bw}}$$

4.9 Animal studies conducted in fasted state

The SN79 compound was administered to male Sprague dawley rats to determine its pharmacokinetic profile. All experimental procedures were approved and performed in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) at the University of Mississippi under Protocol No-08-008. Pre-cannulated Sprague dawley rats (175-199g) were obtained from Harlan Laboratories (Indianapolis, IN). During the study, rats were housed in standard metabolism cages and allowed free movement and water *ad libitum.* Intravenous (IV) formulations and oral (p.o.) formulations were prepared on the same day of the study. The formulations were sonicated to ensure complete solubilization of SN79 before dosing to the animals. The IV solutions were administered via the jugular vein cannula, after which the cannula was flushed with 0.2 ml heparinized saline to ensure complete administration of the dose. The oral (p.o.) formulation was gavage dosed to the rats. After administration, the tube containing the drug solution was rinsed with 0.5 mL saline, which was also administered to the rat. The remaining formulation was assayed using UPLC/MS. Blood samples were collected

using the indwelling cannula. An initial blood volume of 0.05 mL was withdrawn to clear the line of heparinized saline. Using a fresh syringe 0.25 mL of blood was withdrawn and placed in an anti-coagulant coated micro-centrifuge tube. The cannula was then flushed with 0.25 ml of heparinized saline. The blood samples were stored in ice until centrifugation. Rat plasma was separated by centrifugation (Eppendorf 5415C centrifuge) at 3000 g for 10 min at 4°C and stored at -20°C (Kenmore Refrigerator, USA) until analysis by UPLC/MS.

Urine and fecal samples were collected at various time intervals during the study period. All samples were stored at -20°C (Kenmore Refrigerator, USA) until analyzed by UPLC/MS.

4.9.1 Heparinized Saline

A sodium chloride solution with a concentration of 0.9% w/v was prepared in HPLC grade water and auto-claved using the dry heat setting for 20 min. After sterilizing, 5 mg of heparin sodium salt was added to 100mL of sodium chloride solution to obtain a final of 10U/mL concentration of heparin.

4.9.2 Formulations

All the formulations were prepared afresh on the day of the experiment. Both IV and oral formulations were vortex-mixed and sonicated till the compound was completely dissolved in the vehicle.

4.9.2.1 Intravenous formulation

The IV formulation of SN79 was prepared in saline. SN79-*di*-HCl (12.03 mg) was dissolved in 3 mL of saline to produce a final concentration of SN79 equivalent to 4 mg/mL. The solution was

ultra-sonicated for 2 minutes to dissolve the compound completely. The intravenous formulation was administered to rats at a dose of 5 mg/kg.

4.9.2.2 Oral formulation

The oral formulation of SN79 was prepared in saline. SN79-*di*-HCl (28.29 mg) was dissolved in 7 mL of saline to produce a final concentration of SN79 equivalent to 4 mg/mL. The solution was ultra-sonicated for 2 minutes to dissolve the compound completely. The oral formulation was administered at a dose equivalent to 20 mg/kg of SN79.

4.9.3 Formulation stability

The stability of the IV and oral formulations was conducted in rat plasma. During this study we looked for any precipitation or degradation of the compound in presence of saline. A time period of three days was selected, which will encompass the entire animal study period. A five hundred micro-liter of the formulations, both IV and oral, were incubated in 1 mL of rat plasma in capped glass vials at a temperature of 37° C. From this stock, 100 µL of plasma was processed for analysis everyday for 3 days to assess the stability of the compound in rat plasma. Extraction procedure was same as mentioned in Section 3.5.1.

4.9.4 Processing of Plasma samples

4.9.4.1 Standards preparation

SN79 *di*-hydrochloride was used to prepare standard solutions. To prepare a stock solution of concentration 1 mg/ml of SN79, 1.18 mg of SN79 *di*-hydrochloride was dissolved in 1mL of HPLC grade water. Further dilutions were made using HPLC grade water to get final concentrations of 50, 100, 150, 500, 1,000, 2,000, 5,000, 10,000, 30,000 and 50,000ng/ml

respectively. The internal standard, CM156 was prepared at a concentration of 10 µg/ml in water. To prepare the standards, 80 µL of blank rat plasma was taken, to which 10 µL each of standard stock solution was added to get final concentrations of 5, 10, 15, 50, 100, 200, 500, 1,000, 3,000 and 5,000ng/ml. To these standard solutions, 10 µL of the internal standard (10 µg/ml) was added to get a final volume of 100 µL. Chloroform (500 µL) was used as the extraction solvent, and the micro-centrifuge tubes were vortexed for 10 minutes. The samples were then centrifuged for 20 minutes at 10,000 rpm. Supernatant (400µL) was collected and evaporated to dryness in a vacuum oven (Precision, Jouan. Inc, USA). To the dried samples, 100 µL of mobile phase was added and vortexed for 2 minutes. All the samples were filtered with a 0.2µm filter (Waters 13 mm GHP 0.45 µm) and analyzed using UPLC/MS.

Quality Control (QC) samples were prepared separately at concentrations of 5, 15, 200 and 3,000ng/ml to assess accuracy and precision of the assay method. All of the calibration and QC samples were extracted using the method described in the subsequent section and stored at –20°C (Kenmore Refrigerator, USA) until analysis.

4.9.4.2 Sample preparation

An aliquot of 90 µL of rat plasma and 10 µL of the internal standard solution were placed in an eppendorf micro-tube for processing. Chloroform was used as the extraction solvent. Five hundred micro-liter of chloroform was added to these samples and the micro-centrifuge tubes were vortexed for 10 minutes on VWR Signature® pulsing vortex mixer (VWR Scientific Inc., U.S.A). The samples were centrifuged at 3000 rpm for 20 minutes using an Accuspin Micro 17R centrifuge (Fisher Scientific, U.S.A). The organic phase (400 µL) was transferred into 1.5 mLeppendorf tubes (Fisher Scientific, U.S.A) and dried in a vacuum oven (Precision, Jouan. Inc,

USA) at 25 ^{o}C. To the dried samples, 100 µL of mobile phase was added and vortex mixed for 2 minutes. All the samples were filtered with a 0.2 µm syringe filter (Waters 13 mm GHP 0.2 µm) and analyzed using the UPLC/MS.

4.9.5 Processing of Urine samples

4.9.5.1 Standards preparation

SN79- *di*-hydrochloride was used to prepare the standard solutions. To prepare a stock solution of concentration 1 mg/ml of SN79, 1.18 mg of SN79 *di*-hydrochloride was dissolved in HPLC grade water. Further dilutions were made using HPLC grade water to get final concentrations of 50, 100, 150, 500, 1,000, 2,000, 5,000, 10,000, 30,000 and 50,000 ng/ml. The internal standard CM156, was prepared at a concentration of 10 µg/ml in HPLC grade water. To prepare the standards, 80 µL of blank rat plasma was taken, to which 10 µL each of standard stock solution was added to get final concentrations of 5, 10, 15, 50, 100, 200, 500, 1,000, 3,000 and 5,000 ng/ml. To these standard solutions, 10 µL of internal standard (10µg/ml) was added to get a final volume of 100 µL. Chloroform was used as the extraction solvent. Five hundred micro-liter of chloroform was added to these samples and the micro-centrifuge tubes were vortexed for 10 minutes on VWR Signature® pulsing vortex mixer (VWR Scientific Inc., U.S.A). The samples were centrifuged at 3000 rpm for 20 minutes using an Accuspin Micro 17R centrifuge (Fisher Scientific, U.S.A). The organic phase (400 µL) was transferred into 1.5 mLeppendorf tubes (Fisher Scientific, U.S.A) and dried in a vacuum oven (Precision, Jouan. Inc, USA) at 25 ^{o}C. To the dried samples, 100 µL of mobile phase was added and vortex mixed for 2 minutes. All the samples were filtered with a 0.2 µm syringe filter (Waters 13 mm GHP 0.2 µm) and analyzed using the UPLC/MS. Quality Control (QC) samples were prepared separately at concentrations

of 5, 15, 200 and 3000 ng/ml to assess accuracy and precision of the assay method. All calibration and QC samples were extracted with the method described in the section 3.5.1 and stored at -20 °C (Kenmore Refrigerator, USA) until analysis.

4.9.5.2 Sample preparation

The frozen urine samples were thawed at room temperature. Rat urine (90 µL) and 10 µL of the internal standard with 10% of trifluoroacetic acid were transferred into an eppendorf tube and vortex mixed (VWR Signature® pulsing vortex mixer, VWR Scientific Inc., U.S.A) for 2 min. The samples were centrifuged (Accuspin Micro 17R centrifuge, Fisher Scientific, U.S.A) for 10 min at 10,000 rpm and analyzed using UPLC/MS.

4.9.6 Processing of Feces samples

An accurately weighed amount of feces (1 mg) was dried, powdered by mortar and pestle and dissolved in water. After vortex mixing (VWR Signature® pulsing vortex mixer, VWR Scientific Inc., U.S.A) for 10 min, the samples were centrifuged (Accuspin Micro 17R centrifuge, Fisher Scientific, U.S.A) at 10,000 rpm for 20 min and the supernatant was analyzed using UPLC/MS.

4.9.7 Processing of Tissue samples

After allowing the tissues to thaw at room temperature, the tissue samples were washed with saline and homogenized in 10mM ammonium acetate (pH-7.4) containing 0.25M sucrose. The homogenates were centrifuged at 10,000 rpm for 20 min. The supernatant was collected and filtered with a 0.45µm syringe filter (Waters 13 mm GHP 0.45µm) prior to UPLC/MS analysis.

4.10 Animal studies conducted in fed state

In addition to pharmacokinetic studies conducted in fasted state in Section 4.10, studies were also done in fed state laboratory animals. In this study, the animals were given access to food and water *ad libitum* 3.5 hr after drug administration. All experimental procedures were approved and performed in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) at the University of Mississippi under Protocol No-08-008. Pre-cannulated Sprague dawley rats (175-199g) were obtained from Harlan Laboratories (Indianapolis, IN). During the study, rats were housed in standard metabolism cages and allowed free movement and water *ad libitum.*

Intravenous (IV) formulations and oral (p.o.) formulations were prepared (as mentioned in Sections 4.9.2.1 and 4.9.2.2) on the same day of the study. The formulations were sonicated to ensure complete solubilization of SN79 before dosing to the animals. The IV solutions were administered via the jugular vein cannula, after which the cannula was flushed with 0.2 ml heparinized saline to ensure complete administration of the dose. The oral (p.o.) formulation was gavage dosed to the rats. After administration, the tube containing the drug solution was rinsed with 0.5 mL saline, which was also administered to the rat. The remaining formulation was assayed using UPLC/MS.

Blood samples were collected using the indwelling cannula. An initial blood volume of 0.05 mL was withdrawn to clear the line of heparinized saline. Using a fresh syringe 0.25 mL of blood was withdrawn and placed in an anti-coagulant coated micro-centrifuge tube. The cannula was then flushed with 0.25 ml of heparinized saline. The blood samples were stored in ice until centrifugation. Rat plasma was separated by centrifugation (Eppendorf 5415C centrifuge) at

3000 g for 10 min at 4°C and stored at -20°C (Kenmore Refrigerator, USA) until analysis by UPLC/MS.

Urine and faeces samples were collected at various time intervals during the study period. All samples were stored at -20°C (Kenmore Refrigerator, USA) until analyzed by UPLC/MS.

4.10.1 Heparinized Saline

A sodium chloride solution with a concentration of 0.9% w/v was prepared in HPLC grade water and auto-claved using the dry heat setting for 20 min. After sterilizing, 5 mg of heparin sodium salt was added to 100mL of sodium chloride solution to obtain a final of 10U/mL concentration of heparin.

4.10.2 Formulations

All the formulations were prepared afresh on the day of the experiment. Both IV and oral formulations were vortex-mixed and sonicated till the compound was completely dissolved in the vehicle.

4.10.2.1 Intravenous formulation

The IV formulation of SN79 was prepared in saline. SN79-*di*-HCl (12.03 mg) was dissolved in 3 mL of saline to produce a final concentration of SN79 equivalent to 4 mg/mL. The solution was ultra-sonicated for 2 minutes to dissolve the compound completely. The intravenous formulation was administered to rats at a dose of 5 mg/kg.

4.10.2.2 Oral formulation

The oral formulation of SN79 was prepared in saline. SN79-*di*-HCl (28.29 mg) was dissolved in 7 mL of saline to produce a final concentration of SN79 equivalent to 4 mg/mL. The solution was ultra-sonicated for 2 minutes to dissolve the compound completely. The oral formulation was administered at a dose equivalent to 20 mg/kg of SN79.

4.10.3 Processing of Samples

Plasma samples, urine and fecal samples, tissue samples were processed according to the procedures mentioned in Sections 4.9.4, 4.9.5, 4.9.6 and 4.9.7.

CHAPTER V: RESULTS AND DISCUSSION

SN79-*di*-HCl, a novel synthetic compound was studied to examine its potential as an effective agent for attenuating cocaine-induced toxicity and addiction. As part of drug discovery stage, various pre-formulation studies were performed to characterize the physico-chemical properties of the compound. The pharmacokinetic profile of the compound in male Sprague dawley rats was studied to establish its oral bioavailability and to determine the absorption, distribution, metabolism and excretion profile of SN79-*di*-HCl *in vivo*. These results will be presented here.

5.1 cLog P and pKa

The cLog P of SN79-*di*-HCl was calculated using ACD Labs software and was found to be 3.38 indicating that it is a lipophilic compound [270]. The pKa of this compound determined using potentiometric method was found to be 7.9±0.14. This value suggests that it is a weak base (pKa>7.00) respectively [183]. Bases in the pK_a range of 5 to 11 are greatly influenced by changes in pH and hence their absorption is pH dependent [183]. Examples are certain morphine analogs, chloroquine, imipramine and amitriptyline. Weak bases are better absorbed from the relatively alkaline conditions of the intestine where they largely exist in unionized form. Most drugs are given orally and absorption of such drugs is influenced by differences in luminal pH along the GIT, surface area per luminal volume, blood perfusion and the nature of epithelial membranes.

5.2 Aqueous and pH dependent Solubility

The solubility of SN79-*di*-HCl calculated at pH 1.2, 3.0, 5.0, 7.4 and 9.0 was found to be23.9, 26.5, 28.8, 26.6 and 21.0 mg/mL respectively. The solubility of SN79-*di*-HCl in water was found to be 31.5 mg/mL which is 1 part of SN79-*di*-HCl in 32 parts of water. The pH of water is 6.98.The US Pharmacopeia [271] states that a when 1 part of solvent is required to dissolve 30-100 parts of solute, the compendia substance is 'sparingly soluble' in the solvent [271]. Likewise, SN79-*di*-HCl is sparingly soluble in water. The solubility of salt form depends on the size of the counter ion. Generally, size of the counter ion and solubility of salt bear an inverse relationship [183].

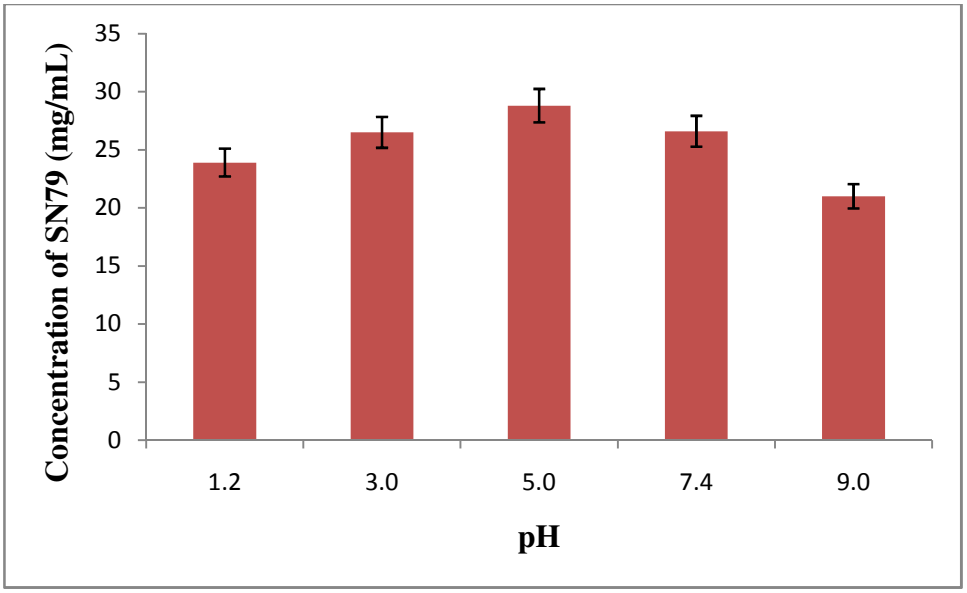


Figure 5.1 pH dependent solubility of SN79

Table 5.1 pH dependent solubility of SN79-di-HCl

pH	Solubility ± SEM
1.2	23.9 ± 0.01
3	26.5 ± 0.03
5	28.8 ± 0.03
7.4	26.6 ± 0.04
9	21.0 ± 0.01

SEM – standard deviation/mean

5.3 pH dependent Stability

Pre-formulation studies consist of all that it takes to characterize a drug substance to enable its formulation into a practical drug delivery system [272]. Drug characterization during pre-formulation stage encompasses various studies including but not limited to determination of solubility, pKa, lipophilicity, stability in aqueous systems etc. Pre-formulation stability studies are usually the first quantitative assessment of chemical stability of a drug. Factors affecting chemical stability critical in rational dosage form design include temperature, pH and excipients. In this study, the effect of temperature and pH on the stability of SN79-*di*-HCl was evaluated to understand the influence of the highly acidic environment of the stomach. The degradation of SN79 in aqueous solutions followed apparent first-order kinetics, since a linear relationship was found between the natural logarithm of the percentage remaining and the storage time ($r>0.990$). Linear plots were obtained using the equation

$$\ln A = -kt + \ln A_0 \qquad \text{(Eq.5.1.)}$$

Where A_0 is the original concentration of the compound, A is the concentration at time t, and k is the rate constant. The experimentally determined k value was obtained from the slope of the plot

by regression analysis. The effect of pH on SN79 was determined using various buffer solutions in the pH range of 1.2 to 9.0. As shown in Figure 5.2., the degradation of SN79 was strongly correlated with the increase of pH. The pH of maximum stability occurs at pH 7.4 when placed in -20°C, pH 1.2 at 25°C and at pH 1.2 and 5 when placed at 37°C. Beyond the minimum, in the pH profile, the *k* value increases with increasing pH until pH 3.0 then decreases until pH 7.4. This might suggest an attack of hydroxyl ion on the cationic species [273]. Between pH 7.4 and 9.0, there is an increase in the k value.

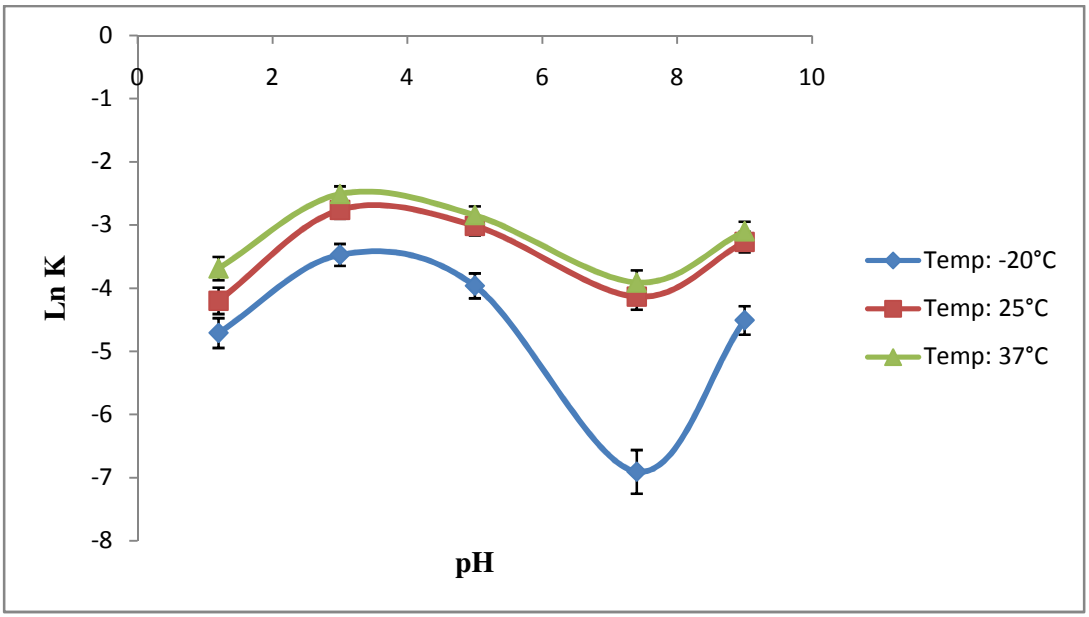


Figure 5.2 Plot of the pH-rate profile of SN79-di-HCl in various buffer solutions; pH ranging from 1.2 to 9.0 and temperatures at -20°C, 25°C and

The thermal effect on SN79-*di*-HCl degradation in buffered solutions (pH range: 1.2 to 9.0) was studied at three different temperatures: -20°C, 25°C and 37°C respectively.

Figure 5.3 represents the Arrhenius plot of the rate (natural logarithm scale) versus the reciprocal of temperature (Kelvin scale), according to the equation:

$$\ln K = \ln S - E_a/RT \qquad \text{(Eq.5.2.)}$$

Where K is the pseudo-first order rate constant, S is the frequency factor, E_a is the energy of activation of the reaction, R is the gas constant (1.985 cal mol^{-1} K^{-1}), and T is the absolute temperature (K°). A linear relationship was found by this plot ($r>0.993$) (Figure 5.3). The activation energy values at pH 1.2, 3.0, 5.0, 7.4 and 9.0 as calculated from the Arrhenius plot are provided in Table 5.2. The $t_{90\%}$ values are provided in Table 5.3. $t_{90\%}$ value gives the time required for 10% degradation of drug at any temperature. The $t_{90\%}$ value was 3 days when the solution was stored at pH = 7.4, T = 25°C and that $t_{90\%}$ value was 105 days when the temperature decreased to -20°C.

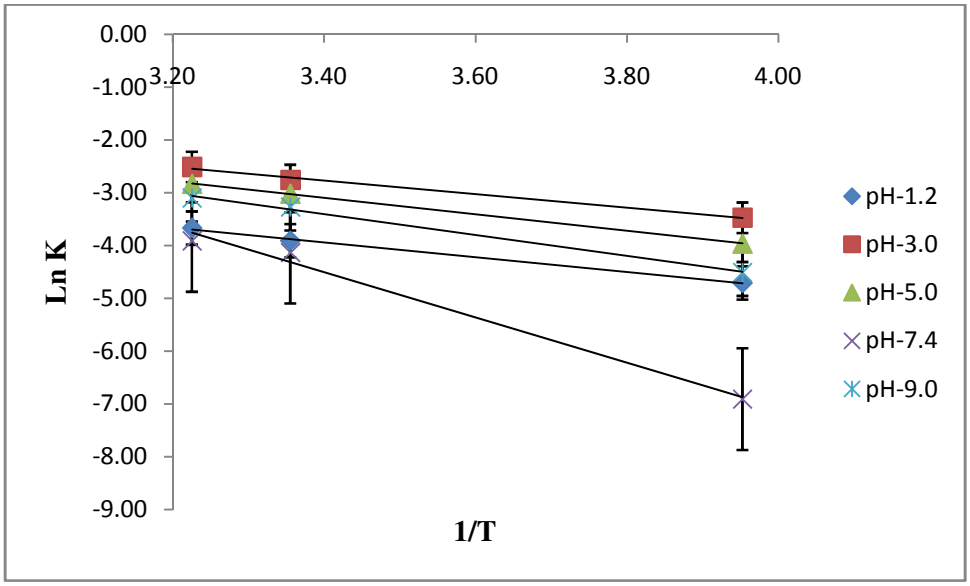


Figure 5.3 Arrhenius plots for the hydrolysis of SN79-di-HCl at various pH values. The pH values indicated here are those at -20°C, 25°C and 37°C.

Table 5.2 Activation energy for the degradation of SN79

pH	Activation energy, E_a kcal/mol
1.2	2.8
3	2.5
5	3.1
7.4	8.5
9	3.9

Table 5.3 Thermal and pH effects on the degradation of SN79

pH	Temperature		
	-20°C	**25°C**	**37°C**
	$t_{90\%}$ **(days)**		
1.2	12	7	2
3.0	3	2	1
5.0	2	2	2
7.4	105	3	0.3
9.0	3	2	1

These results demonstrate that the stability of SN79-*di*-HCl in aqueous systems depends on several factors: temperature, pH, effect of counter-ion and ionic strength of buffers [274].

5.4 Stability in Simulated Gastric and Intestinal Fluids

Oral delivery is the most convenient and desirable route of drug administration. The bioavailability of drugs is influenced by various factors like stability in gastrointestinal fluid and solubility. The stability of a drug substance in gastric and intestinal fluids indicates whether or not drug loss from the gastrointestinal tract is via intestinal permeation or by a degradation

process in the gastrointestinal fluids prior to membrane absorption [266]. Permeability of a compound through the gastrointestinal tract is one of the most important aspects in determining the lead potential of a molecule [275]. In assessing drug substance permeability, the degree of drug degradation in the gastrointestinal fluid prior to intestinal permeation should also be considered. Stability in the gastrointestinal tract may be determined *in vitro* by incubating the drug substance in gastric and intestinal fluids representative of *in vivo* drug exposure to these fluids *viz.* 1 h in SGF and 3 h in SIF. When incubated in simulated gastric and intestinal fluids, SN79-*di*-HCl was stable throughout the duration of study as shown in tables 5.4 and 5.5. The relative difference values were calculated at all time points which showed no significant change in compound's stability from zero time point. Since there is no significant degradation (<5%), the compound can be deemed stable in the gastrointestinal tract and that any drug loss might be a result of membrane permeation instead of gastrointestinal degradation process. Understanding the permeability of compounds across GIT membrane thus becomes an integral part of drug discovery.

Table 5.4 Stability of SN79 in simulated gastric fluids (pH 1.2). Data expressed as mean ± SE (n=3); RD = relative difference

Time (hr)	SN79 (µg/ml)	RD
0	4.456 ± 0.04	--
60	4.458 ± 0.04	-0.04

Table 5.5 Stability of SN79 in simulated intestinal fluids (pH 6.8). Data expressed as mean ± SE (n=2); RD = relative difference

Time (hr)	SN79 (µg/ml)	RD
0	4.555 ± 0.03	--
60	4.567 ± 0.01	-0.25
120	4.599 ± 0.04	-0.96
180	4.547 ± 0.003	0.19

5.5 *In vitro* Plasma Protein Binding Studies

The reversible binding of a drug to plasma proteins is an important determinant of its pharmacokinetic and pharmacodynamic characteristics [276]. For certain drugs, saturable plasma protein binding may occur at therapeutic concentrations which can cause dose-dependent pharmacokinetics. The protein binding profile of SN79-*di*-HCl in rat plasma was evaluated *in vitro*using ultra-filtration. The concentration dependent free fraction (F_u) of SN79 when incubated as SN79-*di*-HCl was studied. At concentrations of 0.5, 1.0, 5.0 and 10µg/mL, the F_u values were 3.9, 4.5, 5.4 and 8.2 respectively. The binding of SN79-*di*-HCl to pooled rat plasma protein showed a concentration dependent behavior (Figure 5.4). This indicates that if an increase in dose is given to subjects this would result in increased peak plasma concentrations of SN79-*di*-HCl.

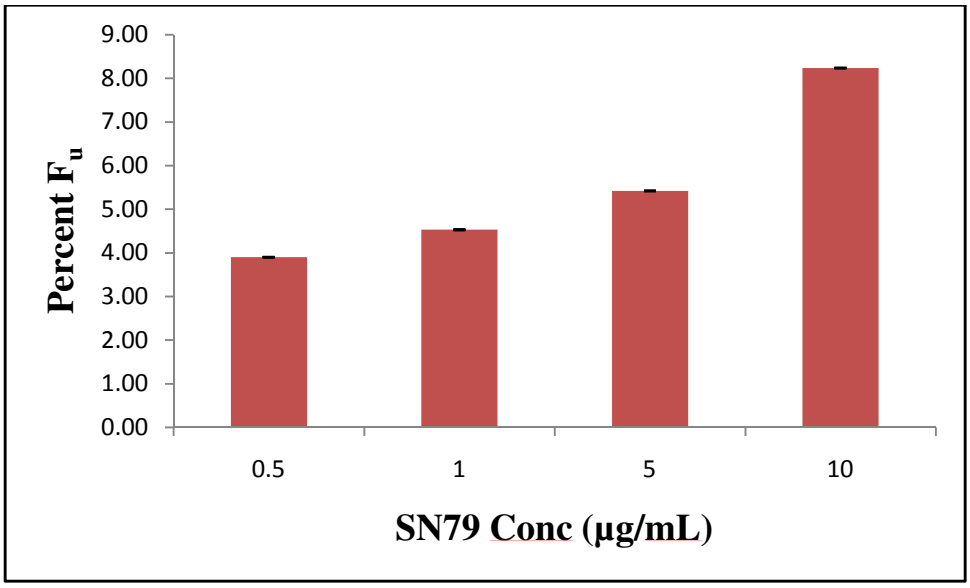


Figure 5.4 Concentration dependent protein binding profile of SN79-di-HCl when incubated in rat plasma

Any changes in the extent of plasma protein binding (expressed as a change in F_u), leads to changes in apparent volume of distribution thereby affecting the terminal $t_{1/2}$.

Concentration dependent protein binding is given by the mathematical equation [277]

$$F_u = 1/ [1+K*P]$$

Where F_u – fraction unbound

K – affinity constant for protein binding

P – concentration of free protein binding sites

According to the above equation, as the concentration of drug increases, the number of free protein binding sites i.e. P decreases thereby increasing the F_u value. Concentration dependent variations in the fraction of unbound drug in plasma are most likely to occur with drugs that

exhibit high association constant (K= 10^5 or 10^6) [278]. Plasma protein binding influences the movement of drug from blood to tissues. It limits drug distribution in that only free or unbound portion of the drug is available to enter the tissues and elicit pharmacological effects. The major binding protein present in plasma is albumin at a concentration of 40 g/L [279]. Since SN79-*di*-HCl is a weak base, its affinity to albumin may not be high since albumin tends to exhibit preferential binding to acidic drugs but may bind weakly to basic drugs [279]. Aside from albumin, alpha-1 acid glycoprotein is another significant plasma protein with affinity for weak bases. Its plasma concentration is about 0.7 g/L. The extent of protein binding of a drug is either drug or protein concentration dependent based on the affinity and capacity of the plasma protein [280]. It is desirable to measure the extent of protein binding *in vitro* at more than two different concentrations, one close to the therapeutic concentration and another one close to a toxic concentration level.

5.6 Animal studies

5.6.1 Pharmacokinetics in fasted state subjects

SN79-*di*-HCl pharmacokinetics was studied following the intravenous (5mg/kg) and oral (20mg/kg) administration of the compound to male Sprague dawley rats. The SN79 concentration-time plots, as obtained following IV and oral dosing, are presented in Fig 5.5 and 5.6. The pharmacokinetic parameters were estimated using non-compartmental analysis with Pharsight ® WinNonlin^{TM} software version 5.2 and are presented in Tables 5.6 and 5.7.After intravenous and oral administration of SN79-*di*-HCl, close and continuous visual monitoring of the animals revealed that there was no severe acute toxicity response as none of the animals showed any signs of behavioral or neurological toxicity during the entire study period.

Table 5.6 Mean plasma pharmacokinetic parameters of SN79-di-hydrochloride after intravenous administration to Sprague dawley rats (n=5)

Compound	C_0 (µg/mL)	AUC (ug-hr/mL)	$T_{1/2}$ (hr)	C_{max} (µg/mL)	V_d (L/kg)	Cl_obs (L/hr/kg)
SN79-*di*-HCl	1.40 ± 0.3	44.5 ± 4.8	1.36 ± 0.04	1.34 ± 0.12	13.7 ± 1.5	0.18 ± 0.01

C_0 – initial concentration obtained by extrapolation on y-axis; AUC – area under the curve; $T_{1/2}$ – plasma half life; C_{max} – maximum plasma concentration; MRT – mean residence time; V_d – volume of distribution; CL – plasma clearance

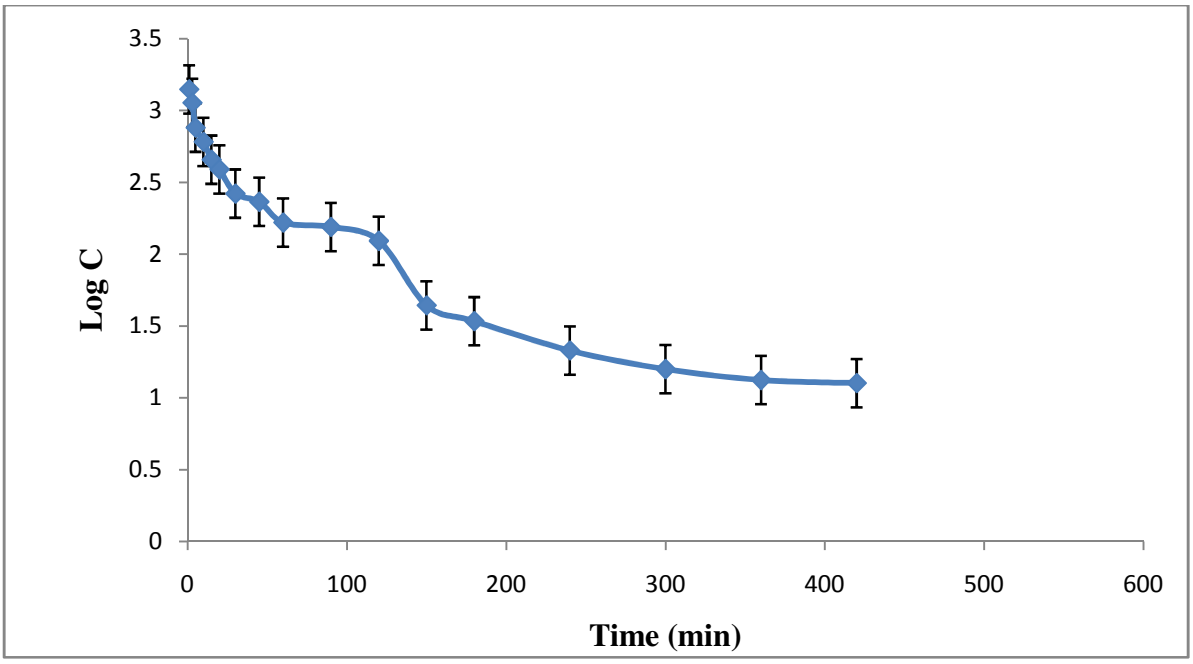


Figure 5.5 Mean plasma concentration time profile of SN79-di-HCl in male Sprague dawley rats after intravenous administration (5mg/kg) (n=5)

Table 5.7 Mean plasma pharmacokinetic parameters of SN79-di-hydrochloride after oral administration to Sprague dawley rats (n=5)

Compound	AUC (ug-hr/mL)	$T_{1/2}$ (hr)	C_{max} (ug/mL)	MRT (hr)	V_d (L/kg)	CL (L/hr/kg)	T_{max}(hr)
SN79-*di*-HCl	117.3± 1.5	7.5± 0.7	0.21± 0.1	9.9± 1.3	115.1± 1.1	0.18± 0.02	1.5± 0.05

AUC – area under the curve; $T_{1/2}$ – plasma half life; C_{max} – maximum plasma concentration; MRT – mean residence time; V_d – volume of distribution; CL – plasma clearance; T_{max} – time of maximum plasma concentration.

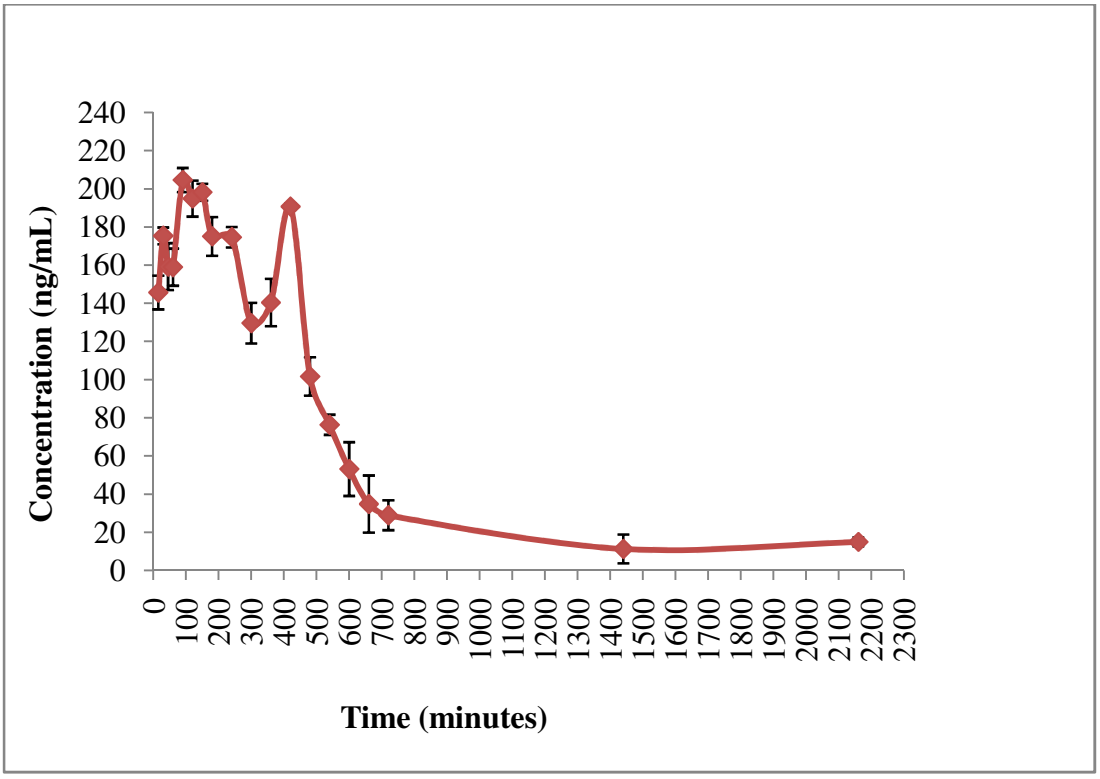


Figure 5.6 Mean plasma concentration time profile of SN79-di-HCl in male Sprague dawley rats after oral administration (20mg/kg) (n=5)

Average half lives of SN79-*di*-hydrochloride after oral and intravenous dosing were found to be 7.51 and 1.36 hrs respectively. The different half-lives observed after IV and oral administration suggest different elimination rates [281]. This might be due to the presence of biologically active metabolites or on-going gastrointestinal absorption [282]. The maximum plasma concentration (C_{max}) was found to be 0.21µg/mL after oral administration. When measured against the oral dose given to rats (20 mg/kg), the $C_{max}$was low. The affinity of this compound to plasma proteins might affect the amount of free drug available for absorption. Results from *in vitro* protein binding studies (Section 5.5), clearly indicate the high affinity of this compound for plasma proteins thereby limiting its availability in the free form. The time required to reach maximum plasma concentration (T_{max}) was 1.5 hours after p.o. dosing.

The C_0 value after intravenous administration was found to be 1.40±0.3 µg/mL. Each rat was given approximately 0.98mg of drug. The plasma concentration determined at one minute after IV administration of SN79-*di*-HCl was 1.34±0.1 µg/mL. The apparent volume of distribution of SN79-*di*-hydrochloride was found to be 115.1 L/kg after oral administration and 13.7 L/kg after intravenous administration. For a drug that is 95% protein-bound with a high volume of distribution [183] it redistributes into a large volume of body fluids and clinical effects may be negligible or insignificant.

The compound clearance value obtained was 0.18 L/hr/kg for both intravenous and oral administration. Only the unbound or free drug is capable of being eliminated. It becomes difficult for the drug-protein complex to penetrate the metabolizing tissue (liver). The large molecular size also prevents it from getting filtered through the glomerulus. Such drugs are eliminated slowly with prolonged elimination half-lives.SN79-*di*-HCl showed a half-life of 7.5 hr. Since SN79-*di*-HCl is a weak base (Log P>3), a major amount of drug was excreted in urine

rather than in feces. The urinary and feces excretion profiles are shown in Figures 5.7, 5.8, 5.9 and 5.10.

Figure 5.7 Urinary excretion profile of SN79-di-HCl in male Sprague dawley rats after IV administration (5mg/kg) (n=5)

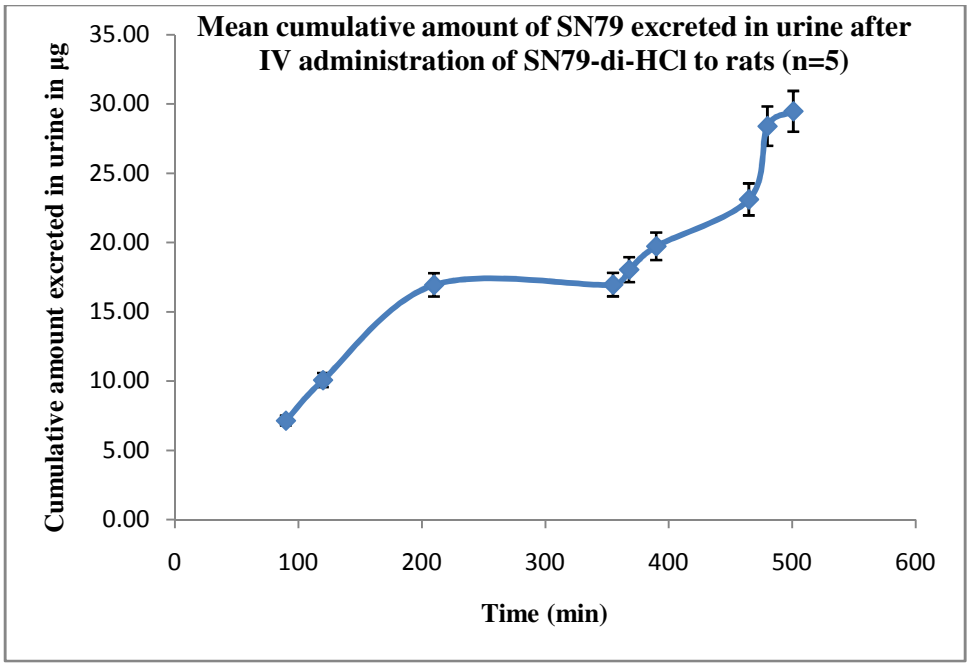


Figure 5.8 Feces excretion profile of SN79-di-HCl in male Sprague dawley rats after IV administration (5mg/kg) (n=5)

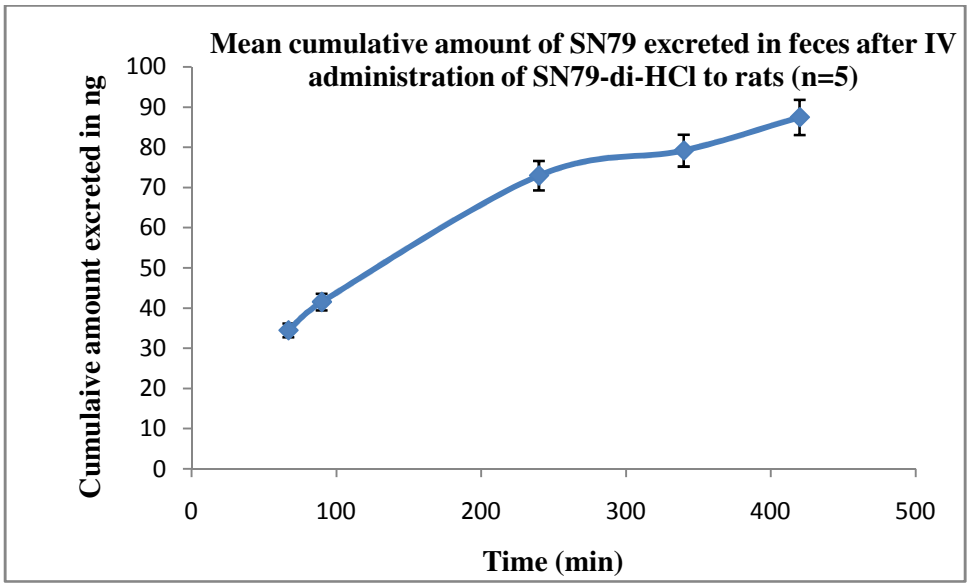


Figure 5.9 Urinary excretion profile of SN79-di-HCl in male Sprague dawley rats after Oral administration (20mg/kg) (n=5)

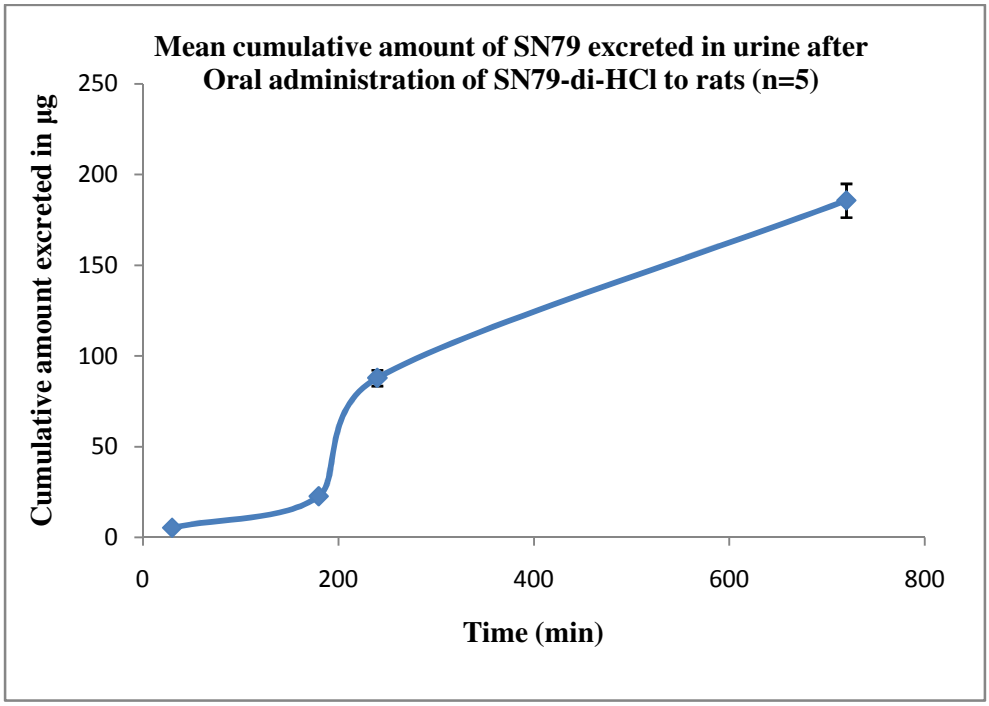


Figure 5.10 Feces excretion profile of SN79-di-HCl in male Sprague dawley rats after Oral administration (20mg/kg) (n=5)

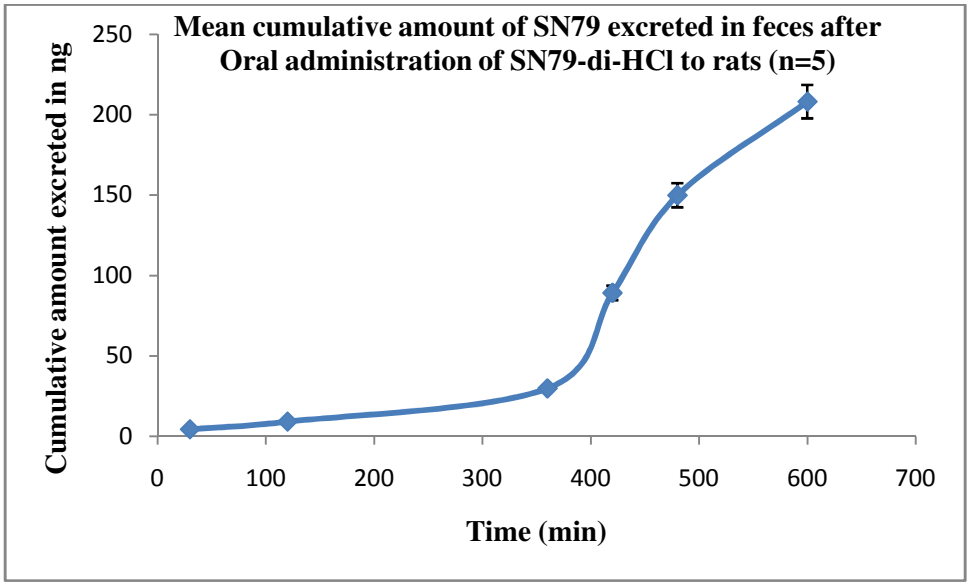


Table 5.8 Tissue distribution of SN79-di-hydrochloride after single dose intravenous administration to Sprague dawley rats at 5mg/kg.

Tissue	Amount (in µg)	% seen in tissue
Heart	0.98	0.10
Liver	3.67	0.37
Pancreas	2.51	0.26
Lungs	2.95	0.30
Kidney	ND	ND

Table 5.9 Tissue distribution of SN79-di-hydrochloride after single dose oral administration to Sprague dawley rats at 20mg/kg.

Tissue	Amount (in µg)	% seen in tissue
Heart	15.7	0.40
Liver	18.9	0.50
Lungs	4.60	0.10
Pancreas	17.0	0.50
Kidney	5.10	0.10

After oral and intravenous dosing, there was a dose-proportional increase in the mean residence time values which were found to be 9.87 and 1.98 hours. When a drug is administered orally, it has to be absorbed into the systemic circulation. Then the drug is distributed such that equilibrium is established between the tissues and blood. Thereafter, the drug is excreted. So the drug tends to stay for longer duration in the body after p.o. dosing. However, when administered intravenously, the drug skips the absorption phase as it is readily available in the systemic circulation thereby reducing its residence time in the body by getting excreted quickly.

From the intravenous and oral dosing of SN79-*di*-hydrochloride, the average oral bioavailability was calculated to be 65.9%. A qualitative visual examination of the data indicated the presence of multiple peaks in the concentration-time profile of oral SN79-*di*-HCl (Figure 5.6). The double peaks were observed at 1.50 and 7.00 hours respectively. Similar multiple peak phenomena have been observed for a number of structurally diverse compounds such as flurbiprofen, penicillamine, aspirin, furosemide and acetaminophen [285-287]. Drug absorption kinetics after oral administration can be influenced by several factors including membrane permeability, drug dissolution and gastrointestinal (GI) motility [288]. Several hypotheses based on region-

dependent variation in absorption [289], enterohepatic recirculation [290], variable gastric emptying and intestinal transit rates [291] and intestinal bacterial reconversion of biliary metabolite [292] have been proposed to explain this behavior. *Enterohepatic recycling* refers to the circulation of bile from the liver, where it is produced, to the small intestine, where it aids in digestion of fats and other substances, back to the liver [293]. It has been cited as the most probable reason for the secondary peaks noticed following the p.o. dosing of amprenavir[294]. Enterohepatic recirculation was ruled out here because it would be expected that a second peak would also occur following intravenous administration. The absorption of cimetidine, a histamine H_2-receptor antagonist has been shown to be lower in rat jejunum than in duodenum and ileum [295] and has been postulated as a possible explanation for plasma level double peaks in humans [296]. The time that a drug spends in the stomach and along the intestine is dictated to a large extent by gastric emptying and gastrointestinal motility. Relatively lower amount of most drugs are absorbed from the stomach compared to the small intestine, and drug is retained in the stomach until it is delivered to the small intestine where it is absorbed [293]. For drugs with high water solubility, part of the dose could be delayed in the stomach, a phenomenon called as *Delayed Gastric Emptying*. Many drugs such as cimetidine, nifedipine, avitriptran, ^{13}C-octanoate and epinastine are known to have shown double peak phenomena owing to delayed gastric emptying. Cimetidine has always shown double peak phenomenon when gastric pH is low, but very rarely when it is high. The possible role of gastrointestinal motility as a major determinant of the phenomena of secondary maxima occurring only in the fasted state has been previously addressed [297-298]. Such a possibility is quite plausible since gastrointestinal motility patterns are unique in the fasted state in humans and dogs and is altered drastically in the fed state in the two species. Gastrointestinal motility in the fasted state is characterized by cyclical fluctuations

in contractile activity of the stomach and intestine and is composed of four phases [299]. This cyclical pattern of contractile activity is termed as the migrating motility complex (MMC). Phase-I known as basal phase is characterized by complete lack of contractions followed by a 'preburst activity', which is phase-II. The contractions increase in number and activity in phase-II. Large amplitude contractions constitute phase-IIIand they occur at the maximum frequency observable. Phase-IV is an intermediate phase, that is often times present or absent, acting as a transition period between the intense activity in phase-III and the basal quiescent phase-I. Woodtli and Owyang[300] reported that duodenal pH is altered with MMC phases that could directly affect drug absorption via a pH dependent absorption effect or indirectly modulate absorption by affecting phase-II duration and overall MMC. The most common reason for occurrence of multiple peak phenomenon following p.o. dosing is *Variability of Absorption* within different regions of the gastrointestinal tract – especially zero or minimal absorption from jejunum, followed by absorption from duodenum or ileum.

The cause for appearance of multiple peak phenomenon needs further investigation. To understand whether or not food has any effect on the absorption of SN79-*di*-HCl from the gut, a similar pharmacokinetics study was conducted in fed state male Sprague dawley rats.

5.6.2 Influence of food on the multiple peak phenomena observed after oral administration

In addition to pharmacokinetic studies conducted in fasted state, studies were also done in fed state laboratory animals. In this study, the animals were given access to food and water *ad libitum* 3.5 hr after drug administration. Animals were given a p.o. dose of 20 mg/kg and IV dose of 5 mg/kg. Dosing, blood collection and sample processing was done as mentioned in section

4.10. The SN79 concentration-time plots, as obtained following IV and oral dosing, are presented in Fig 5.12 and 5.13. The pharmacokinetic parameters were estimated using non-compartmental analysis with Pharsight ® WinNonlin™ software version 5.2 and are presented in Tables 5.10 and 5.11. After intravenous and oral administration of SN79-*di*-HCl, close and continuous visual monitoring of the animals revealed that there was no severe acute toxicity response as none of the animals showed any signs of behavioral or neurological toxicity during the entire study period.

As shown in Fig 5.11, the multiple peak phenomena was retained in fed state subjects. As a result, it can be concluded that food has no effect with minimal or insignificant differences in the pharmacokinetic parameters (Tables 5.11 and 5.12).

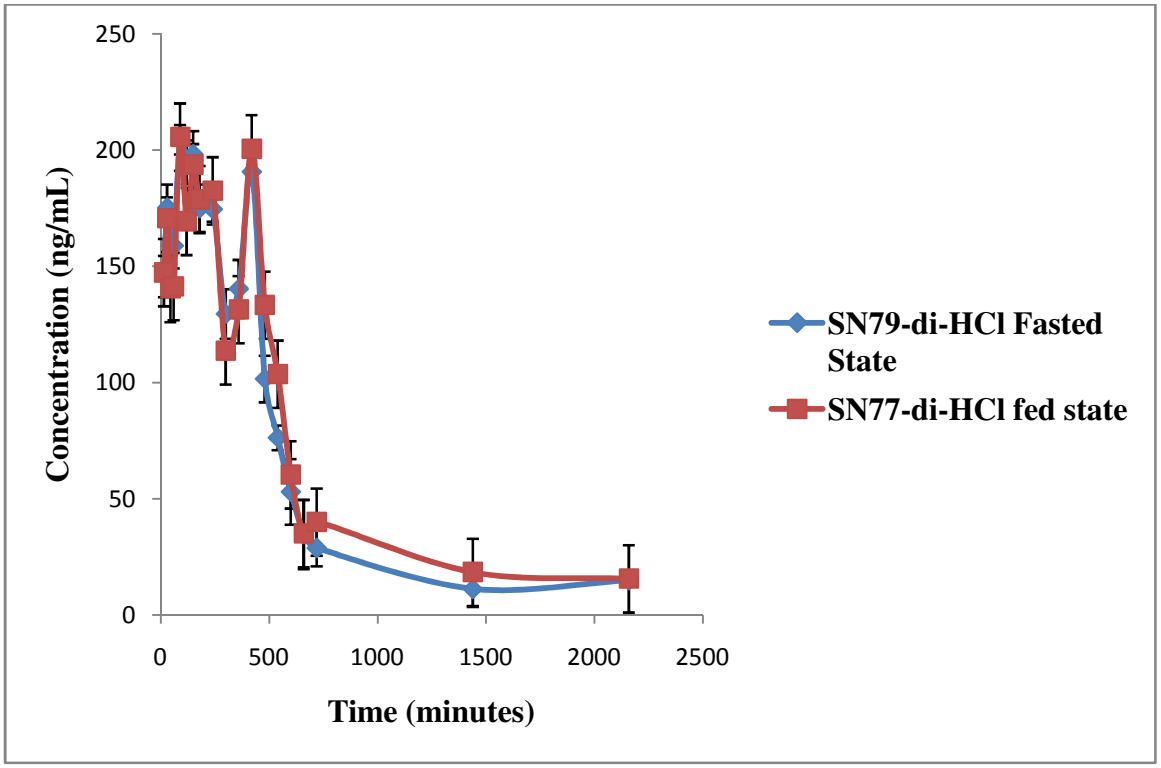


Figure 5.11 Mean plasma concentration time profiles of SN79-di-HCl in fasted and fed state male Sprague dawley ratsafter oral administration (20mg/kg)

There was no significant change in AUC in fed state rats (111 µg-hr/mL) and fasted rats (117µg-hr/mL). Likewise, there was no significant change in the pharmacokinetic parameters in presence/absence of food as shown in Table 5.10.

Table 5.10 Comparison of pharmacokinetic parameters in fasted and fed state rats

Pharmacokinetic parameter	Fasted State	Fed State
Half-life (hr)	7.50	7.80
Volume of distribution (L/kg)	115	114
Clearance (L/hr/kg)	0.18	0.13
T_{max}(hr)	1.50	1.52

A comparison of both the pharmacokinetic profiles, in fasted and fed state led us to conclude that food in the gastrointestinal tract did not alter the extent of absorption of oral SN79-*di*-HCl in male Sprague dawley rats; however the effect of food on absorption depends on the dosage form. These results suggest there may be other factors that induce the multiple peak phenomenons in the oral pharmacokinetic profile of SN79-*di*-HCl. Therefore, this multiple peak phenomenon might be due to inhibition of gastrointestinal transit but not due to fasted or fed condition of the rats (as discussed in Section 5.6.1.).

Table 5.11 Mean plasma pharmacokinetic parameters of SN79-di-hydrochloride after intravenous administration to fed state male Sprague dawley rats (5mg/kg) (n=6)

Compound	C_0 (µg/mL)	AUC (ug-hr/mL)	$T_{1/2}$ (hr)	C_{max} (µg/mL)	V_d (L/kg)	Cl_obs(L/hr/kg)
SN79-*di*-HCl	2.03 ± 0.30	49.8 ± 0.26	1.53 ± 0.19	1.72 ± 0.23	13.5 ± 0.25	0.11 ± 0.22

C_0 – initial concentration obtained by extrapolation on y-axis; AUC – area under the curve; $T_{1/2}$ – plasma half life; C_{max} – maximum plasma concentration; MRT – mean residence time; V_d – volume of distribution; CL – plasma clearance

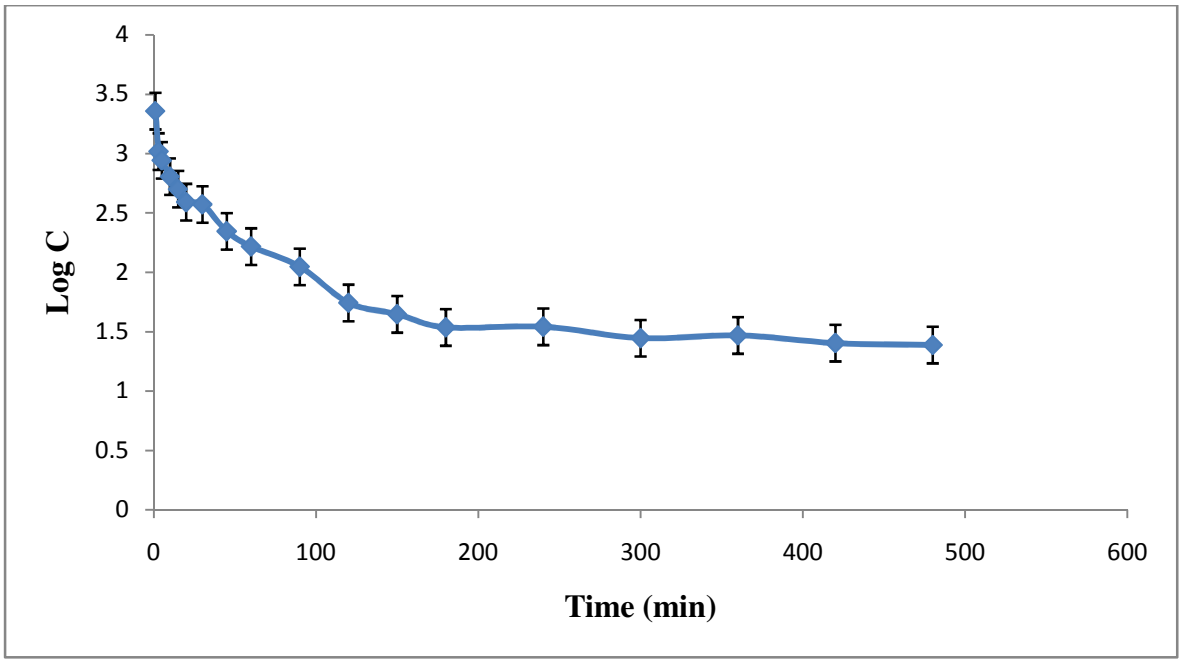


Figure 5.12 Mean plasma concentration time profile of SN79-di-HCl in fed state male Sprague dawley rats intravenous administration (5mg/kg) (n=6)

Table 5.12 Mean plasma pharmacokinetic parameters of SN79-di-hydrochloride after oral administration to fed state male Sprague dawley rats (20mg/kg) (n=6)

Compound	AUC (ug-hr/mL)	$T_{1/2}$ (hr)	C_{max} (ug/mL)	MRT (hr)	V_d (L/kg)	CL (L/hr/kg)	T_{max}(hr)
SN79-*di*-HCl	111.3 ± 0.12	7.8 ± 0.7	0.21 ± 0.21	10.1 ± 1.3	113.7 ± 1.1	0.13 ± 0.11	1.52 ± 0.05

AUC – area under the curve; $T_{1/2}$ – plasma half life; C_{max} – maximum plasma concentration; MRT – mean residence time; V_d – volume of distribution; CL – plasma clearance; T_{max} – time of maximum plasma concentration.

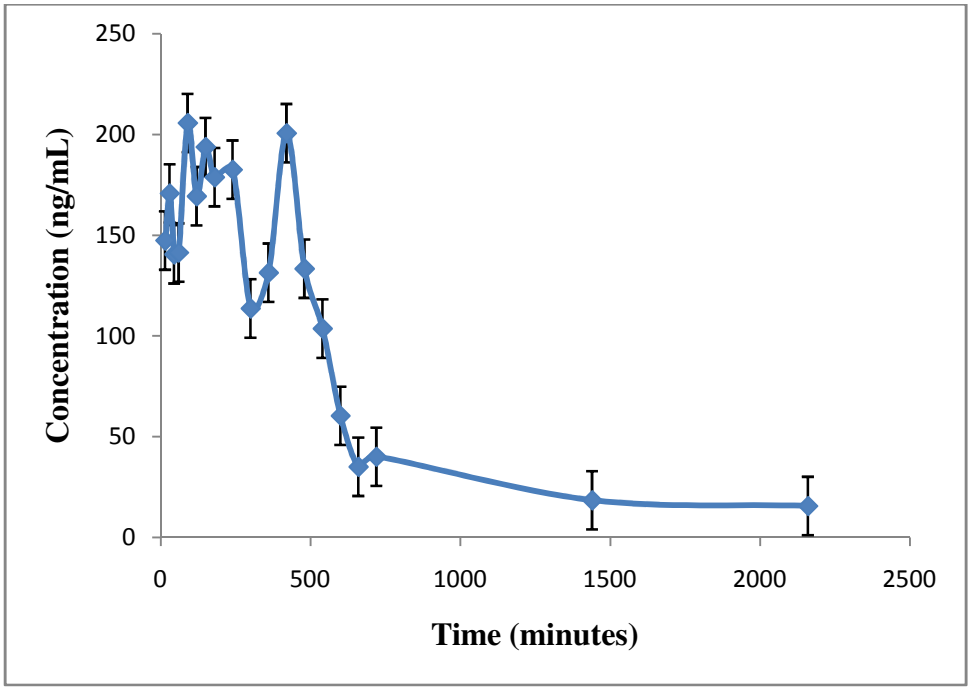


Figure 5.13 Mean plasma concentration time profile of SN79-di-HCl in fed state male Sprague dawley rats oral administration (20mg/kg) (n=6)

5.7 *In vitro* GIT Permeation Studies

The optimization of the bioavailability of drugs administered via the oral route is one of the most important aims for the pharmaceutical industry during the development phase of new products [301]. Bioavailability is characterized by both the amount of drug absorbed from the dosage form reaching the systemic circulation and the rate of this process. For the oral route, the quantity of drug reaching the systemic circulation is influenced by many factors. These include physicochemical interactions with the environment in the intestinal lumen, as well as biological interactions with the physiology and anatomy of the intestinal membrane, which either interfere or aid in the transport process [270]. For most drugs, the absorption from the gastrointestinal tract is mediated by passive diffusion from the lipid membrane.

The absorption pattern of SN79-*di*-HCl was studied across the gastrointestinal tract. Various sections of the gut including duodenum, jejunum and ileum were used to determine the permeability of SN79. Mass transport across a barrier, can be caused by diffusion or migration. Permeability was calculated from the measured flux of diffusion and the concentration gradient. The permeation of SN79 from mucosal side to serosal (A-B) and from serosal to mucosal side (B-A) was studied (Table 5.13). Permeability of SN79-*di*-HCl from A-B across duodenum, jejunum and ileum was 2.53×10^{-6} cm/sec, 2.33×10^{-6} cm/sec and 2.47×10^{-6} cm/sec respectively. There was no significant difference in these values. Similarly, the permeability of SN79-*di*-HCl from B-A across duodenum, jejunum and ileum was 1.64×10^{-5} cm/sec, 1.73×10^{-5} cm/sec and 1.61×10^{-5} cm/sec.

Table 5.13 Mean apparent permeability of SN79-di-HCL from mucosal to serosal (A-B) and from serosal to mucosal side (B-A)

Section of GIT	SN79-*di*-HCl		
	(A-B) P_{app} x 10^{-6} cm/sec	(B-A) P_{app} x 10^{-5} cm/sec	(B-A)/(A-B)
Duodenum	2.53	1.64	0.65
Jejunum	2.33	1.73	0.74
Ileum	2.47	1.61	0.65

P_{app} – apparent permeability coefficient of SN79-di-HCl

5.8 *In vitro* Metabolic Stability

The liver poses particular problems in constructing physiologically-based pharmacokinetic models since this organ is not only a distribution site for drugs/chemicals but frequently the major site of metabolism [302]. The importance of hepatic drug metabolism is substantial and also it is crucial to the success of the model that the *in vitro* data on the kinetics of metabolism be incorporated in a judicious manner. The key parameter in *in vitro* drug metabolism is intrinsic clearance (CL_{int}), which is a pure measure of enzyme activity towards a substrate and is independent of other physiological determinants such as blood flow or drug binding within the matrix [302]. Apparent intrinsic clearance of SN79 was measured using rat liver microsomes. The determination of *in vitro* intrinsic clearance (Cl_{int}) for drug candidates in the early discovery stage is a common practice in pharmaceutical industry [269]. From toxicological and pharmacological points of view, it is desirable to design a "safer" drug that undergoes predictable metabolic inactivation or no metabolism [303]. The *in vitro* half life of SN79 when incubated in rat liver microsomes was found to be 0.25 hr, which is less than *in vivo* half life

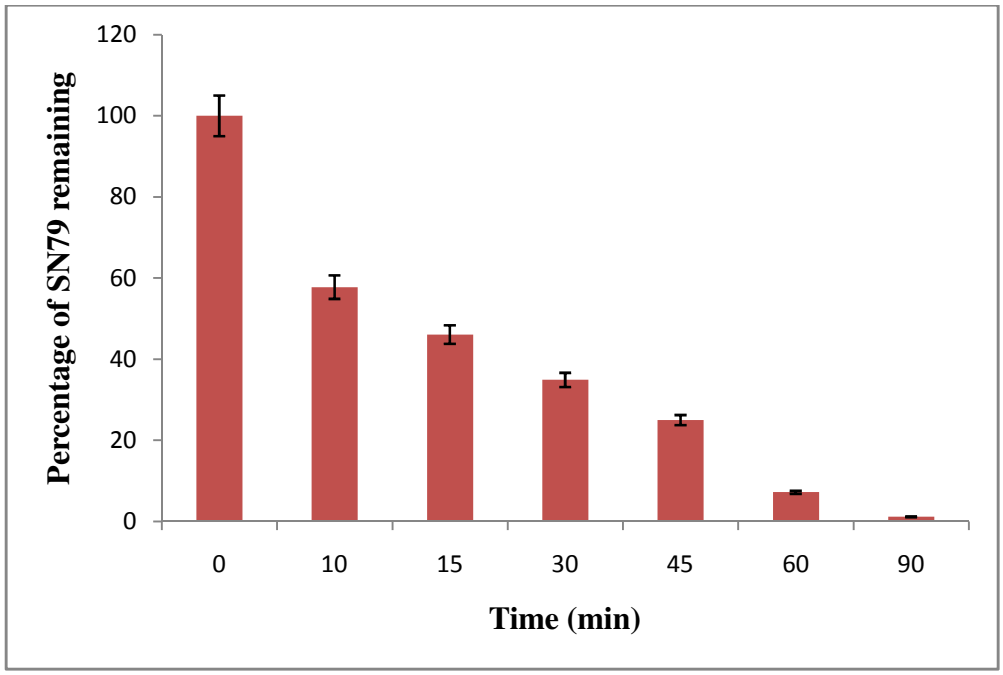


Figure 5.14 Metabolic stability of SN79-di-HCl incubated in rat liver microsomes (n=3)

Table 5.14 Metabolic stability of SN79-di-HCl incubated in rat liver microsomes (n=3)

Time (min)	% SN79 Remaining	SEM
0	100	0
10	57.8	0.03
15	46.1	0.02
30	34.9	0.03
45	25	0.09
60	7.2	0.02
90	1.2	0.02

SEM = standard deviation/mean

value, 1.36 hr after intravenous administration (Table 5.6). The apparent intrinsic clearance derived from *in vitro* $t_{1/2}$ value is 4.66 L/hr/kg. This value is higher than the *in vivo* clearance value, 0.18 L/Hr/kg after intravenous administration (Table 5.6). This might be attributed to the high protein binding nature of SN79-*di*-HCl. Owing to its high affinity for plasma proteins (Section 5.5), the compound maybe less available for metabolizing enzymes. The *in vivo* $t_{1/2}$

(1.36 hr) is prolonged since larger drug-protein complex cannot easily penetrate the metabolizing organ, liver. SN79-*di*-HCl is considered metabolically unstable since less than 20% of the compound was remaining at sixty minutes [304].

CHAPTER VI: CONCLUSIONS

With the advance of medicinal chemistry, many new tools have been developed to help discover better drugs faster. In pursuit of a selective sigma-2 receptor ligand, SN79 (6-acetyl-3-(4-(4-(4-fluorophenyl) piperazin-1-yl) butyl) benzo[*d*] oxazol-2 (3H)-one) with a molecular weight of 411.3 daltons was synthesized by Dr. Christopher R. McCurdy's group from the Department of Medicinal Chemistry at the University of Mississippi. This compound was selected for further development as a potential lead candidate. Absorption, distribution, metabolism and excretion were determined as these processes play a critical role in selecting high quality hits and leads for drug discovery programs.

Ultra performance liquid chromatography-mass spectrophotometry (UPLC-MS) was the analysis tool of choice throughout this dissertation. A simple, sensitive and accurate UPLC-MS method using single ion reaction mode was developed in rat plasma that is capable of rapid separation and selective identification of SN79 in rat plasma. The extraction procedure is simple and short, allowing sufficient sample-throughput to be applied to pharmacokinetic studies of SN79. The method was validated in accordance with FDA guidelines and was shown to be reproducible and accurate. The volume of injection was optimized to 10 µL, thereby making it beneficial considering the limitation of sample volumes obtained during pharmacokinetic studies. Thus, the method was successfully applied to *in vivo* studies conducted in rats.

As part of drug discovery and development, there is a need to better understand the physicochemical properties of SN79. Assays were performed to determine important physicochemical properties that influence oral absorption, membrane permeation and a host of other critical ADME processes. Log P and pK_a values, solubility in aqueous and buffered media, influence of temperature and pH on stability were determined. Based on these results, it was concluded that SN79 is basic and lipophilic.

Understanding the impact stability continues to have on drug discovery and development, a number of *in vitro* studies including stability testing in simulated gastric and intestinal fluids and metabolic stability in rat liver microsomes were also determined. SN79 emerged stable in the gastrointestinal fluids suggesting no precipitation or degradation occurs during absorption from the gut, while it exhibited susceptibility to metabolism such that less than 20% remained after 60 minutes incubation with rat liver microsomes.

With the required physiochemical data and knowledge of *in vitro* behavior, single dose pharmacokinetic studies were conducted in fasted state male Sprague Dawley rats. The average oral bioavailability was ~66% in fasted state subjects. A qualitative visual examination of the data indicated the presence of multiple peaks in the concentration-time profile of oral SN79-*di*-HCl. Similar profiles were noticed for several compounds in literature and one of the plausible reason was the presence of absorption windows along the gastrointestinal tract. Consequently, a similar pharmacokinetic study was performed at the same dose in rats to establish the role of food on absorption of SN79-*di*-HCl from the gut. It was concluded that results from this study had no impact on the appearance of multiple peaks because the same phenomena was observed in the oral profile of fed state rats also. In support of this data, *in vitro* permeation in the gastrointestinal tract and plasma protein binding nature of SN79-*di*-HCl were also studied. The

results revealed that SN79-*di*-HCl is highly protein bound (~93%) with concentration dependence. The permeation of SN79-*di*-HCl along the three segments of the gut, duodenum, jejunum and ileum was found to be similar, thus, ruling out the possibility of presence of absorption windows for SN79 along GIT.

In conclusion, a novel synthetic compound, SN79 was researched as part of drug discovery and development with different focuses. Despite low *in vitro* metabolic stability and high protein binding affinity, the compound did have good oral bioavailability and showed no severe acute toxicity response in rats at the doses administered, thereby paving way to take the compound for further investigations. Structural modifications to the chemical moiety might help improve the metabolic stability without compromising bioavailability.

CHAPTER VII: FUTURE STUDIES

- The underlying cause for appearance of multiple peaks in oral concentration-time profile of SN79-*di*-HCl must be determined. Based on data published in literature, studies can be conducted to see if variable gastric emptying rates and intestinal flow rates has a role in inducing multiple peaks.
- One major determinant of pharmacokinetics of SN79-*di*-HCl is its disposition in the brain. Since SN79 is a drug that acts on the central nervous system, it is imperative to establish the amount of compound that crosses the blood brain barrier. This can be achieved by harvesting the brain of the rat after termination of the pharmacokinetic study and analyzing it for the presence of SN79.
- Drug efflux pumps such as P-glycoprotein and multidrug resistance proteins are known to influence the pharmacokinetics of drugs administered intravenously and orally. To ascertain whether or not these transporters have a bearing on the intestinal absorption and tissue distribution of SN79, pharmacokinetic studies can be performed in presence and absence of transporter inhibitors and compare the difference in oral bioavailabilities thus obtained.
- For certain drugs, *in vivo* metabolites tend to have similar therapeutic response as the parent drug or in some cases, induce a toxic response. Hence, identification and quantitative determination of metabolites in rat urine might be helpful in understanding the pathways of metabolism, thereby mitigating any unwanted effects.

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Appendix A

Table A.1. Calibration curve of SN79-*di*-HCl for intraday accuracy and precision of SN79 in rat plasma.

Conc (ng/ml)	Area
5	390
10	766
50	4008
100	7703
500	40026
1000	72193
5000	401532

Initial Conc (ng/mL)	Area	Response	Conc (ng/mL)	Accuracy	RSD
5	346.0	0.0026	5.069	101.38	0.03
	354.1	0.0025	4.935	98.71	
	355.3	0.0026	5.050	101.01	
	359.3	0.0026	5.005	100.10	
	357.0	0.0025	4.886	97.71	
	353.6	0.0024	4.696	93.93	
15	1077	0.0077	14.95	99.6	0.02
	1099	0.0078	15.16	101.0	
	1092	0.0076	14.74	98.2	
	1085	0.0076	14.92	99.50	
	1091	0.0079	15.40	102.65	
	1086	0.0077	15.09	100.6	
200	14504	0.0976	190.4	95.22	0.03
	15085	0.1019	198.8	99.4	
	14557	0.1039	202.7	101.36	
	14750	0.1028	200.7	100.34	
	15263	0.1061	207.1	103.57	
	14885	0.1032	201.4	100.7	
3000	230753	1.5233	2973	99.10	0.02
	230937	1.6016	3126	104.2	
	229580	1.6188	3159	105.32	
	222925	1.5979	3119	103.95	
	228500	1.5662	3057	101.89	
	225442	1.6183	3158	105.28	

RSD = standard deviation/mean accuracy = (conc/spiked conc)*100

Table A.2. Calibration curve of SN79-*di*-HCl for intraday accuracy and precision of SN79 in rat plasma after 4 hr.

Conc (ng/ml)	Area
5	417
10	752
50	3825
100	7815
500	38293
1000	74235
5000	425320

Initial Conc (ng/mL)	Area	Response	Conc (ng/mL)	Accuracy	RSD
5	345.6	0.0025	4.882	97.63	0.02
	335.7	0.0024	4.748	94.96	
	324.2	0.0025	4.885	97.71	
	333.8	0.0024	4.733	94.66	
	332.4	0.0025	4.907	98.15	
	337.6	0.0024	4.707	94.13	
15	1165	0.0084	16.32	108.8	0.05
	1236	0.0080	15.70	104.6	
	1030	0.0077	15.04	100.3	
	1019	0.0073	14.31	95.38	
	1099	0.0075	14.67	97.80	
	1125	0.0081	15.83	105.6	
200	13705	0.0980	191.2	95.59	0.03
	13795	0.1043	203.6	101.8	
	13699	0.1016	198.3	99.15	
	13703	0.1020	199.1	99.56	
	13599	0.1006	196.3	98.14	
	13741	0.1055	206.0	103.0	
3000	189435	1.4387	2808	93.60	0.04
	209750	1.5591	3043	101.4	
	194008	1.4735	2876	95.86	
	200329	1.4836	2896	96.52	
	185615	1.4076	2747	91.57	
	185121	1.4081	2748	91.60	

RSD = standard deviation/mean accuracy = (conc/spiked conc)*100

Table A.3. Calibration curve of SN79-*di*-HCl for intraday accuracy and precision of SN79 in rat plasma after 6 hr.

Conc (ng/ml)	Area
5	350
10	730
50	3429
100	7329
500	33772
1000	77708
5000	308510

Initial Conc (ng/mL)	Area	Response	Conc (ng/mL)	Accuracy	RSD
5	257.8	0.0019	4.157	83.14	0.06
	255.9	0.0019	4.201	84.03	
	258.8	0.0019	4.336	86.72	
	242.2	0.0018	4.031	80.62	
	253.8	0.0018	4.092	81.84	
	222.9	0.0016	3.603	72.06	
15	876	0.0063	14.24	94.9	0.01
	890	0.0063	14.20	94.7	
	892	0.0064	14.29	95.3	
	880	0.0063	14.11	94.06	
	885	0.0065	14.59	97.28	
	881	0.0064	14.26	95.0	
200	12724	0.0911	204.5	102.27	0.02
	12191	0.0877	196.8	98.4	
	11911	0.0874	196.2	98.08	
	12327	0.0881	197.7	98.87	
	11756	0.0894	200.6	100.30	
	12777	0.0913	204.8	102.4	
3000	188208	1.3563	3044	101.46	0.01
	182920	1.3488	3027	100.9	
	189935	1.3743	3084	102.80	
	187775	1.3578	3047	101.57	
	189942	1.3743	3084	102.81	
	187121	1.3420	3012	100.39	

RSD = standard deviation/mean accuracy = (conc/spiked conc)*100

Table A.4. Calibration curve of SN79-*di*-HCl for intraday accuracy and precision of SN79 in rat plasma after 8 hr.

Conc (ng/ml)	Area
5	371
10	688
50	3764
100	6895
500	35483
1000	66826
5000	339499

Initial Conc (ng/mL)	Area	Response	Conc (ng/mL)	Accuracy	RSD
5	334.7	0.0025	5.037	100.73	0.02
	324.9	0.0024	4.914	98.28	
	322.9	0.0024	4.982	99.64	
	314.1	0.0024	4.815	96.30	
	319.2	0.0024	4.839	96.79	
	320.9	0.0025	5.043	100.86	
15	1089	0.0080	16.39	109.2	0.02
	1098	0.0079	16.16	107.7	
	1083	0.0078	15.88	105.9	
	1055	0.0076	15.61	104.05	
	1052	0.0079	16.24	108.25	
	1067	0.0076	15.64	104.3	
200	13585	0.0976	199.7	99.86	0.03
	14282	0.1019	208.5	104.2	
	13569	0.0975	199.5	99.76	
	13430	0.0962	196.8	98.41	
	13278	0.0949	194.2	97.11	
	13811	0.1018	208.3	104.2	
3000	204942	1.4720	3011	100.38	0.01
	202909	1.4777	3023	100.8	
	202276	1.5061	3081	102.71	
	200055	1.5015	3072	102.39	
	206572	1.4813	3030	101.01	
	208576	1.4949	3058	101.95	

RSD = standard deviation/mean accuracy = (conc/spiked conc)*100

Table A.5. Calibration curve of SN79-*di*-HCl for interday accuracy and precision on day one.

Conc (ng/ml)	Area
5	377
10	604
50	3720
100	6073
500	35143
1000	65817
5000	388304

Initial Conc (ng/mL)	Area	Response	Conc (ng/mL)	Accuracy	RSD
5	354.2	0.0025	5.046	100.93	0.02
	349.3	0.0025	4.987	99.74	
	355.9	0.0025	5.053	101.07	
	334.8	0.0024	4.785	95.70	
	349.1	0.0025	4.987	99.73	
	342.7	0.0025	4.880	97.61	
15	973	0.0071	14.17	94.5	0.02
	954	0.0069	13.64	90.9	
	968	0.0070	13.93	92.9	
	952	0.0073	14.58	97.22	
	939	0.0071	14.19	94.59	
	959	0.0069	13.74	91.6	
200	14255	0.1102	218.9	109.47	0.04
	13940	0.1004	199.4	99.7	
	13828	0.1015	201.8	100.88	
	13397	0.1010	200.7	100.37	
	13685	0.0982	195.1	97.56	
	13244	0.1024	203.4	101.7	
3000	200970	1.4494	2880	96.01	0.05
	211646	1.4521	2886	96.2	
	209203	1.5737	3127	104.25	
	210913	1.6266	3233	107.75	
	216475	1.4773	2936	97.86	
	211500	1.4967	2974	99.14	

RSD = standard deviation/mean accuracy = (conc/spiked conc)*100

Table A.6. Calibration curve of SN79-*di*-HCl for interday accuracy and precision on day two.

Conc (ng/ml)	Area
5	429
10	810
50	4653
100	10510
500	40843
1000	79450
5000	396903

Initial Conc (ng/mL)	Area	Response	Conc (ng/mL)	Accuracy	RSD
5	406.1	0.0030	5.098	102.0	0.02
	413.1	0.0031	5.339	106.8	
	410.0	0.0031	5.283	105.7	
	411.4	0.0030	5.154	103.1	
	413.3	0.0031	5.229	104.6	
	405.9	0.0030	5.061	101.2	
15	1163	0.0083	14.14	94.29	0.01
	1164	0.0085	14.38	95.87	
	1185	0.0085	14.40	96.01	
	1121	0.0086	14.60	97.34	
	1190	0.0087	14.71	98.10	
	1101	0.0084	14.26	95.07	
200	17126	0.1231	209.1	104.5	0.04
	15200	0.1132	192.3	96.16	
	16860	0.1233	209.6	104.8	
	17083	0.1230	208.9	104.5	
	15356	0.1144	194.4	97.21	
	15550	0.1138	193.3	96.63	
3000	245492	1.8604	3161	105.4	0.02
	242173	1.8021	3062	102.1	
	248652	1.7821	3028	100.9	
	247487	1.8740	3184	106.1	
	242558	1.7702	3007	100.2	
	237068	1.8029	3063	102.1	

RSD = standard deviation/mean accuracy = (conc/spiked conc)*100

Table A.7. Calibration curve of SN79-*di*-HCl for interday accuracy and precision on day three.

Conc (ng/ml)	Area
5	390
10	766
50	4008
100	7703
500	40026
1000	72193
5000	341532

Initial Conc (ng/mL)	Area	Response	Conc (ng/mL)	Accuracy	RSD
5	336.3	0.0025	5.053	101.1	0.02
	324.1	0.0025	4.989	99.77	
	345.3	0.0025	5.034	100.7	
	318.9	0.0025	4.906	98.12	
	347.0	0.0026	5.237	104.7	
	343.6	0.0025	5.022	100.4	
15	1078	0.0082	16.51	110.1	0.05
	1000	0.0076	15.22	101.5	
	1042	0.0075	14.94	99.57	
	1025	0.0073	14.66	97.74	
	1011	0.0073	14.63	97.51	
	1016	0.0073	14.58	97.21	
200	13306	0.1019	203.9	101.9	0.01
	12785	0.0983	196.7	98.37	
	12857	0.0988	197.7	98.87	
	13050	0.1000	200.2	100.1	
	13263	0.0991	198.4	99.20	
	13285	0.0990	198.1	99.05	
3000	200753	1.4818	2966	98.86	0.03
	210937	1.5719	3146	104.9	
	200580	1.4346	2871	95.72	
	210325	1.5076	3018	100.6	
	210600	1.4947	2992	99.73	
	208642	1.4977	2998	99.93	

RSD = standard deviation/mean accuracy = (conc/spiked conc)*100

Table A.8. Calibration curve of SN79-*di*-HCl for short term freeze thaw stability in rat plasma.

Conc (ng/ml)	Area
5	390
10	766
50	4008
100	7703
500	40026
1000	72193
5000	341532

Short-term stability of SN79-*di*-HCl after 4 hours of incubation in rat plasma (n=3)

Initial Conc (ng/mL)	Area	Response	Conc (ng/mL)	Accuracy
5	751.8	0.0057	4.399	87.97
	770.3	0.0057	4.454	89.08
	770.9	0.0058	4.654	93.08
15	1421	0.0105	13.96	93.05
	1412	0.0102	13.32	88.81
	1441	0.0107	14.49	96.57
200	13554	0.0999	192.8	96.38
	13776	0.0992	191.5	95.74
	13916	0.1011	195.2	97.60
3000	205705	1.5137	3020	100.7
	197986	1.5767	3146	104.9
	199359	1.4726	2938	97.94

RSD = standard deviation/mean accuracy = (conc/spiked conc)*100

Short-term stability of SN79-*di*-HCl after 6 hours of incubation in rat plasma (n=3)

Initial Conc (ng/mL)	Area	Response	Conc (ng/mL)	Accuracy	RSD
5	763.2	0.0056	4.120	82.39	6.24
	772.7	0.0058	4.626	92.52	
	775.5	0.0056	4.213	84.25	
15	1389	0.0101	13.16	87.70	1.54
	1381	0.0101	13.12	87.46	
	1385	0.0102	13.49	89.93	
200	11937	0.0907	174.4	87.20	3.83
	11565	0.0843	161.6	80.78	
	11834	0.0872	167.3	83.66	
3000	168592	1.1990	2391	79.7	0.60
	169405	1.2131	2419	80.6	
	167470	1.2039	2401	80.03	

RSD = standard deviation/mean accuracy = (conc/spiked conc)*100

Short-term stability of SN79-*di*-HCl after 8 hours of incubation in rat plasma (n=3)

Initial Conc (ng/mL)	Area	Response	Conc (ng/mL)	Accuracy	RSD
5	747.0	0.0056	4.150	83.00	1.55
	732.7	0.0056	4.183	83.66	
	752.7	0.0055	4.059	81.18	
15	1357	0.0100	13.10	87.33	3.89
	1337	0.0096	12.15	81.01	
	1326	0.0097	12.42	82.83	
200	11751	0.0865	166.0	83.01	3.93
	11904	0.0905	173.9	86.96	
	11707	0.0839	160.9	80.43	
3000	165908	1.2317	2456	81.88	2.16
	163912	1.1948	2383	79.42	

RSD = standard deviation/mean accuracy = (conc/spiked conc)*100

Table A.9. Calibration curve of SN79-*di*-HCl for freeze thaw cycle in rat plasma.

Conc (ng/ml)	Area
5	251
10	474
50	2572
100	4545
500	26544
1000	46427
5000	239845

Stability of SN79-*di*-HCl in rat plasma after three freeze thaw cycles in rat plasma (n=3)

Initial Conc (ng/mL)	Area	Response	Conc (ng/mL)	Accuracy	RSD
5	245.7	0.0018	5.175	103.5	1.39
	246.0	0.0018	5.037	100.7	
	247.5	0.0018	5.139	102.8	
15	758.6	0.0054	15.21	101.4	1.48
	755.7	0.0055	15.53	103.5	
	740.6	0.0056	15.65	104.3	
200	9423	0.0705	197.6	98.81	1.43
	9588	0.0690	193.3	96.63	
	9481	0.0708	198.5	99.26	
3000	137081	0.9940	2786	92.86	2.13
	135077	1.0369	2906	96.87	
	132086	1.0113	2834	94.48	

RSD = standard deviation/mean accuracy = (conc/spiked conc)*100

Table A.10. Calibration curve of SN79-*di*-HCl for 30 days stability in rat plasma.

Conc (ng/ml)	Area
5	436
10	974
50	4694
100	9095
500	41304
1000	83955
5000	423645

Stability of SN79-*di*-HCl in rat plasma after 30 days (n=3)

Initial Conc (ng/mL)	Area	Response	Conc (ng/mL)	Accuracy	RSD
5	390.2	0.0029	4.729	94.58	1.73
	384.8	0.0029	4.632	92.64	
	380.1	0.0028	4.529	90.58	
15	1111	0.0084	13.59	90.58	3.26
	1132	0.0087	14.08	93.85	
	1098	0.0083	13.54	90.29	
200	15741	0.1188	193.0	96.48	4.31
	15517	0.1145	186.0	93.01	
	14376	0.1090	177.0	88.52	
3000	203441	1.4777	2400	80.02	0.94
	230399	1.7375	2823	94.08	
	234513	1.6797	2729	90.95	

RSD = standard deviation/mean accuracy = (conc/spiked conc)*100

Appendix B

Table.B.1. pKa determination of SN79-*di*-HCl. Data presented as volume of HCl added vs pH of the resulting SN79 solution. (n=2)

Volume of NaOH added (in µL)	pH
100	6.16
200	6.69
300	7.26
400	8.2
500	8.51
600	8.79
700	9.18
800	9.15
900	9.35
1000	9.49
1100	9.6
1200	9.65
1300	9.7
1400	9.77
1500	9.82
Volume of HCl added (in µL)	**pH**
100	9.52
200	9.26
300	8.19
400	7.52
500	7.23
600	7.05
700	6.89
800	6.73
900	6.59
1000	6.43
1100	6.24
1200	6.02
1300	5.65
1400	5.07
1500	4.62
1600	4.37
1700	4.22
1800	4.1
1900	4.01
2000	3.93
2100	3.87
2200	3.81
2300	3.77

Volume of NaOH added (in µL)	pH
100	6.37
200	7.28
300	8.33
400	8.86
500	9.14
600	9.12
700	9.17
800	9.28
900	9.33
1000	9.38
Volume of HCl added (in µL)	**pH**
100	9.16
200	8.68
300	7.72
400	7.29
500	6.92
600	6.72
700	6.48
800	6.29
900	5.99
1000	5.61
1100	5.11
1200	4.69
1300	4.44
1400	4.29
1500	4.18
1600	4.08
1700	4.01
1800	3.94
1900	3.88
2000	3.84

Table B.2. Calibration curve of SN79-di-HCl for solubility studies.

Conc (ng/mL)	Area
5	399
10	759
25	2047
50	4209
100	7884
200	15938
500	38451
1000	72987
3000	218359
5000	407577

pH dependant solubility of SN79-di-HCl in buffers prepared as per the USP guidelines (n=3).

pH	Area	Conc (ng/mL)	Dilution Factor	Conc (ng/mL)	Conc (mg/mL)	Average	SEM
1.2	93299	1196	20000	23914866	23.9	23.9	0.01
	91526	1173	20000	23468879	23.5		
	94319	1209	20000	24171485	24.2		
3	101434	1298	20000	25961263	26.0	26.5	0.03
	106970	1368	20000	27353792	27.4		
	102468	1311	20000	26221356	26.2		
5	110469	1412	20000	28233933	28.2	28.8	0.03
	111200	1421	20000	28417809	28.4		
	116382	1486	20000	29721293	29.7		
7.4	107548	1375	20000	27499182	27.5	26.6	0.04
	99172	1270	20000	25392278	25.4		
	105146	1345	20000	26894982	26.9		
9	80639	1037	20000	20730474	20.7	21.0	0.01
	82923	1065	20000	21304993	21.3		
	81737	1050	20000	21006666	21.0		

SEM = standard deviation/average

Table B.3. Stability of SN79-*di*-HCl in stimulated gastric fluid (pH = 1.2) prepared as per USP guidelines (n=3)

Time (min)	Area	Conc (ng/mL)	Conc (µg/mL)	Mean Conc (µg/mL)	SEM	RD
0	910213	4520.6	4.52	4.46	0.04	0
	881696	4379.1	4.38			
	899613	4468.0	4.47			
60	904972	4494.6	4.49	4.46	0.04	-0.04
	881715	4379.2	4.38			
	906013	4499.8	4.50			

SEM = standard deviation/average; RD = [(initial conc-conc at time t)/initial conc]*100

Table B.4. Stability of SN79-*di*-HCl in stimulated intestinal fluid (pH = 1.2) prepared as per USP guidelines (n=2)

Time (min)	Area	Conc (ng/mL)	Conc (µg/mL)	Mean Conc (µg/mL)	SEM	RD
0	923610	4587.1	4.59	4.56	0.03	0
	910800	4523.6	4.52			
60	921231	4575.3	4.58	4.57	0.01	-0.25
	917681	4557.7	4.56			
120	933605	4636.7	4.64	4.60	0.04	-0.96
	918517	4561.9	4.56			
180	916136	4550.0	4.55	4.55	0.003	0.19
	914804	4543.4	4.54			

SEM = standard deviation/average; RD = [(initial conc-conc at time t)/initial conc]*100

Table B.5. Calibration curve of SN79-*di*-HCl for protein binding study

Conc(ng/mL)	Drug Area
5	1001
10	2802
25	4907
50	8941
100	18231
200	37123
500	93934
1000	208675
3000	608090
5000	1009967

Concentration dependant protein binding study of SN79-*di*-HCl incubated in rat plasma (n=3)

Incubated Conc (ug/mL)	Area	Concug/mL	Mean Conc (ug/mL)	F_u	F_u %	SEM
0.5	2895	0.019	0.019	0.039	3.90	0.006
	2903	0.019				
	2938	0.019				
1	7593	0.042	0.043	0.045	4.53	0.029
	8060	0.044				
	8006	0.044				
5	50288	0.253	0.257	0.054	5.42	0.030
	50325	0.253				
	52972	0.266				
10	153593	0.763	0.761	0.082	8.24	0.002
	153060	0.760				
	153006	0.760				

SEM = standard deviation/average; RD = [(initial conc-conc at time t)/initial conc]*100

Table B.6. Calibration curve for determination of SN79 content in rat plasma samples after administration of SN79-*di*-HCl to fasted state male Sprague Dawley rats

Conc (ng/mL)	Ratio
5	0.0034
10	0.0058
25	0.0153
50	0.0304
100	0.0573
250	0.1250
300	0.1563
500	0.2274
1000	0.5120
2500	1.2387
3000	1.4221
5000	2.1916

Determination of SN79 content in rat plasma samples after IV administration of SN79-*di*-HCl to fasted state Sprague Dawley rats (5mg/kg) - Rat 1

Time (min)	Drug Area	IS Area	Ratio	Conc (ng/mL)
1	75998	124576	0.61	1392
3	51497	106189	0.48	1106
5	48688	115329	0.42	963
10	26641	109322	0.24	556
15	21136	122032	0.17	395
20	19460	114843	0.17	387
30	13289	119084	0.11	255
45	12097	114319	0.11	241
60	11388	118356	0.10	219
90	6699	126714	0.05	121
120	2866	136586	0.02	48
150	2176	137453	0.02	36
180	1584	127064	0.01	28
240	1473	130338	0.01	26
300	1189	117070	0.01	23
360	993	141152	0.01	16
420	895	131568	0.01	16
480	850	133052	0.01	15

Determination of SN79 content in rat plasma samples after IV administration of SN79-*di*-HCl to fasted state Sprague Dawley rats (5mg/kg) - Rat 2

Time (min)	Drug Area	IS Area	Ratio	Conc (ng/mL)
1	91793	118336	0.78	1769
3	71257	141920	0.50	1145
5	46861	125929	0.37	849
10	43100	139799	0.31	703
15	31178	140848	0.22	505
20	24993	138601	0.18	411
30	19980	126571	0.16	360
60	18854	126424	0.15	340
90	12093	134638	0.09	205
120	10218	74464	0.14	313
150	2787	114096	0.02	56
180	1925	132230	0.01	33
240	1453	132138	0.01	25
300	1438	138531	0.01	24
360	1057	136133	0.01	18
420	963	141834	0.01	15
480	799	132122	0.01	14

Determination of SN79 content in rat plasma samples after IV administration of SN79-*di*-HCl to fasted state Sprague Dawley rats (5mg/kg) - Rat 3

Time (min)	Drug Area	IS Area	Ratio	Conc (ng/mL)
1	73645	148165	0.50	1134
3	44712	152349	0.29	669
5	36840	145382	0.25	578
10	36662	131785	0.28	635
15	29201	151034	0.19	441
20	25853	154501	0.17	382
30	16762	151104	0.11	253
45	15286	129051	0.12	270
60	11509	151502	0.08	173
90	7498	129894	0.06	132
120	4959	127697	0.04	89
150	3353	141429	0.02	54
180	2592	166453	0.02	36
240	1905	131674	0.01	33
300	1541	136474	0.01	26
360	1403	141500	0.01	23
420	923	152985	0.01	14
480	733	157022	0.005	11

Determination of SN79 content in rat plasma samples after IV administration of SN79-*di*-HCl to fasted state Sprague Dawley rats (5mg/kg) - Rat 4

Time (min)	Drug Area	IS Area	Ratio	Conc (ng/mL)
1	82258	142829	0.58	1314
3	68813	125980	0.55	1246
10	33270	119369	0.28	636
15	23660	138029	0.17	391
20	18636	137527	0.14	309
30	15472	134626	0.11	262
45	15253	150298	0.10	231
60	6805	158665	0.04	98
90	5511	129904	0.04	97
120	4159	135634	0.03	70
150	1819	150266	0.01	28
180	1654	131344	0.01	29
240	938	141375	0.01	15
300	879	137283	0.01	15
360	710	112066	0.01	14
420	483	129848	0.004	8
480	412	126564	0.004	10

Determination of SN79 content in rat plasma samples after IV administration of SN79-*di*-HCl to fasted state Sprague Dawley rats (5mg/kg) - Rat 5

Time (min)	Drug Area	IS Area	Ratio	Conc (ng/mL)
1	67201	138599	0.48	1106
3	44276	132513	0.33	762
5	35969	130333	0.28	630
10	26858	134592	0.20	455
15	17950	124994	0.14	328
20	16268	130554	0.12	284
30	12070	130196	0.09	211
45	11754	130542	0.09	205
60	7530	151003	0.05	114
90	6248	127518	0.05	112
120	3113	132660	0.02	54
150	2947	147527	0.02	46
180	2708	133342	0.02	46
240	1222	125014	0.01	22
300	974	125751	0.01	18
420	589	150914	0.004	9
480	409	118934	0.003	8

Determination of SN79 content in rat plasma samples after oral administration of SN79-*di*-HCl to fasted state Sprague Dawley rats (20mg/kg) - Rat 1

Time (min)	Drug Area	IS Area	Ratio	Conc (ng/mL)
15	11170	168336	0.07	151
30	13208	122661	0.14	325
45	9042	179422	0.09	212
60	7543	179231	0.08	177
90	12756	121404	0.11	240
120	11783	125972	0.14	313
150	9781	159550	0.10	234
180	8165	129293	0.09	202
240	9445	126827	0.11	248
300	4504	123754	0.05	123
360	6228	148330	0.06	144
420	5600	148747	0.06	129
480	7120	105882	0.07	153
540	7386	106590	0.07	158
600	4612	102549	0.04	103
660	2599	145138	0.03	62
720	969	174742	0.01	30
1440	388	112335	0.35	18
2160	101	126473	0.80	8

Determination of SN79 content in rat plasma samples after oral administration of SN79-*di*-HCl to fasted state Sprague Dawley rats (20mg/kg) - Rat 2

Time (min)	Drug Area	IS Area	Ratio	Conc (ng/mL)
15	7945	111727	0.07	162
30	13616	117916	0.12	278
45	8899	119460	0.07	170
60	8968	123543	0.07	166
90	10631	113522	0.09	214
120	10638	120228	0.09	202
150	15245	123068	0.12	283
180	12365	121316	0.10	232
240	12273	126015	0.10	222
300	4640	164130	0.07	165
360	5483	158424	0.09	214
420	15756	159836	0.26	601
480	5160	163703	0.08	185
540	2187	160144	0.04	83
600	2146	167955	0.03	72
660	1001	147531	0.02	48
720	1085	157401	0.02	43
1440	263	168206	0.004	13
2160	349	162403	0.01	9

Determination of SN79 content in rat plasma samples after oral administration of SN79-*di*-HCl to fasted state Sprague Dawley rats (20mg/kg) - Rat 3

Time (min)	Drug Area	IS Area	Ratio	Conc (ng/mL)
15	6283	110268	0.06	130
30	6658	116122	0.06	131
45	5648	109967	0.05	117
60	6145	102198	0.06	137
90	8272	115461	0.07	163
120	8377	101372	0.08	188
150	6602	106108	0.06	142
180	7717	134616	0.06	131
240	8037	132423	0.06	138
300	6353	118994	0.05	122
360	6286	120660	0.05	119
420	5339	119752	0.04	102
480	4803	119105	0.04	92
540	5880	140455	0.04	96
600	2584	127128	0.02	46
660	1485	117351	0.01	29
720	1373	115485	0.01	27
1440	887	116791	0.01	17
2160	218	132764	0.002	4

Determination of SN79 content in rat plasma samples after oral administration of SN79-*di*-HCl to fasted state Sprague Dawley rats (20mg/kg) - Rat 4

Time (min)	Drug Area	IS Area	Ratio	Conc (ng/mL)
15	10362	139895	0.07	169
30	10626	120782	0.09	201
45	8997	138006	0.07	149
60	7855	137838	0.06	130
90	11400	129154	0.09	201
120	7668	115219	0.07	152
150	8045	123779	0.06	148
180	8466	116968	0.07	165
240	7542	126376	0.06	136
300	5536	129267	0.04	98
360	5583	126820	0.04	100
420	3304	121492	0.03	62
480	2572	127154	0.02	46
540	1413	122696	0.01	26
600	1176	133420	0.01	20
660	617	110108	0.01	13
720	924	107360	0.01	20
1440	550	116177	0.005	11
2160	663	102927	0.01	15

Determination of SN79 content in rat plasma samples after oral administration of SN79-*di*-HCl to fasted state Sprague Dawley rats (20mg/kg) - Rat 5

Time (min)	Drug Area	IS Area	Ratio	Conc (ng/mL)
15	6745	132863	0.05	116
30	7860	132186	0.09	194
45	8267	126980	0.07	149
60	10567	130359	0.08	185
90	10795	120260	0.09	205
120	7369	140641	0.05	120
150	10778	133202	0.08	185
180	6909	108586	0.06	145
240	7291	129670	0.06	128
300	7015	113727	0.06	141
360	6092	111921	0.05	124
420	3304	126061	0.03	60
480	1713	122260	0.01	32
540	1154	140177	0.01	19
600	1493	139624	0.01	24
660	1186	122942	0.01	22
720	1424	129818	0.01	25
1440	1167	122623	0.01	22

Table B.7. Calibration curve for determination of SN79 content in rat plasma samples after administration of SN79-*di*-HCl to fed state male Sprague Dawley rats

Conc(ng/mL)	Ratio
5	0.0038
10	0.0083
25	0.0183
50	0.0402
100	0.0081
200	0.0185
500	0.3733
1000	0.8810
3000	2.3564
5000	3.8771

Determination of SN79 content in rat plasma samples after intravenous administration of SN79-*di*-HCl to fed state Sprague Dawley rats (5mg/kg) - Rat 1

Time (min)	Drug Area	IS Area	Ratio	Conc (ng/mL)
1	43339	158110	0.75	1500
3	24264	152799	0.46	927
5	20563	147702	0.43	870
10	14425	167046	0.30	439
15	12968	143766	0.22	601
20	9225	164889	0.16	293
30	8676	155032	0.14	324
45	6134	164574	0.09	198
60	5121	174527	0.07	146
90	4812	173380	0.07	139
120	1566	169648	0.02	53
150	928	165263	0.01	37
180	806	164798	0.01	33
240	541	171535	0.01	23
300	421	176166	0.01	19
360	409	160134	0.01	22
420	354	146410	0.01	23
480	316	161264	0.01	19

Determination of SN79 content in rat plasma samples after intravenous administration of SN79-*di*-HCl to fed state Sprague Dawley rats (5mg/kg) - Rat 2

Time (min)	Drug Area	IS Area	Ratio	Conc (ng/mL)
1	66005	159793	1.10	2216
3	30912	164288	0.52	970
5	29143	156424	0.48	1041
10	17060	153764	0.34	643
15	14343	142067	0.32	690
20	10647	166302	0.21	329
30	9063	142407	0.16	436
45	7739	163907	0.12	250
60	5967	159871	0.10	208
90	3163	162180	0.05	110
120	2131	168345	0.03	71
150	1693	164054	0.03	61
240	871	164618	0.01	35
300	504	158543	0.01	25
360	437	148586	0.01	26
420	360	145083	0.01	24
480	358	126394	0.01	35

Determination of SN79 content in rat plasma samples after intravenous administration of SN79-*di*-HCl to fed state Sprague Dawley rats (5mg/kg) - Rat 3

Time (min)	Drug Area	IS Area	Ratio	Conc (ng/mL)
1	46557	152767	0.88	1773
3	22014	148991	0.45	907
5	16599	151282	0.32	656
10	14106	151360	0.27	557
15	12468	157063	0.22	445
20	9842	161112	0.16	330
30	8270	160710	0.14	281
45	6353	162173	0.10	213
60	4524	167407	0.07	142
90	2667	166868	0.04	88
120	1692	172428	0.02	55
150	1393	167452	0.02	49
180	758	164265	0.01	32
240	725	168338	0.01	29
300	708	158654	0.01	32
360	578	158874	0.01	28
420	574	171860	0.01	24
480	522	155650	0.01	27

Determination of SN79 content in rat plasma samples after intravenous administration of SN79-*di*-HCl to fed state Sprague Dawley rats (5mg/kg) - Rat 4

Time (min)	Drug Area	IS Area	Ratio	Conc (ng/mL)
1	44576	175195	2.52	1194
3	32804	163198	0.59	1046
5	21584	160235	0.52	725
10	16238	165306	0.36	505
15	11729	153607	0.28	446
20	10428	137631	0.25	562
30	8393	164635	0.22	268
45	5062	151056	0.13	206
60	4482	146237	0.10	202
90	2595	166526	0.10	86
120	1669	161874	0.04	62
150	1489	154194	0.03	63
180	991	135473	0.03	64
240	747	151864	0.03	37
300	674	152931	0.01	34
360	627	144830	0.01	36
420	570	163851	0.01	26

Determination of SN79 content in rat plasma samples after intravenous administration of SN79-*di*-HCl to fed state Sprague Dawley rats (5mg/kg) - Rat 5

Time (min)	Drug Area	IS Area	Ratio	Conc (ng/mL)
1	55209	157246	0.96	1937
3	38427	162828	0.61	1231
5	35748	168213	0.52	1056
10	23248	160595	0.38	776
15	17684	164529	0.27	556
20	13568	163330	0.21	437
30	9396	150126	0.19	383
45	7366	160888	0.12	250
60	4653	146934	0.10	206
90	792	129595	0.03	62
120	303	128476	0.01	29
150	270	123314	0.01	31
180	264	126690	0.01	28
300	225	127371	0.01	25
360	193	125473	0.01	23
420	139	123615	0.01	20
480	123	131751	0.004	16

Determination of SN79 content in rat plasma samples after intravenous administration of SN79-*di*-HCl to fed state Sprague Dawley rats (5mg/kg) - Rat 6

Time (min)	Drug Area	IS Area	Ratio	Conc (ng/mL)
1	18022	130859	0.58	1176
3	16258	135051	0.46	936
5	10590	132425	0.33	661
10	9244	135331	0.26	531
15	7100	138814	0.19	374
20	4139	123066	0.18	367
30	3321	117474	0.18	388
45	2389	134257	0.07	148
60	1220	131546	0.04	86
90	1195	146350	0.03	60
120	568	124626	0.02	54
150	544	135586	0.02	39
180	333	135516	0.01	27
240	252	137166	0.01	22
360	221	140913	0.01	19
420	166	141801	0.004	16
480	102	133439	0.003	14

Determination of SN79 content in rat plasma samples after oral administration of SN79-*di*-HCl to fed state Sprague Dawley rats (20mg/kg) - Rat 1

Time (min)	Drug Area	IS Area	Ratio	Conc (ng/mL)
30	4261	139700	0.11	223
45	4763	144014	0.11	225
60	3403	137567	0.09	189
90	5103	142425	0.12	249
120	5511	144411	0.12	256
150	3929	132831	0.12	248
180	4455	147292	0.09	197
240	1573	132810	0.05	104
300	1307	136569	0.04	80
360	1124	131039	0.04	81
480	2933	122452	0.13	269
540	1725	136968	0.05	101
600	1086	137226	0.03	67
660	590	143913	0.01	35
720	476	132127	0.01	38
2160	106	143213	0.002	13

Determination of SN79 content in rat plasma samples after oral administration of SN79-*di*-HCl to fed state Sprague Dawley rats (20mg/kg) - Rat 2

Time (min)	Drug Area	IS Area	Ratio	Conc (ng/mL)
15	1700	134232	0.05	108
30	3076	145118	0.07	145
45	2895	142816	0.07	143
60	2553	141703	0.06	131
90	3042	148035	0.06	135
120	3778	147199	0.08	168
150	3369	142593	0.08	166
180	3287	141176	0.08	168
240	3277	147877	0.07	145
300	2651	139518	0.07	142
360	3018	140274	0.07	158
420	2041	136344	0.06	121
480	2291	139365	0.06	125
540	3017	149166	0.06	131
600	1009	141986	0.02	56
660	5449	137323	0.15	300
720	849	127853	0.03	69
1440	527	142230	0.01	33
2160	96	138933	0.002	13

Determination of SN79 content in rat plasma samples after oral administration of SN79-*di*-HCl to fed state Sprague Dawley rats (20mg/kg) - Rat 3

Time (min)	Drug Area	IS Area	Ratio	Conc (ng/mL)
15	2617	129324	0.08	187
30	3514	148827	0.09	152
45	3971	156864	0.07	148
60	2067	131509	0.08	139
90	2272	135021	0.13	138
120	3370	143364	0.12	164
150	3507	146862	0.10	158
180	1528	129719	0.10	111
240	1195	131469	0.07	84
300	1185	130963	0.08	85
360	2029	137943	0.08	115
420	1862	148453	0.09	85
480	3495	148446	0.05	152
540	2489	141537	0.04	128
600	1015	136274	0.02	64
660	503	129297	0.01	43
720	418	129302	0.01	37
1440	454	117889	0.003	23
2160	167	143985	0.001	16

Determination of SN79 content in rat plasma samples after oral administration of SN79-*di*-HCl to fed state Sprague Dawley rats (20mg/kg) - Rat 4

Time (min)	Drug Area	IS Area	Ratio	Conc (ng/mL)
15	2699	134942	0.08	163
30	4505	151640	0.09	183
45	2302	133656	0.07	145
60	3535	147554	0.07	157
90	3895	151340	0.08	160
120	1595	125984	0.06	131
150	694	123380	0.03	68
180	543	122700	0.02	56
240	1049	125434	0.04	91
300	1429	128175	0.05	110
360	1632	131115	0.05	113
420	2528	128741	0.09	184
480	1285	131016	0.04	91
540	571	123495	0.02	57
600	462	125451	0.02	44
660	405	130471	0.01	35
720	408	131304	0.01	34
1440	241	131914	0.01	23

Determination of SN79 content in rat plasma samples after oral administration of SN79-*di*-HCl to fed state Sprague Dawley rats (20mg/kg) - Rat 5

Time (min)	Drug Area	IS Area	Ratio	Conc (ng/mL)
15	2699	134942	0.08	163
30	4505	151640	0.09	183
45	2302	133656	0.07	145
60	3535	147554	0.07	157
90	3895	151340	0.08	160
120	1595	125984	0.06	131
150	694	123380	0.03	68
180	543	122700	0.02	56
240	1049	125434	0.04	91
300	1429	128175	0.05	110
360	1632	131115	0.05	113
420	2528	128741	0.09	184
480	1285	131016	0.04	91
540	571	123495	0.02	57
600	462	125451	0.02	44
660	405	130471	0.01	35
720	408	131304	0.01	34
1440	241	131914	0.01	23

Determination of SN79 content in rat plasma samples after oral administration of SN79-*di*-HCl to fed state Sprague Dawley rats (20mg/kg) - Rat 6

Time (min)	Drug Area	IS Area	Ratio	Conc (ng/mL)
15	1565	133442	0.05	102
30	1481	134099	0.04	95
45	1693	131168	0.05	117
60	1654	132761	0.05	109
90	1595	125325	0.06	134
120	2295	133983	0.07	143
150	1739	131745	0.05	118
180	1454	130667	0.05	103
240	722	132806	0.02	52
300	716	130670	0.02	55
360	1355	132022	0.04	93
420	1665	141176	0.04	89
480	3199	136795	0.09	182
540	1894	132990	0.06	123
600	1508	138948	0.04	86
660	293	129547	0.01	28
720	299	135240	0.01	25
2160	116	120426	0.006	20

Table B.8. Calibration curve for SN79-*di*-HCl for determination of SN79 content in gastrointestinal permeability samples from mucosal to serosal side

Conc (ng/mL)	Drug Area
5	1005
10	2220
25	4834
50	9405
100	19367
200	39844
500	99739
1000	198174
3000	601001
5000	1014556

Determination of SN79 content in permeability study from mucosal to serosal side in rat duodenum

Time (min)	Area	Conc (ng/mL)	Cumulative amt (ng)	Accumulation (ug/cm^2)	Flux ($ug\ cm^{-2}\ min^{-1}$)	Permeability (cm/min) x 10^{-4}	P_{app} (cm/sec) X 10^{-6}
15	350	8	8	0.11			
30	1238	12	13	0.19			
60	2926	20	24	0.34			
90	8923	50	58	0.82	0.038	1.5	2.53
120	28628	147	165	2.34			
150	38434	196	243	3.44			
180	50155	254	340	4.81			

P_{app} – apparent permeability coefficient

Determination of SN79 content in permeability study from mucosal to serosal side in rat jejunum

Time (min)	Area	Conc (ng/mL)	Cumulative amt (ng)	Accumulation (ug/cm^2)	Flux (ug cm^{-2} min $^{-1}$)	Permeability (cm/min) x 10^{-4}	P_{app} (cm/sec) x 10^{-6}
15	385	8	8	0.11	0.035	1.4	2.33
30	1291	12	14	0.19			
60	2342	17	21	0.30			
90	8658	49	56	0.79			
120	29130	150	167	2.36			
150	38759	197	244	3.46			
180	41901	213	299	4.24			

P_{app} – apparent permeability coefficient

Determination of SN79 content in permeability study from mucosal to serosal side in rat ileum

Time (min)	Area	Conc (ng/mL)	Cumulative amt (ng)	Accumulation (ug/cm^2)	Flux (ug cm^{-2} min $^{-1}$)	Permeability (cm/min) x 10^{-4}	P_{app} (cm/sec) x 10^{-6}
15	371	8	8	0.11	0.037	1.5	2.47
30	1249	12	14	0.19			
60	2313	17	21	0.30			
90	10098	56	63	0.89			
120	29334	151	169	2.40			
150	41450	211	259	3.67			
180	45012	228	319	4.51			

P_{app} – apparent permeability coefficient

Table B.9. Calibration curve for SN79-*di*-HCl for determination of SN79 content in gastrointestinal permeability samples from serosal to mucosal side

Conc (ng/mL)	Drug Area
5	945
10	2324
25	6962
50	9261
100	23568
200	43502
500	94811
1000	150435
3000	283169
5000	982640

Determination of SN79 content in permeability study from serosal to mucosal side in rat duodenum

Time (min)	Area	Conc (ng/mL)	Cumulative amt (ng)	Accumulation (ug/cm^2)	Flux ($ug\ cm^{-2}\ min^{-1}$)	Permeability (cm/min) x 10^{-3}	P_{app} (cm/sec) x 10^{-5}
15	13202.79	137	137	1.94	0.246	0.98	1.64
30	21894.03	188	215	3.05			
60	42196.94	306	371	5.25			
90	82878.03	542	668	9.46			
120	108782.3	693	927	13.13			
150	202969.4	1240	1613	22.83			
180	314304.9	1887	2508	35.50			

P_{app} – apparent permeability coefficient

Determination of SN79 content in permeability study from serosal to mucosal side in rat jejunum

Time (min)	Area	Conc (ng/mL)	Cumulative amt (ng)	Accumulation (ug/cm^2)	Flux (ug cm^{-2} min $^{-1}$)	Permeability (cm/min) x 10^{-3}	P_{app} (cm/sec) x 10^{-5}
15	18639	169	169	2.39	0.259	1.04	1.73
30	21457	185	219	3.10			
60	38797	286	357	5.05			
90	81653	535	663	9.39			
120	107092	683	918	12.99			
150	213606	1302	1674	23.69			
180	328157	1967	2599	36.79			

P_{app} – apparent permeability coefficient

Determination of SN79 content in permeability study from serosal to mucosal side in rat ileum

Time (min)	Area	Conc (ng/mL)	Cumulative amt (ng)	Accumulation (ug/cm^2)	Flux (ug cm^{-2} min $^{-1}$)	Permeability (cm/min) x 10^{-3}	P_{app} (cm/sec) x 10^{-5}
15	15775	152	152	2.16	0.241	0.96	1.61
30	23728	199	229	3.24			
60	42074	305	375	5.31			
90	83045	543	674	9.55			
120	100895	647	887	12.55			
150	210115	1282	1651	23.37			
180	303398	1824	2449	34.67			

P_{app} – apparent permeability coefficient

Table B.10. Metabolic stability of SN79 incubated as SN79-*di*-HCl in rat liver microsomes.

Time (min)	Area	% Remaining	Average	SEM
0	88776	100	100	0
	87414	100		
	84644	100		
10	50107	56.4	57.8	0.03
	50321	57.6		
	50238	59.4		
15	40002	45.1	46.1	0.02
	40198	46.0		
	40015	47.3		
30	30549	34.4	34.9	0.03
	30076	34.4		
	30478	36.0		
45	20004	22.5	25.0	0.09
	22669	25.9		
	22538	26.6		
60	6276	7.1	7.2	0.02
	6384	7.3		
	6181	7.3		
90	1070	1.2	1.2	0.02
	1023	1.2		
	1032	1.2		

Percent remaining = (area at time t×100)/area at time zero; SEM = standard deviation/average

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VITA

HarshaVinnakota was born in Hyderabad, Andhra Pradesh, India on April 5th, 1985. She is the only daughter of Dr. RevathyVinnakota and Mr. Narasimha RaoVinnakota. She graduated with a Bachelor's degree in Pharmacy in May 2006 from Birla Institute of Technology and Science, Pilani, Rajasthan, India. She received the Prathibha award, a state government certificate of academic excellence by the Government of Andhra Pradesh and the National Merit Certificate awarded by the Government of India.

With a strong desire to pursue higher education to supplement her knowledge, she joined the Department of Pharmaceutics at the University of Mississippi in August 2006. During her tenure as a graduate student under the able guidance of Dr. Bonnie A. Avery, Harsha received several honors. In April 2008, she was inducted into the Honor Society of Phi Kappa Phi, the nation's oldest, largest and most selective collegiate honor society for academic excellence. The same year she was inducted into Rho-Chi for intellectual achievement in pharmacy. She received an award for outstanding poster presentation during the American Association of Pharmaceutical Scientist-southern regional discussion group (AAPS-SRDG), PharmForum 2008 held at University of Arkansas for Medical Sciences. Harsha was also the recipient of Graduate School Council Grant and Graduate School Summer Fellowship awarded by the Graduate School, University of Mississippi in 2009-2010. She was selected for Who's Who among Students in American Colleges and Universities in 2010-2011.

Harsha is a current member of AAPS. During the course of her study at the University of Mississippi, she served in various leadership roles in different capacities for a number of organizations. An interest in cultural affairs motivated her to become the Cultural Secretary of India Association at the University of Mississippi from 2006-2007. Thereafter, she spearheaded the India Association as the first woman President from 2008-2009. Harsha represented the department of Pharmaceutics as the Senator in the Graduate Student Council at the University of Mississippi in 2007-2008. She served as the Chair-Elect (2007-2008) and Chair (2008-2009) of the AAPS-University of Mississippi Student Chapter. She was a core member and the Chair of the planning committee for PharmForum 2009, an annual meeting of AAPS-SRDG. She was invited to serve as the student representative of AAPS-Student Postdoc Outreach department from 2009-2010. Harsha received the Doctor of Philosophy degree in Pharmaceutics in May 2011.