Metabolic Stability And Pharmacokinetics In Lead Optimization Of Potential Anti-Psychostimulant Pharmacotherapies

Seshulatha Jamalapuram

University of Mississippi

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METABOLIC STABILITY AND PHARMACOKINETICS IN LEAD OPTIMIZATION
OF POTENTIAL ANTI-PSYCHOSTIMULANT PHARMACOTHERAPIES

A Dissertation
presented in partial fulfillment of requirements for the degree of
Doctor of Philosophy in Pharmaceutical Sciences
in the Department of Pharmaceutics
The University of Mississippi

by
SESULATHA JAMALAPURAM

9:00 AM NOVEMBER 30 2012
ABSTRACT

Drug addiction is a chronic disorder characterized by obsessive and uncontrollable drug-seeking behaviors. It is a major public health problem globally as well as in the United States. Apart from causing severe medical complications it also leads to several socioeconomic problems like healthcare expenditures, lost earnings and increase in drug-related crime. The discovery and development of potential pharmacotherapies to treat cocaine dependence has been a high priority for more than two decades but still there is US-FDA approved medication. This illustrates the need for the development of effective medication to treat cocaine and methamphetamine abuse. Sigma receptors have recently been identified as potential targets for the development of novel therapeutics. The affinity of cocaine and methamphetamine to sigma receptors represents that targeting these receptors using selective antagonists will be an effective strategy in the development of novel medications. This dissertation is primarily focused on the role of metabolism and pharmacokinetics in the lead optimization of novel sigma receptor antagonists to treat abuse of cocaine and methamphetamine.

Taking this in to consideration, we first determined in vivo and in vitro properties of CM156, [3-(4-(4-cyclohexylpiperazin-1-yl)butyl)benzo[d]thiazole-2(3h)-thione] a highly selective sigma receptor antagonist using a validated UPLC/MS method. However due to its poor in vivo and in vitro performance further research was halted. Several analogs were then synthesized by blocking the metabolic soft spots of CM156. We performed a preliminary screening of a series of the high affinity sigma receptor antagonists using in vitro metabolism studies and selected AZ66, 3-(4-(4-cyclohexylpiperazine-1-yl)pentyl)-6-fluorobenzo[d]thiazole
2(3h)-one, as a lead compound. AZ66 is an optimized sigma receptor ligand with high metabolic stability that has been shown to mitigate behavioral effects of methamphetamine, suggesting that it can become a potential candidate to treat methamphetamine abuse. We determined its physicochemical properties such as solubility, pKa, Log $P_{O/W}$ and Log $D_{PBS, (pH \ 7.4)}$. We have also assessed its in vitro metabolic stability and in vivo pharmacokinetic parameters and absolute bioavailability in rats using a validated UPLC-MS/MS method. We conducted repeated dose pharmacokinetic studies and estimated the plasma steady state concentrations. More importantly we estimated its extent of central nervous system penetration using brain to plasma ratio studies in rats. The results of these in vitro and in vivo studies will lead to development of successful pharmacotherapies for cocaine and methamphetamine abuse.
DEDICATION

Dedicated to my parents, Satyanarayana Rao Jamalapuram and Suguna Jamalapuram, and
my husband Pradeep kumar Vuppala
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<thead>
<tr>
<th>Abbreviation</th>
<th>Meaning</th>
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<tr>
<td>ADME</td>
<td>Absorption, distribution, metabolism and elimination</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under the curve</td>
</tr>
<tr>
<td>B/P</td>
<td>Brain to plasma ratio</td>
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<tr>
<td>BBB</td>
<td>Blood-brain barrier</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic Adenosine Mono Phosphate</td>
</tr>
<tr>
<td>CID</td>
<td>Collision induced dissociation</td>
</tr>
<tr>
<td>CL</td>
<td>Clearance</td>
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<tr>
<td>CL&lt;sub&gt;int&lt;/sub&gt;</td>
<td>Intrinsic clearance</td>
</tr>
<tr>
<td>C&lt;sub&gt;max&lt;/sub&gt;</td>
<td>Maximum plasma concentration</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CYP 450</td>
<td>Cytochrome P 450</td>
</tr>
<tr>
<td>DA</td>
<td>Dopamine</td>
</tr>
<tr>
<td>DAT</td>
<td>Dopamine Transporters</td>
</tr>
<tr>
<td>DBH</td>
<td>Dopamine β-hydroxylase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>---------------</td>
<td>-----------</td>
</tr>
<tr>
<td>DTG</td>
<td>1,3-di-O-tolylguanidine</td>
</tr>
<tr>
<td>ESI</td>
<td>Electro spray ionization</td>
</tr>
<tr>
<td>F</td>
<td>Oral bioavailability</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>FMO</td>
<td>Flavin monooxygenase</td>
</tr>
<tr>
<td>GABA</td>
<td>γ-amino butyric acid</td>
</tr>
<tr>
<td>GIT</td>
<td>Gastro intestinal tract</td>
</tr>
<tr>
<td>GMP</td>
<td>Good manufacturing practice</td>
</tr>
<tr>
<td>GLU</td>
<td>Glutamate</td>
</tr>
<tr>
<td>HILIC</td>
<td>Hydrophilic interaction liquid chromatography</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>HLM</td>
<td>Human liver microsomes</td>
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<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>IACUC</td>
<td>Institutional Animal Care and Use Committee</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IS</td>
<td>Internal standard</td>
</tr>
<tr>
<td>IV</td>
<td>Intravenous</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<td>--------------</td>
<td>-----------------------------------------------</td>
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<tr>
<td>LC</td>
<td>Locus ceruleus</td>
</tr>
<tr>
<td>LC-MS/MS</td>
<td>Liquid chromatography/mass spectrometer</td>
</tr>
<tr>
<td>LLOQ</td>
<td>Lower limit of quantification</td>
</tr>
<tr>
<td>LOD</td>
<td>Lower limit of detection</td>
</tr>
<tr>
<td>MA</td>
<td>Methamphetamine</td>
</tr>
<tr>
<td>MRM</td>
<td>Multiple reaction monitoring</td>
</tr>
<tr>
<td>MRT</td>
<td>Mean residence time</td>
</tr>
<tr>
<td>NADP</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NCE</td>
<td>New chemical entity</td>
</tr>
<tr>
<td>NE</td>
<td>Norepinephrine</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartic acid</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCP</td>
<td>Phencyclidine</td>
</tr>
<tr>
<td>PET</td>
<td>Positron Emission Tomography</td>
</tr>
<tr>
<td>PK</td>
<td>Pharmacokinetics</td>
</tr>
<tr>
<td>pKa</td>
<td>Acid dissociation constant</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>QC</td>
<td>Quality control</td>
</tr>
<tr>
<td>RDS</td>
<td>Reward-deficiency syndrome</td>
</tr>
<tr>
<td>RE</td>
<td>Relative error</td>
</tr>
<tr>
<td>RSD</td>
<td>Relative standard deviation</td>
</tr>
<tr>
<td>S/N</td>
<td>Signal to noise ratio</td>
</tr>
<tr>
<td>SARs</td>
<td>Structure-activity relationships</td>
</tr>
<tr>
<td>SIR</td>
<td>Selected ion recording</td>
</tr>
<tr>
<td>SIM</td>
<td>Selected ion monitoring</td>
</tr>
<tr>
<td>$t_{1/2}$</td>
<td>Half-life</td>
</tr>
<tr>
<td>$T_{\text{max}}$</td>
<td>Time of maximum concentration</td>
</tr>
<tr>
<td>UDPGA</td>
<td>Uridine 5'-diphospho-glucuronic acid</td>
</tr>
<tr>
<td>UNODC</td>
<td>United Nations Office on Drugs and Crime</td>
</tr>
<tr>
<td>UPLC/MS/MS</td>
<td>Ultra performance liquid chromatography/mass spectrometer</td>
</tr>
<tr>
<td>US-FDA</td>
<td>United States Food and Drug Administration</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra violet</td>
</tr>
<tr>
<td>$V_d$</td>
<td>Volume of distribution</td>
</tr>
<tr>
<td>5-HT</td>
<td>Serotonin</td>
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ACKNOWLEDGMENTS

First and foremost, I would like to express my deep and sincere gratitude to my advisor, Dr. Bonnie A. Avery. I have been amazingly fortunate to have an advisor who gave me the freedom to explore on my own and at the same time the guidance to recover when my steps faltered. Under her supervision I have been motivated to strive for and achieve exceptionally high standards in research. Her patience and endless support helped me overcome many crisis situations and finish this dissertation. Especially, the support I got from her during last semester is unforgettable in my life.

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Last but not the least, I am ever grateful to God, the creator and the guardian, and to whom I owe my very existence.
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3.2. EXPERIMENTAL

3.3. RESULTS AND DISCUSSION

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CHAPTER - 1
INTRODUCTION

1.1. Cocaine abuse and addiction

Drug addiction is a chronically relapsing disorder characterized by (1) compulsion to seek and use the drug, (2) loss of control for intake of the drug, and (3) the emergence of a negative emotional state (e.g., dysphoria, anxiety, and irritability) reflecting a withdrawal syndrome when access to the drug is prevented. Drug addiction is conceptualized as a chronic disorder that involves both impulsivity and compulsivity that yield a composite addiction cycle of three stages: ‘intoxication’, ‘withdrawal/negative affect’, and ‘preoccupation/anticipation’ (craving) [1, 2].

Impulse control disorders are characterized by an increasing sense of tension compelling an impulsive act and pleasure, or relief at the time of committing the act. Impulse control disorders are largely associated with positive reinforcement mechanisms. In contrast, compulsive disorders are characterized by anxiety and stress before a compulsive repetitive behavior and relief from the stress by performing the compulsive behavior. Compulsive disorders are greatly associate with negative reinforcement mechanisms [3]. Cocaine addiction is characterized by impulsivity, impaired social relationships, and abnormal mesocorticolimbic reward processing [4]. Mesocorticolimbic circuits, involving the dopaminergically innervated ventral and dorsal striatum as well as orbitofrontal and anterior cingulate cortices, are crucially involved in reward processing, and dysregulation in these circuits is implicated in both impulsivity and cocaine
dependence [5]. A possible explanation for diminished mesocorticolimbic activation is the reward-deficiency syndrome (RDS) hypothesis [6], which assumes that drugs of abuse, due to their potent dopaminergic effects, normalize ventral striatal dopamine levels, leading RDS individuals to consume cocaine or other abused drugs [1, 7].

Despite several efforts, the treatment of cocaine abuse has proven to be insubstantial and there is currently no US FDA-approved drug to treat cocaine addiction. The primary effect of cocaine on the central nervous system (CNS) is reuptake blockade of monoamines. Cocaine binds with dopamine transporters (DAT) in the same binding pocket as dopamine (DA) and prevents reuptake of DA. As a result of this, the short-term effects of cocaine, like euphoria, begin [8]. The long-term effect of cocaine addiction involves cocaine-induced changes in the brain’s communication system, including the rapid upregulation of DAT expression on the cell surface. A single use of cocaine will increase the surface DAT expression for at least a month, due to the increase of the surface DAT expression with chronic use, there are less DA molecules available in the synapse for signaling, which might contribute to the drug seeking or carving [9].

In general, two aspects have to be taken into consideration for the treatment of drug addiction: antagonizing the stimulant and toxic effects of the abused drug and restoring the functions of brain back to normal. Without antagonizing the effects of cocaine, it may prove difficult to bring the function of the brain to normal. The main reason behind this is when cocaine is taken it will increase expression of the surface DAT for at least a month and the function of the brain cannot be normal [10]. Henceforth, it is necessary to antagonize the stimulant effects of cocaine for the treatment of cocaine-addiction.
Cocaine addiction and overdose are major medical and public health problems that challenge the treatment. The traditional approaches to treat addiction include targeting a specific subtype of transporters/receptors that affect various neurotransmitter systems, such as dopaminergic, serotonergic, noradrenergic, cholinergic, glutamatergic, GABAergic and opioidergic pathways, and modulate neurological processes [10].

Cocaine is available primarily in two forms: cocaine hydrochloride salt and the free base. The salt form is a white powder and is water soluble, so it can be taken intravenously or intranasally. The free base, also called as “crack” is white to light brown in color and is insoluble in water. “Crack” is derived from the crackling sound produced when it is heated. Crack cocaine is often smoked or injected [11]. Cocaine available as non pharmaceutical or illicit drug on the streets contains many adulterants. A recent finding has shown that 70 % of the cocaine that is seized in the USA contains levamasole, an anti-helminthic drug. Cocaine adulterated with levamasole has been responsible for many deaths and emergency cases. However, the particular reason behind the adulteration with this specific drug was not identified [12].

1.2. Pharmacokinetics of cocaine

After entering in to the body and systemic circulation, cocaine is metabolized by the liver and plasma cholinesterases into pharmacologically inactive metabolites such as benzoylecgonine and eagonine methyl ester. Urinary metabolites constitute 90% of the actual cocaine dose and a very small percentage is excreted as unchanged compound in the feces [12]. Cocaine is consumed usually via oral, intranasal, intravenous and pulmonary routes. The rate of entry of cocaine in to the systemic circulation depends on the route of administration, the quicker being the smoked or intravenous (i.v.) administration. After oral or intranasal administration plasma
concentration increases slowly. These differences in the entry rate explain the greater reinforcing effects produced by the i.v or smoked compared to oral or intranasal cocaine. Rapid increases in plasma concentrations of cocaine are directly correlated to its physiological and subjective effects [11].

1.3. Pharmacology of cocaine

The mesocorticolimbic reward system consists of dopaminergic cells in the ventral tegmental area (VTA) and their projections to the nucleus accumbens (NAc) and prefrontal cortex (PFC), as well as glutamate (GLU) projections from the PFC to both the VTA and NAc. Other important brain structures associated drug dependence are amygdala, hippocampus and hypothalamus [13]. Cocaine rapidly increases dopamine levels in the PFC and NAc. Even though various neurotransmitter systems are involved, many studies suggest that activation of the mesocorticolimbic system is primarily responsible for the reinforcing effects produced by cocaine in humans [14].

Despite the fact that cocaine exhibits numerous physiological and neurological effects on this system, the primary mechanism underlying its reinforcing effects is inhibition of reuptake of DA [15]. Figure 1.0 gives pictorial representation of mechanism of cocaine. Many studies have shown that cocaine’s reinforcing properties are related to its ability to increase synaptic DA by binding to DAT. In fact, rendering DAT insensitive to cocaine by molecular engineering had proven to abolish its potent reinforcing effects [16]. Several studies have associated DA neurotransmission explicitly with cocaine induced euphoria.

Dopamine receptor antagonists attenuate the positive subjective effects of cocaine in humans partially by interrupting the DA neurotransmission [17]. However, complete blockade
was not achieved by selective pacification of DA suggesting the involvement of other transmitter systems. In addition many, compounds that are promising for dependence alter norepinephrine (NE) neurotransmission through a number of mechanisms [18, 19].

**Figure 1.1:** Classical mechanism of action of cocaine

### 1.4. Cocaine pharmacotherapies

#### 1.4.1. Dopaminergic medications

Currently, dopamine receptors are classified in to two types of receptor families: dopamine D1-like family and dopamine D2-like family. D1 and D5 receptors that initiate cyclic adenosine mono- phosphate (cAMP) formation fall under D1-like family. The D2-like family that includes D2, D3 and D4 receptors inhibits the cAMP formation [20]. The acute and chronic effects of cocaine are proposed to be mediated by both D1 and D2-like receptors. Some of the
dopaminergic compounds that were tested for treating cocaine addiction are modafinil, mazindol, disulfiram, nepicastat, methylphenidate, selegelline, amantadine and bromocriptine. The following paragraphs provide a brief out-line of the few of these dopaminergic agents that are in clinical trials.

Methylphenidate is used for the treatment of narcolepsy and attention-deficit hyperactivity disorder (ADHD). Methylphenidate was proposed as an ‘agonist treatment’ for use in cocaine addiction because it stimulates the release of dopamine and inhibits reuptake of dopamine, norepinephrine and serotonin [21].

Disulfiram is an aldehyde dehydrogenase inhibitor as well as a dopamine-beta-hydroxylase (DBH) inhibitor. It is currently approved for treatment of alcohol dependence. Dopamine-beta-hydroxylase converts dopamine to norepinephrine leading to increased DA levels in the brain and inhibition of DBH leads to a decreased neuronal and synaptic norepinephrine levels compared to dopamine [22, 23]. A recent study has shown that the co-administration of disulfiram with cocaine increased the plasma cocaine concentrations 3 to 6 fold, which perhaps contributed to the decreased craving and increased dysphoria observed during the study. In a double-blind randomized clinical study of disulfiram for the treatment of cocaine dependence in methadone-stabilized individuals, it was observed that there was a considerable decrease in cocaine-positive urines in the group receiving 250 mg/day compared to lower doses like 62.5 mg and 125 mg, of the medication or placebo [24]. These findings suggest that disulfiram may be a potential medication for treating cocaine abuse. Nonetheless, additional studies with disulfiram are needed to determine the optimal dose and duration of treatment.
Nepicastat, like disulfiram, is a novel selective DBH-inhibitor and is currently under investigation treating cocaine dependence. In preclinical studies, nepicastat has shown to decrease brain noradrenaline levels but increase dopamine synaptic levels. These results suggest that nepicastat, would be a potential candidate to treat cocaine dependence [25].

Modafinil promotes wakefulness and is used in the treatment of hypersomnia and narcolepsy. Even though little is known about its mechanism of action, there is evidence that shows that modafinil acts as a central alpha-1 adrenergic agonist, and increases both dopamine and glutamate release in specific areas of the brain [26]. As chronic cocaine use is associated with depletion of extra-cellular glutamate levels and glutamatergic synaptic strength in the nucleus accumbens, modafinil might serve as a promising candidate for treatment of cocaine dependence. Modafinil pretreatment at two oral dosages (200 mg and 400 mg) demonstrated significant attenuation of euphoria caused by the cocaine intravenous administration [27]. Modafinil reduces, craving for cocaine, cocaine use among sub-populations of cocaine users and it has partial reinforcing effects and thus continues to represent as a promising candidate for the treatment of cocaine dependence.

Since cocaine elicits its effects by increasing the synaptic DA levels, for several years research has been focused on discovering a therapeutic candidate that blocks DA receptors. Unfortunately, this strategy has not been fruitful. As a result the current researchers are focusing more on other neurotransmitter systems such as norepinephrine (NE).

1.4.2. Cocaine effects on norepinephrine neurotransmission

Not only does cocaine bind to DAT, it also blocks the reuptake of 5-hydroxytryptamine (5-HT) and NE and elevates synaptic levels of these neurotransmitters. Even though NE plays a
greater role in regulating increasing in DA, several studies indicate that 5-HT also contributes to
some of the behavioral effects generated by cocaine [28]. It was suggested that modifying the NE
production through genetic alterations or by administering drugs that facilitate NE release
cocaine’s effects could be altered significantly [18, 29]. Nevertheless, the precise role of NE in
mediating psychostimulant behavioral effects in humans still remains uncertain. Cocaine binds to
norepinephrine transporters the dopamine transporter, indicating that the modulation of
mesocorticolimbic dopaminergic transmission can be achieved through alternative pathways
[30].

Bupropion is a norepinephrine and dopamine re-uptake inhibitor currently FDA approved
as an antidepressant and also to help smoking cessation. It was shown that in a 12-week placebo-
controlled trial on cocaine-abusers bupropion reduce cocaine use in depressed patients. In a
recent study, the combination of bupropion administered at a dose of 300 mg/day with
contingency management was found to significantly decrease the proportion of cocaine-positive
urine samples [31].

Venlafaxine is a mixed norepinephrine and serotonin inhibitor. When administered at a
dose of 225 mg/day was shown to produce a small but obvious reduction in euphoria induced by
cocaine. A double-blind, placebo-controlled design was conducted with nefazodone, an
antidepressant, which has serotonin and norepinephrine re-uptake inhibition as well as 5-HT2 a
receptor antagonist effects. It was observed that urine benzoylecgonine, the major metabolite of
cocaine, declined quickly in the nefazodone group compared to the placebo group [32].

Atomoxetine is a long-acting selective norepinephrine re-uptake inhibitor that is
approved for the therapy of ADHD in children and adults. This is currently in trials as a potential
therapeutic compound for treatment of cocaine abuse and addiction in patients suffering with ADHD [30].

**1.4.3. Adrenoceptor antagonists**

Cocaine also activates the adrenergic system, which includes the central noradrenergic system and its peripheral counterpart the sympathoadrenal system. It has been shown that the adrenergic system plays a role in mediating the physiological response to cocaine including increases in heart rate, blood pressure and arousal. Adrenoceptor antagonists have inhibited some of the behavioural and toxic effects of cocaine in rats, suggesting its role in mediating cocaine effects. Propranolol, carvedilol and labetalol are few of the adrenoreceptor antagonists tested for cocaine treatment [33].

A recent study has shown that an acute treatment with 50 mg of carvedilol attenuated cocaine induced changes in heart rate along with systolic and diastolic blood pressure. However, self-administration of cocaine was found to be decreased when subjects received carvedilol at a dose of 25 mg rather than 50 mg. Based on these results, it was proposed that β-adrenoceptor antagonists may attenuate the reinforcing effects of cocaine. However, further studies are needed to confirm the use of adrenoceptor antagonists for the treatment of cocaine addiction [20].

Cocaine users usually exhibit reduced cerebral blood flow and deficits in the cortical perfusion with areas of both hypo and hyper perfusion. This reduced cerebral blood flow is likely to be associated with some of the neuropsychological impairments in verbal learning, memory and attention commonly observed in cocaine addiction [34]. In a recent study, cocaine users \( (n=9) \) with cerebral blood flow deficits on single proton emission computerized tomography (SPECT) scans were compared with cocaine users \( (n=6) \) having no deficits after 2-day treatment
with isradipine administered at a dose of 15 mg/day. Isradipine, is a calcium channel blocker used in treatment of hypertension. It was found that isradipine improved cerebral blood flow in areas of hypoperfusion. It also attenuated various cocaine-induced responses like ischemic changes in the brain and altered systolic blood pressure [35]. These early studies suggest that vasodilators may be helpful in reversing cerebral hypoperfusion observed in cocaine users.

1.4.4. Glutamate medications

Glutamate neurotransmission plays a significant role in reward and reinforcement caused by drugs of abuse. Glutamate receptors may be one of the potential therapeutic targets for the treatment of cocaine dependence [36, 37]. Glutamate, the most widely spread excitatory neurotransmitter in the brain, is essential for a numerous processes including long term depression, extinction, and reward-related learning [38, 39]. Various preclinical studies have shown that projections of glutamate neurotransmitter from the PFC to the NAc are critical for stress- and cocaine-primed restoration of turned off cocaine self-administration in animals. Cocaine self-administration was reinstated when glutamate ionotropic receptor agonist AMPA (alpha-amino-3-hydroxy-5-methyl-4- isoxazolepropionic acid) was infused into the NAc, while the effect was successfully attenuated by blocking the translation and expression of the AMPA receptor subunit using antisense oligonucleotides, glutamate receptor 1(GLuR1) [40].

Glutamate levels in the brain positively correlated with chronic use of cocaine suggesting that the continuous exposure to cocaine brought changes in glutamate. These studies suggest glutamate receptors may become one of the pharmacotherapeutic targets for cocaine dependence. Few ionotropic ligands have been introduced for clinical use in neurodegenerative disorders and are now being tested for pharmacotherapy of cocaine addiction [41].
Various N-methyl-D-aspartic acid (NMDA) receptor antagonists including competitive (memantine), non-competitive (modafinil, lamotrigine, and ibogaine) antagonists were shown to counteract relapse and drug-seeking behavior in animals. These drugs inhibit dopamine receptor sensitization, which usually seen in cocaine addiction [42]. Memantine, was shown to inhibit cocaine self-administration in rats and in a dose-dependent fashion it also suppresses cocaine-conditioned motor response [43]. In humans memantine has been shown to augment the feeling of pleasure induced by cocaine [44].

In poly drug users, ibogaine was reported to reduce craving for cocaine and heroin. It also enhances glutamate effects by blocking its uptake by glial cells, and by increasing the release from cortical synaptosomes. In series of trials Mash et al. found that ibogaine significantly decreased craving for cocaine and heroin during inpatient detoxification [45]. However, it is a potentially addictive compound, which is reported to induce neurotoxic effects and its use for treatment of drug addiction is always questionable.

Modafinil, an alpha-1-adrenergic agonist and a noradrenaline reuptake inhibitor, is a safer agent acting on glutametergic pathways, which is currently approved for narcolepsy. It is also a glutamate agonist. Modafinil was well tolerated, thus leading to valid treatment retention. It was proposed that modafinil promotes cocaine abstinence owing to its ability to restore normal glutamate baseline levels in the nucleus accumbens by reversing cocaine induced dopamine dysregulation [46].

Lamotrigine, an approved anticonvulsant which is used in the long-term treatment of bipolar disorder is one more drug acting on glutametergic pathways. Lamotrigine has a broad range of effects on brain cells. It decreases sustained, repetitive, high-frequency firing of voltage-
dependent sodium channels that may result in a preferential decreased release of presynaptic glutamate. A study showed that lamotrigine administered at a dose of 300 mg/day was effective in decreasing craving for cocaine; however it was not effective in patients diagnosed with bipolar disorder [47].

1.4.5. GABA system

Gamma-amino butyric acid (GABA) system is presently receiving increased attention as a potential target to treat cocaine dependence. GABA is a widely distributed neurotransmitter that primarily mediates inhibition in the brain [48]. GABA receptors are classified into two types GABA<sub>A</sub> and GABA<sub>B</sub>. GABA<sub>A</sub> receptors belong to the superfamily of ligand-gate ion channels. Their activation decreases neuronal excitability by increasing chloride influx while GABA<sub>B</sub> receptors mediate the slow inhibitory response to GABA [49]. GABA<sub>B</sub> receptors are coupled to G-proteins and are found both pre- and postsynaptically. GABA<sub>B</sub> receptors antagonists block calcium channels and act as autoreceptors, diminishing neurotransmitter release presynaptically. Postsynaptically they open the potassium channels, leading to reduced neuronal excitability and hyperpolarisation [50].

Evidences suggest that GABA modifies the dopaminergic system and cocaine effects. These include; (1) striatum and nucleus accumbens which contain dopaminergic neurons also contain GABAergic synapses, this suggests the anatomical relationship between GABA and dopaminergic systems. (2) Chronic cocaine intake changes the functional coupling of GABA receptors and also the subunit composition of GABA<sub>A</sub> receptors. (3) Treatment with vigabatrin, (gamma-vinyl-GABA) that acts by inhibiting the breakdown of GABA attenuates cocaine-induced locomotor hyperactivity and release of DA in the nucleus accumbens in rats [51, 52].
GABA medications that are being tested for pharmacotherapy of cocaine are topiramate, valproic acid, baclofen, gabapentin, tiagabine and progesterone. Baclofen, a selective GABA\(_B\) receptor agonist, is used widely as a treatment of spasticity in spinal cord injuries or multiple sclerosis. It has shown efficacy in preclinical trials for treatment of cocaine dependence. Baclofen was shown to attenuate cocaine seeking behavior and reduced self-administration of cocaine dose-dependently in rats. It also inhibited cocaine-induced release of dopamine in the nucleus accumbens [53]. A clinical trial showed significant reductions in cocaine use in baclofen treated subjects than those receiving the placebo. However, baclofen did not demonstrate a statistically significant impact on cocaine craving but it can be used in patients who need treatment for relapse prevention [54]. Baclofen appears to be a well tolerated drug with no side effects but its short half-life (3–4 h) is the major concern as it leads to poor patient compliance.

Topiramate, is an approved antiepileptic drug, that controls dopamine release in the nucleus accumbens by enhancing GABAergic inhibitory activity and by blocking the excitatory effects of glutamatergic neurons [55]. Topiramate antagonizes glutamatergic afferents to the meso-corticolimbic dopaminergic system. In a randomized, double-blind, placebo-controlled study it was shown that topiramate could not reduce cocaine use significantly. But after week eight of the study, it was found from the urine benzoylecgonine test that subjects treated with topiramate were more likely to be abstinent from cocaine compared to the subjects given a placebo [55]. Clinical trials with tiagabine, gabapentin and baclofen in cocaine users resulted in promising findings suggesting that the GABA system may be a potential target for cocaine pharmacotherapy.

1.4.6. Serotonergic system
In addition to its interaction with the dopamine and norepinephrine transporters, cocaine also binds to serotonin transporters (SERT), resulting in the inhibition of presynaptic uptake of this monoamine. Cocaine has high affinity for the serotonin transporters (Ki = 217 nM). The higher dopamine transmission in the nucleus accumbens during acute cocaine intoxication is accompanied by enhanced release of serotonin [56]. Also cocaine withdrawal is characterized by reduced levels of serotonin in the nucleus accumbens and depletion of serotonin levels in the brain.

It was reported that the management of the serotonergic system mitigates drug craving in humans. Although the results were inconclusive, these studies suggested that serotonergic receptors might become potential targets for the drug development to treat cocaine addiction [57]. Many studies have shown 5-HT3 receptor activation mediates the reinforcing effects of cocaine partially in posterior ventral tegmental area [58]. Initial preclinical studies in rats demonstrated that serotonin-enhancing compounds were associated with reduced self-administration of cocaine, but in human clinical trials of selective serotonin reuptake inhibitors varied results were observed [59].

Ibogaine, an indole alkaloid was found to be promising for the treatment of dependence caused due to multiple drugs like cocaine, alcohol, opium, and methamphetamine. Ibogaine has an affinity for a number of receptor sites including sigma-1 and sigma-2, opioid (kappa, mu, and delta), nicotinic, DAT, NMDA and SERT. Ibogaine and its metabolite noribogaine have been found to increase 5HT in the brain extracellularly and both of them have shown to reduce cocaine self-administration appreciably [60, 61].

1.4.7. Immunotherapies
One of the approaches for the persistent reduction of the reinforcement effects of cocaine is to prevent its access to the brain. This blockade precludes the occupation of the receptors in the brain which is required to elicit pleasurable and reinforcing effects of abused drugs like cocaine and methamphetamine. To understand the underlying mechanism, the antibody affinity as well as the total amount of the antibody in circulation must be known [62]. The use of vaccinations for treating cocaine addiction effectively requires a complete understanding of the mechanisms of antibody blockade and the immunological factors that control the progress of antibody responses.

Currently, the vaccines for treating cocaine and methamphetamine are in the clinical and preclinical development stages, respectively. The cocaine vaccine, TA-CD is a conjugate of cocaine hapten and inactivated cholera toxin B that stimulates antibody production. The generated antibodies are specific to cocaine [63]. When cocaine reaches the blood, it binds to TA-CD resulting in the creation of larger immune complexes that are unable to cross the blood-brain-barrier. These molecules are then metabolized by cholinesterases into inactive metabolites which are excreted in urine [64]. In a phase I clinical study (n=34), it was observed that human subjects receiving TA-CD generated immunologic response which resulted in the creation of cocaine-specific antibodies [65]. Higher doses resulted in higher mean antibody levels. In all the studies, the vaccine exhibited a favorable safety profile. Currently, TA-CD is under large-scale, multisite, phase IIb clinical testing [22].

However, a number of investigations on noradrenergic, adrenergic, serotonergic and muscarinic mechanisms propose that due to limited efficacy and unfavorable side effect they may be less favorable targets for development of therapeutic candidates to treat cocaine abuse. Therefore, current studies more focused on the sigma receptors.
1.5. Methamphetamine abuse and pharmacotherapies

Methamphetamine (MA) abuse is a serious social and medical problem globally and in the United States as well [66]. Methamphetamine is developed from the parent drug amphetamine and was used originally in nasal decongestants. Commonly it is known as “meth”, “speed”, and “chalk”. The smoked form is referred as “crystal”, “crank”, and “glass”. The major routes of administration of MA are oral, intranasal, inhalation and injection. Methamphetamine has multiple effects on the central nervous system. Like cocaine it produces euphoria and increased activity. However, the effects of MA last longer than (6- 8 h) cocaine and amphetamine [67]. It is a highly potent releaser of monoamines by increasing cytoplasmic concentrations of dopamine and serotonin and also norepinephrine, adrenaline and histamine. Preclinical and clinical studies have demonstrated that its use can produce irreversible neuronal damage and serious neuropsychiatric consequences. The acute use in higher quantities may cause a serious psychotic syndrome known as the MA psychosis. Discontinuation after the chronic use frequently results in a withdrawal syndrome including increased appetite, sleep disturbances, dysphoric mood and fatigue [67, 68]. Despite the seriousness, currently there are no FDA-approved medications for MA addiction. Some of the promising medications for treating MA addiction include rivastigmine, modafinil, bupropion and lobeline [69].

Bupropion, a DA reuptake inhibitor is currently approved for the treatment of depression and nicotine dependence. Extended use of MA reduces dopamine concentrations in the synapse. Bupropion reinstates the dopamine homeostasis by increasing the dopamine concentration in the synaptic cleft. In preclinical studies bupropion has reduced the neurotoxic effects produced by a single large dose of MA. In a double-blind, placebo-controlled trial, it was found that bupropion was effective in increasing the time of abstinence in male patients with MA dependence [70].
Modafinil is an antidepressant that may beneficial in treating the dysphoria associated with MA withdrawal. It is likely to decrease MA craving and may also improve the cognitive functions in MA dependent patients, Therefore it may be used to improve the response to behavioral therapies. Currently, modafinil is under clinical trials for the treatment of MA dependence [71].

Acetylcholine is implicated in the reinforcing and locomotor effects caused by the use of MA. Therefore, acetylcholinesterase (AChE) inhibitors may play a vital role to bring down methamphetamine seeking behavior. Donepezil, a reversible acetylcholinesterase inhibitor, is currently used for the treatment of dementia attenuated the reinstatement of methamphetamine-seeking behavior stimulated after self-administration in rats [72].

Rivastigmine, another AChE inhibitor, has an affinity for dopaminergic, adrenergic, muscarinic or opioid receptors. In a two week human laboratory study 3 mg of rivastigmine substantially reduced methamphetamine-induced rise in diastolic blood pressure and also craving and anxiety [73]. However, there are no recent reports of clinical trials of rivastigmine to treat methamphetamine dependence.

Lobeline is currently in clinical trials for methamphetamine abuse treatment. Lobeline interacts with nicotinic receptor subtypes, DAT, and vesicular monoamine transporters [74]. It was shown that pretreatment with lobeline (0.3–3.0 mg/kg) in rats has decreased the response for d-methamphetamine reinforcement. Furthermore, increased dose of d-methamphetamine did not surmount the lobeline-induced decrease in responding for MA. These results suggest that lobeline may be an effective, novel pharmacotherapy for methamphetamine abuse [75].
Principles of drug discovery

The hunt for new drugs can be divided into two stages: discovery and development. Drug discovery includes generating a hypothesis of the target receptor for a particular disorder and screening the in vitro and/or in vivo biological activities of the new drug candidates. Drug development involves the assessment of efficacy and toxicity of the new drug candidates. To aid in a discovery program, accurate data on pharmacokinetics and metabolism must be available as early as possible since it ultimately contributes to the eventual success or failure of the compound. The advent of early absorption, distribution, metabolism and excretion (ADME) screening has dramatically decreased the proportion of compounds failing in clinical trials due to these reasons. The main aim of preclinical ADME is to eliminate weak drug candidates in the early stages of drug development which allow resources to be focused on fewer and more-likely-to-succeed drug candidates. Withdrawing a drug from the market is a huge loss economically, therefore an early recognition of potential problems results in a substantial savings [74, 75]. Early ADME not only provides the necessary data for selecting preclinical candidates, it can also accelerate the timeline for investigational new drug applications and subsequently new drug application submission to the FDA [76].

The prime determinant of efficacy and unexpected toxicity of a drug is how it penetrates biological barriers such as intestinal wall, or blood-brain barrier (BBB). This is notably true in central nervous system (CNS) drugs, because candidates that have in vitro efficacy but cannot penetrate the BBB do not show in vivo efficacy. Thus, the entry of drugs in to the CNS is limited by the presence of the BBB. The BBB effectively isolates the brain from the blood because of the presence of tight junctions connecting the endothelial cells of the brain vessels. In addition, drug metabolizing enzymes and efflux pumps, such as P-glycoprotein (P-gp) and the multi-drug-
resistance protein located within the endothelial cells, push out the exogenous molecules from the brain [77]. As a result of these complications, CNS drugs under development have a notoriously huge failure rate [78]. Many of these failures perhaps occur due to lack of BBB permeability. With in vitro tools (such as brain micro dialysis and brain to plasma ratio) promising drug candidates without effective BBB penetration could be improved at the earliest stages of development.

A molecule needs to overcome many physicochemical barriers to reach the target site successfully. The first and foremost important barrier is solubility and permeability. Solubility and permeability assessments are crucial in ruling in or out the potential of a compound to be a drug. A preliminary solubility screen will provide information about the solubility of the new chemical entity (NCE) in solvents well-suited with administration to humans. A compound that lacks both solubility and permeability is unlikely to become a potential drug candidate even if it is potent in the primary screening assay [79]. The next barrier is chemical and metabolic stability. Chemical stability in buffers, simulated gastric and intestinal fluids, and metabolic stability in plasma, hepatocytes or liver microsomes of different species predicts a compound’s stability in different environments which will be encountered in the human body. After this, the next step is to define absorption properties of the compounds. Measurement of plasma protein binding indicates the degree of availability of the free compound in the blood circulation. Only free drug is active pharmacologically, available for metabolism and can cross the BBB measurement of protein binding is very important. Metabolism and drug-drug interaction issues can be detected by screening for inhibition of CYP-450 liver enzymes as CYP-450’s play crucial role (around 70% of metabolism) in the metabolism of xenobiotics. All these assays allow
chemists and biologists to obtain actionable information early, allowing them to gain understanding of structure-activity and structure-property relationships [76].

**Figure 1.2** Drug discovery process from identification and validation through to filing of a compound

A new drug approval is preceded by three steps, discovery, preclinical development, and clinical trial. A target for a particular disease state is identified, and several new molecules are designed and screened for toxicity and efficacy in the development stage. The results of the preliminary pharmacology and toxicology tests contribute to selection of a lead candidate. Once a lead compound is identified, the normal preclinical development process consists of: manufacture of drug substance/active pharmaceutical ingredient; preformulation and formulation (dosage design); analytical and bioanalytical methods development and validation; metabolism and pharmacokinetics in rodents; toxicology and good manufacturing practice (GMP) manufacture and documentation of drug product for use in clinical trials [80].

1.6.1. Analytical and bioanalytical methods
Analytical chemistry applications are found throughout the drug development process including the initial drug discovery phase. These applications are categorized into pharmaceutical analysis and bioanalysis. Pharmaceutical analysis is the measurement of an analyte in a clean sample or formulation, whereas bioanalysis involves the quantification of an analyte in a biological matrix, such as plasma, serum, urine and tissues [80].

Reliable analytical methods are required to test and qualify in-coming materials, in-process methods, equipment, formulations, drug substances, and drug products. The analytical methods are critical for analyzing various formulations that may be investigated for a final dosage form and are also integral part of quality control in GLP and GMP. In addition, FDA and International Conference on Harmonisation (ICH) guidelines require stability testing on each lot of drug substance and drug product. Therefore, sensitive, robust and specific analytical methods need to be developed for a variety of materials [80].

Bioanalytical support plays a vital role during the lead optimization stages [81]. Arrays of bioanalytical methods are required to completely describe the pharmacokinetics behavior in laboratory animals as well as in humans. Bioanalytical tools can play a significant role for the progress in drug discovery and development [82]. Physiologic fluids such as blood, serum, plasma, urine and tissues are analyzed to determine the absorption and disposition of a drug candidate administered to a test animal. The major goal of the bioanalysis is to assess the over-all ADME characteristics of the NCE. Often the concentration of the NCE in the biological matrix changes with time, and perhaps fall below nanogram level, therefore, quantification limits for the bioanalytical methods should be much lower than those required for analytical methods [81]. Appropriate bioanalytical methods are required to detect drug candidate at low nanogram levels, at the same time linearly over three orders of magnitude. Effects from the endogenous materials
(such as plasma proteins) of the biological matrix and stability issues make the accurate analysis difficult. Methods developed to analyze the pharmacokinetic study samples need complete separation of the analytes from matrix components [83]. The performance of the bioanalytical assay can be improved by removing interferences from the matrix through complex sample preparation steps, and concentrating the analyte of interest. Common methods of sample preparation include protein precipitation, liquid-liquid extraction and solid phase extraction [84].

1.6.2. Drug metabolism and pharmacokinetics in drug discovery

Pharmacokinetics, toxicokinetics, and metabolism are key determinants in the selection of a good drug candidate. Pharmacokinetic parameters are extrapolated from measurements of drug concentration in the plasma, blood, or other biological matrix over a selected time period. Pharmacokinetic data provides information that can guide future animal and clinical studies for the selection of the dose levels and frequency of administration. Oral and intravenous administrations are compared to determine the oral bioavailability of the drug if an oral route is anticipated for the clinic [85].

Undesirable pharmacokinetic properties, such as poor absorption, too long or too short half-life ($t_{1/2}$), and extensive first-pass metabolism majorly contribute to the failure of many drug candidates in early stages of drug development programs. The failure rate is very high because of these reasons. This demonstrates the significance of pharmacokinetics in drug discovery and development. To be successful a drug candidate needs to possess good bioavailability and a desirable $t_{1/2}$. Therefore, a precise estimation and complete understanding of the pharmacokinetic parameters in the early stages lead to good drug design [86].
Comprehensive information on the metabolism and pharmacokinetics of the new drug candidate is required by regulatory agencies [86]. Many of the approved drugs generate metabolites which possess biological activity. These active metabolites may have different pharmacology and PK properties than the parent drug. A thorough understanding of the properties of active metabolite is important for estimating toxicity and therapeutic outcome [87]. It is ideal to assess the metabolism of new drugs in vitro before proceeding to clinical studies. It is always advantageous to compare the metabolism of the drug in animals and humans in the initial stages of the drug development process as it can give information about the animal species selection for toxicity studies. Early information about the enzymes involved in the drug metabolism is very useful in the design of drug-drug interactions studies [88, 89].

In addition, the invention of liquid chromatography-mass spectrometry and liquid chromatography-nuclear magnetic resonance techniques resulted in potential to study the metabolism and PK of new drugs in the early drug discovery stage [90].

1.7. A brief introduction to the present research work

Sigma receptors have recently been identified as potential targets for the development of novel therapeutics to treat both cocaine and MA addiction and abuse. Earlier studies have demonstrated that BD1008 (N-[2-(3,4-dichlorophenyl)ethyl]-N-methyl-2-(1-pyrrolidinyl)ethylamine) a sigma receptor ligand possess anticocaine activity [91]. It was shown pretreatment with antagonists selective to sigma receptor such as AC927 (N-phenethylpiperidine oxalate) attenuated methamphetamine induced hyperthermia, dopaminergic and serotonergic neurotoxicity. Cocaine and MA interact with sigma receptors at physiologically relevant concentrations. The affinity of cocaine and MA to sigma receptors represents that targeting these
receptors using selective antagonists will be an effective strategy in the development of novel medications [92].

This dissertation is primarily focused on the metabolism and pharmacokinetics of novel sigma receptor antagonists. A series of sigma ligands were evaluated for in vitro and in vivo biological activity using different ADME tools. The in vitro metabolic stability and the in vitro half-lives of various analogs will be discussed. Further comprehensive studies were conducted for the compounds that demonstrated high in vitro metabolic stability. Development of bioanalytical methods to characterize the preclinical DMPK parameters of the compounds will be discussed. The dissertation also describes in detail the PK parameters and the factors affecting the parameters along with their possible routes of biotransformation.
CHAPTER - 2

METABOLISM AND PHARMACOKINETICS of 3-(4-(4-CYCLOHEXYLPIPERAZIN-1-YL)BUTYL)BENZO[D]THIAZOLE-2(3H)-THIONE, A NOVEL MIXED AFFINITY SIGMA RECEPTOR ANTAGONIST

2.1. Introduction

Cocaine abuse continues as a major problem in the United States due to its powerful psychological addictive properties [93]. The most common manifestations of drug toxicity are agitation, kindling (seizures, psychosis), neurotoxicity and stroke damage. Cocaine has been shown to block the reuptake of dopamine from the synapse by inhibiting the dopamine transporter and thus causing euphoria [94]. There has been a wide spread research effort to develop new molecular entities against the rewarding and adverse effects of cocaine by targeting dopamine, adrenergic and glutamate receptors. However, because of its innumerable action sites, currently no approved medication is available to treat cocaine toxicity [93]. Therefore new compounds that can mitigate the actions of cocaine are needed for the treatment of cocaine addiction [95] Interactions of cocaine with sigma receptors were revealed by the blockade of locomotor stimulant effects, attenuation of convulsions and rewarding effects of cocaine by selective sigma receptor antagonists like BD 1008 [96-98]. Also, cocaine has been shown to bind to sigma receptors with an affinity of about 2 µM, suggesting that sigma receptors are likely to represent promising targets for the development of anti-cocaine agents [93, 99-102].
Sigma receptors, discovered in 1976 by Martin and co-workers, were originally thought to be a class of opioid receptors; however numerous studies have proved them as unique receptors distinct from other proteins [93, 99, 102, 103]. To date, two subtypes of sigma receptors have been identified; sigma-1 and sigma-2, based on the binding and drug discrimination studies [99, 104]. The sigma-1 receptor exhibits high affinity and stereoselectivity for the (+)-isomers of benzomorphans, in contrast sigma-2 receptor prefers the (-)-stereoisomers. The sigma-1 receptor was cloned in 1996 [13, 14]. However, the sigma-2 receptor has not yet been cloned, and the plausible reason may be lack of selective sigma-2 ligands [105, 106]. Sigma receptors are widely distributed in the CNS with the highest concentrations found in the substantia nigra, and the cerebellum. These regions are engaged in reward, addiction and motor control. In addition, sigma receptors are distributed in peripheral organs such as the heart, liver and gastrointestinal tract [93, 94, 107].

Several studies have shown that many antidepressant and antipsychotic drugs bind to sigma-1 receptors [104, 108]. Thus selective sigma-1 receptor ligands have been proposed as potential candidates in the treatment of neuropsychological disorders such as psychotic major depression, Alzheimer’s disease and Schizophrenia [109]. A major concern with the currently existing sigma receptor ligands is that most of them are not purely sigma selective. Other than sigma receptors, they bind to dopamine transporters or NMDA receptors. Thus studies with these non sigma-prefering ligands can further complicate the understanding of the role of sigma receptors. Therefore, highly selective sigma ligands play a vital role in the development of therapeutic agents to treat cocaine abuse and various psychological disorders [11, 107].

Compound CM156 (3-((4-(4-cyclohexylpiperazin-1-yl)butyl)benzo[d]thiazole-2(3H)-thione), a cyclohexylpiperazine derivative, is a highly selective sigma receptor antagonist shown
to possess high affinity for sigma-1 and sigma-2 receptors in the nanomolar and subnanomolar range [99, 105]. Pharmacological studies demonstrated that CM156 was the best sigma receptor ligand available to date with highest selectivity and preferential affinity to sigma receptors. CM156 was shown to significantly attenuate the expression of cocaine induced behavioral sensitization and place conditioning behaviors possibly by interfering with access of cocaine to sigma receptors [103]. This study suggests the involvement of sigma receptors in the subchronic effects of cocaine such as sensitization and the reward properties [107, 110]. Cocaine also initiates multiple signal transduction pathways that modify the activities of neurotransmitter systems such as glutamatergic, dopaminergic and cholinergic systems. These systems can be regulated by sigma receptors. The novel sigma receptor antagonist CM156 is anticipated to modulate and reduce the actions of these neurotransmitter systems activated by cocaine.

In order to better understand the pharmacokinetic characteristics of CM156 we developed and validated a rapid, sensitive and reliable UPLC/MS method for the quantitative determination of CM156 in plasma. A high sample throughput was achieved by simple sample preparation and short chromatographic run times under isocratic conditions using this method. The developed bioanalytical method was validated for specificity, linearity, precision, accuracy and lower limit of quantification. This method was ultimately used in a pilot study to assess the pharmacokinetic parameters of CM156 in Sprague-Dawley rats after a single i.v administration of the compound. Using the data obtained from the pilot study, this method will be further used to investigate the ADME properties of CM156 in the future.

2.2. Experimental

2.2.1. Chemicals and reagents
Compound CM156 (Fig 2.1) was synthesized previously by Christophe Mésangeau et al. The internal standard (IS), aripiprazole, was purchased from Sigma-Aldrich (St. Louis, MO, USA). Formic acid was obtained from Mallinckrodt Baker Inc. (Phillipsburg, NJ, USA). Acetonitrile and water were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Ammonium formate was purchased from Sigma-Aldrich (St. Louis, MO, USA). Rat plasma was purchased from Innovative Research (Peary Court Novi, MI, USA). All solvents used were HPLC grade.

2.2.2. Preparation of calibration standards and quality control (QC) samples

The stock solution of CM156 was prepared by dissolving an accurately weighed amount (0.91 mg in 910 µL) of the compound in water to obtain a final concentration of 1 mg/mL. A 40 µL of the stock solution (1 mg/mL) was transferred to a clean eppendorf tube and to this 960 µL of acetonitrile was added using a pipette to make a series of working standard solutions, at concentrations ranging from 50 to 40,000 ng/mL. The IS stock solution (1 mg/mL) was prepared by weighing 0.63 mg of IS and dissolving in 630 µL methanol and from this primary stock solution, a working standard solution of IS (3 µg/mL) was prepared by diluting 3 µL of stock solution with 1 mL of acetonitrile. All stock solutions were stored at -20 °C (Kenmore) and used within one week of preparation. Calibration standards in plasma were prepared freshly at concentrations of 5, 10, 50, 100, 500, 1000, 2000 and 4000 ng/mL by spiking blank rat plasma with the working standard solutions. Blank rat plasma (100 µL) was transferred in to a 1.5 mL eppendorf tube with a pipette and to this 10 µL of working standard solutions of concentrations ranging from 50–40,000 ng/mL were added. Quality Control samples were prepared by transferring 10 µL of working standard solutions of concentrations 100, 4000, 30,000 ng/mL to an eppendorf tubes containing 100 µL of blank plasma to get 10, 400, and 3000 ng/mL standards, respectively.
2.2.3. Sample preparation

A liquid-liquid extraction method was used to extract CM156 from all of the rat plasma samples including calibration standards and QC samples. Prior to extraction, the rat plasma samples (100 µL) were thawed at room temperature, spiked with 10 µL of IS (3 µg/mL) and vortexed for 30 s. The mixture was then extracted with chloroform. A volume of 800 µL of chloroform was added to each sample, vortexed (VWR Scientific Inc., Radnor, PA, USA) for 15 min and centrifuged at 10,000 x g for 10 min at 4 °C. A fixed aliquot (750 µL) of the organic phase was transferred using a pipette into an eppendorf tube (Fisher Scientific, Pittsburgh, PA, USA) and dried in a vacuum oven (Precision Scientific, Winchester, VA, USA) at 25 °C. The resulting residue was reconstituted with 100 µL of acetonitrile and transferred into a micro sample insert (Microsolv Technology Corp., Eatontown, NJ, USA) that was pre-installed in a 1.5 mL auto-sampler vial for analysis.

2.2.4. Liquid chromatographic and mass spectrometric conditions

The chromatographic separations were performed on an Acquity UPLC (Waters Corp., Milford, MA, USA) equipped with a binary solvent manager, vacuum degasser, temperature controlled column compartment, and an auto sampler. Chromatographic separations were performed on a Waters Acquity UPLC™ BEH HILIC column (1.7 μm, 2.1 × 50 mm) using a mobile phase of 10 mM ammonium formate containing 0.1% formic acid and acetonitrile (10:90, v/v). The flow rate was set at 0.2 mL/min and this resulted in a total run time of 4 min. The injection volume was set at 10 µL and the column temperature was held constant at 25 °C. The mass spectrometric detection was carried out on a Micromass Quattro Micro™ system (Waters Corp., Manchester, UK) in positive ion mode.
Fig. 2.1. Structures of CM156 and Aripiprazole (IS)

The following MS parameters were selected for optimal detection of the CM156 compound: a capillary voltage of 4.74 kV; a cone voltage of 36 V; an extractor voltage of 5 V; a RF lens voltage of 0.5 V; a source temperature of 60 °C and a desolvation temperature of 250 °C. The desolvation and cone gas flows were set at 500 and 72 L/hr, respectively. Quantification was carried using selected ion monitoring (SIM) for CM156 m/z 390 and IS m/z 448, with a dwell time of 500 ms. Data acquisition and data processing were performed using Masslynx 4.1 software (Micromass, Manchester, UK) and Microsoft Excel.

2.2.5. Method validation

Analytical method validation assays were performed as per the United States Food and Drug Administration (US-FDA) Bioanalytical Method Validation Guidance [111]. The validation of the UPLC/MS method included linearity, lower limit of quantification (LLOQ), limit of detection (LOD), precision, accuracy, selectivity, recovery, matrix effect and stability.

2.2.5.1. Linearity and sensitivity
An eight-point calibration curve 5, 10, 50, 100, 500, 1000, 2000 and 4000 ng/mL was constructed by plotting the ratio of the analyte peak area/ IS peak area versus analyte concentration. The linearity of the calibration curve was evaluated by linear regression analysis (MS Excel 2007). The sensitivity of the developed method was determined using LLOQ, the lowest concentration on calibration curve with a relative standard deviation, (R.S.D.) and relative error (RE) of less than 20%. The LOD is defined as the analyte concentration which gives rise to peak whose height is 3 times that of baseline noise.

2.2.5.2. Selectivity

The selectivity of the developed method was investigated for the assessment of potential interferences of the analyte and IS from endogenous substances. This was evaluated by comparing the chromatograms of six different lots of blank rat plasma with the corresponding spiked plasma samples with CM156 and the IS.

2.2.5.3. Recovery and matrix effect

The extraction recovery of CM156 from rat plasma was determined at concentrations of 10, 400, and 3000 ng/mL by comparing the peak area ratios of the compound and IS. Recovery was calculated by comparing the plasma samples spiked with the compound and IS before extraction with the plasma samples to which the compound and IS were added after extraction.

Matrix effect is the effect (such as ion suppression) caused by the components other than the analyte (such as metabolites or endogenous substances) present in the sample on an analytical method. The matrix effect, due to co-eluting plasma components, was evaluated by spiking six different lots of blank rat plasma with the QC solutions. The matrix effect of CM156 was
determined at three QC levels (10, 400, 3000 ng/mL) by comparing the peak area ratios of standards prepared in plasma with peak area ratios of standards prepared in acetonitrile.

Matrix effect = \frac{\text{Peak area of standards prepared in plasma}}{\text{Peak area of standards prepared in acetonitrile}} \times 100

### 2.2.5.4. Precision and accuracy

The precision and accuracy of the assay were determined by analyzing QC samples at three different concentrations (10, 400, 3000 ng/mL). To evaluate intra-day accuracy and precision, QC samples were analyzed in six replicates at each concentration level. The inter-day accuracy and precision was determined by analysis of QC samples on three consecutive days. The concentrations were calculated based on calibration curve. The accuracy of the assay was expressed as relative error (RE),

\[
\text{RE} = \frac{\text{Observed concentration} - \text{Spiked concentration}}{\text{Spiked concentration}} \times 100\%.
\]

The precision of the developed method was expressed as relative standard deviation (R.S.D.),

\[
\text{R.S.D} = \frac{\text{Standard deviation}}{\text{Mean}} \times 100
\]

The intra-day and inter-day precisions were required to be below 15%, and the accuracy to be within ±15%.

### 2.2.5.5. Stability

The stability of CM156 in rat plasma was determined by the analysis of six replicates of QC samples (10, 400, 3000 ng/mL) exposed to various storage conditions including freeze/thaw
short-term temperature, room temperature, long-term temperature stability and the stability of CM156 in reconstituted samples (auto-injector stability). For freeze-thaw stability studies, unprocessed QC samples (100 µL) were subjected to three freeze-thaw cycles. Each sample was stored at -20 °C (Kenmore) for 24 h and thawed at room temperature, after which the samples were refrozen for 24 h under the same conditions. At the end of each cycle, the samples were processed as explained in the section 1.2.3, analyzed and compared with the freshly prepared QC samples. For the short-term temperature stability study, unprocessed CM156 and IS QC samples were kept at room temperature for 12 h, which exceeds the routine preparation time of the samples. At different time points (0, 3, 6 & 12 h) the samples were processed, analyzed and compared with the freshly prepared QC samples. The post operative stability during storage in the auto sampler was assessed by re-injecting the samples that were held in the auto sampler at 25 °C for 24 h. To determine long-term stability, QC samples were stored at -20 °C for 1 month (30 days) which exceeds the time between sample collection and sample analysis.

2.2.6. Physicochemical characterization

2.2.6.1. Determination of the acid dissociation constant (pKa)

pKa of the compound was determined by Potentiometric titrations and ACD/Labs (Algorithm Version: v12.1.0.3346, Toronto, ON, Canada). For potentiometric titrations, a 1 mM solution of CM156 was prepared by dissolving 11 mg of compound in 25 mL of water. To this solution 25 µL of 0.01 M sodium hydroxide solution was added using a 200 µL pipette (Fisher Scientific, NJ, USA) and the change in pH was recorded using a Mettler Toledo S20 SevenEasy pH Meter (Fisher Scientific, NJ, USA). The procedure was repeated until a constant pH was obtained. Thereafter, 25 µL of 0.01 M hydrochloric acid was added continuously and the change in pH was recorded until a constant value is reached. The titration curves were then constructed.
by plotting the recorded pH versus volume of base/acid added. The intersection point of the curves was noted as the pKa of the compound.

2.2.6.2. Determination of Log \( P_{O/W} \), Log \( D_{PBS,pH\,7.4} \) and Solubility

The octanol/water (pH 6.4) and octanol/PBS (pH 7.4) partition coefficients (Log P and Log D\(_{7.4}\)) of CM156 were determined by Stir flask method. For the determination of Log P and Log D, both phases (octanol, water/PBS) were saturated with one another before the experiment for 12 h. A portion of CM156 (0.54 mg) was accurately weighed and transferred to a clean glass vial (4 mL) and then to this 1 mL of the aqueous phase saturated with octanol was added using a 1 mL pipette (Eppendorf, Hamburg, Germany). To the above solution 1 mL of octanol added and stirred for 24 h on a VWR Dylastir magnetic stirrer (Bridgeport, NJ, USA) using a magnetic bead. An aliquot of 100 µL of the aqueous phase was then analyzed by UPLC/MS/MS. Calibration curves prepared in phosphate buffer and water were used for the estimation of the concentration in aqueous phase. For the preparation of calibration curve, 0.91 mg of compound was dissolved in 910 µL of acetonitrile to get a stock solution of 1 mg/mL. From this solution calibration standards were prepared by diluting with buffer.

The solubility of CM156 was determined in distilled water and in phosphate buffer saline (pH 7.4) using the standard shake flask method. An excess amount (20 mg) of CM156 was added to 100 µL of the solvent in 2 mL glass vials. The vials were constantly agitated (100 rpm) at room temperature (25 °C) for 24 h in reciprocating shaking water bath (Precision Scientific, VA, USA) for uniform mixing. After 24 h, the samples were collected using a pipette and centrifuged at 10,000 x g for 5 min. The supernatant was then analyzed using UPLC/MS/MS.

2.2.7. Metabolic stability of CM156 in rat, mouse and human liver microsomes
Biotransformation of xenobiotics is divided into two types: phase I (hydrolysis, oxidation, and reduction) and phase II (conjugation). The cytochrome P450 (CYP 450) enzyme superfamily is predominantly responsible for phase I metabolism, and phase II conjugation is mainly catalyzed by UDP-glucuronyltransferases [112, 113]. CM156 was incubated with liver microsomes from male rats, mouse and humans in Tris buffer (pH 7.4). The standard incubation mixture contained 0.1 M MgCl₂, 1 mM NADPH regenerating system (NADP 1 mM, glucose-6-phosphate 5 mM, and glucose-6-phosphate dehydrogenase 1 Unit/mL), 1 mg of microsomal protein and 5 µM substrate. The final volume was adjusted to 1 mL with the appropriate amount of buffer. Incubations were performed at 37 °C and 100 rpm in shaking water bath (Precision Scientific, VA, USA) for 30 min. Metabolic stability was assessed quantitatively by measuring the disappearance of the substrate during the incubation period. Reactions were initiated by the addition of NADPH regenerating system after 5 min of pre-incubation and terminated at predetermined time points (0, 5, 10, 15, 20 and 30 min) by the addition of equal volumes (1 mL) of ice-cold acetonitrile. Samples were vortexed for 30 sec, then centrifuged at 10,000 x g for 10 min finally the supernatant was analyzed by UPLC/MS/MS. The separation was achieved on a C₁₈ (2.1 mm x 50 mm, 1.7 µm) column by elution with mobile phase consisting of 0.1% formic acid in water and acetonitrile (23:77 v/v). The mobile phase was pumped at a flow rate of 0.15 mL/min. For Phase II metabolism studies, CM156 was incubated with rat liver microsomes in presence of UDPGA (Sigma-Aldrich, USA). The substrate concentration was 5 µM. Incubations of CM156 without NADPH and UDPGA were run as control.

2.2.8. Plasma protein binding studies

The extent of protein binding of CM156 in rat plasma was determined by ultrafiltration using the Centrifree micropartition system (Amicon, MA, USA) at specific concentrations of 300
and 2000 ng/mL. A stock solution (1 mg/mL) of CM156 was prepared by dissolving 0.45 mg in 450 µL. From this stock solution working standards of concentrations 3 and 20 µg/mL were prepared by diluting with buffer. The plasma samples of concentrations 300 and 2000 ng/mL were then prepared by diluting the working standards with rat plasma. The samples (500 µL) were then transferred using 1 mL pipette to Centrifree® devices and incubated at 37 °C. After 15 min of incubation, the plasma samples were subjected to centrifugation in Beckman rotor centrifuge at 1500 x g for 10 min and ultrafiltrates were collected in to a 1.5 mL eppendorf tube from the Centrifree devices using a pipette. To an aliquot (100 µL) of the ultrafiltrates, 10 µL of internal standard was added and analyzed by UPLC/MS/MS. A control experiment was done with ammonium acetate buffer (pH 7.4) to estimate the nonspecific binding of the substrate. Protein binding was calculated using the following equation.

\[
\text{Plasma protein binding (\%)} = 100 - \left( \frac{\text{Concentration of ultrafiltrate} \times 100}{\text{Concentration added}} \right)
\]

2.2.9. In vivo pharmacokinetics in rats

The developed and validated UPLC/MS method was used to determine the pharmacokinetic parameters of CM156 in rats after the intravenous administration of the compound. The animal experimental protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Mississippi. Six male Sprague-Dawley rats (180-200 g) were obtained from Harlan Laboratories (Indianapolis, IN, USA), which had already inserted polyethylene cannulas into the right jugular vein. The rats were housed in metabolic cages and allowed free movement and access to water during the whole experiment. The rats were fasted for 12 h before dosing and for the first 4 h after dosing. A small amount of CM156 (4 mg) was dissolved in saline (2 mL, measured using a pipette) for i.v administration. A single
intravenous bolus (0.5 mL) of CM156 was injected at a dose of 5 mg/kg through the jugular vein cannula in less than 30 s. The formulation was filtered through a 0.2 µm filter prior to administration. The i.v. solution was administered via the jugular vein cannula using a syringe (1 mL), after which the cannula was flushed with 0.2 mL heparinized saline to ensure complete administration of the dose.

Blood samples were collected using a syringe (1 mL) through the indwelling cannula into heparinized micro centrifuge tubes at 0, 2, 5, 10, 15, 20, 30, 45, 60, 90, 120, 150, 180, 240, 360, 480 min. An initial blood volume of 0.05 mL was withdrawn to clear the line of heparinised saline. A fresh syringe was then used to withdraw a 0.25 mL blood sample that was placed in a micro-centrifuge tube. After each blood sampling, 0.2 mL of a heparin-saline (10 I.U./mL) solution was used to flush the catheter. Blood samples were immediately centrifuged at 10,000 \( \times g \) for 20 min at 4 °C using an Accuspin Micro 17R centrifuge (Fisher Scientific, Pittsburgh, PA, USA). The plasma was separated from the solid components and transferred into a 1 mL micro centrifuge tubes. These samples were frozen (Kenmore) at -20 °C until they were analyzed. The pharmacokinetic parameters were calculated by non compartmental analysis using WinNonlin 5.2 (Pharsight, Mountain View, CA, USA).

**Urine sampling**

A solution of CM156 was intravenously administered to rats \((n=6)\) at a dose of 5 mg/kg. Urine was collected in to BD vacutainer containers at specified time intervals (0-2, 2-4, 4-8 h) after the administration of compound. Blank urine (urine before administering the test compound) that was collected in to the graduated urine collection tube of the metabolic cage was also transferred to BD vacutainer®. The volume of urine collected in graduated containers was
noted during each time interval and kept frozen at -20 °C until analyzed. Urine samples were analyzed by LC/MS/MS for the quantification of CM156 in urine and to detect phase I and phase II metabolites.

2.2.10. Brain to plasma ratio

Brain to plasma ratio gives information about the extent of penetration of the compound into the brain [114]. For the determination of brain to plasma ratio of CM156, brains were thawed at room temperature and transferred to a 20 mL glass vial. To this 3 mL of water was added and the tissues were homozinized using a tissue homozinizer, (Tekmark, OH, USA). The homogenate was then transferred to a fresh eppendorf tube (1.5 mL) and centrifuged at 10,000 x g for 10 min to remove the entrapped air. An aliquot (100 µL) of the homogenate was transferred using a pipette in to a clean eppendorf tube, to this 10 µL of IS was added and vortexed for 30 s. The mixture was then extracted with chloroform (800 µL) by vortexing for 10 min and centrifuged using an Accuspin Micro 17R centrifuge at 10,000 x g for 7 min. A fixed aliquot (725 µL) of the lower organic layer was collected after centrifugation and subjected to evaporation in a vacuum oven (Precision Scientific, Winchester, VA, USA). The residue was reconstituted in 100 µL of water and aliquot of 5 µL was injected into the UPLC/MS/MS for the quantification. The concentration of CM156 in brain samples was then determined from standard curves prepared in blank brains (Pel-Freeze, AR, USA).

Calibration curve

The preparation of calibration standards is similar to standards prepared in plasma, explained in section 1.2.3. Briefly, for the construction of a standard graph seven concentrations 0.1, 0.5, 1, 2, 5, 7 µg/mL were used. The standards were prepared in blank brain samples by
spiking 10 µL of solutions of concentrations 1, 5, 10, 20, 50 and 70 µg/mL. To this mixture, 10 µL of the IS (3 µg/mL) was added, vortexed for 30 s and extracted with chloroform (800 µL). A fixed aliquot of the organic phase (725 µL) was separated using a pipette after centrifugation and was evaporated to dryness in a vacuum oven. The dried samples were reconstituted with 100 µL of water and analyzed using UPLC/MS/MS.

Atenolol and Imipramine were used negative and positive controls. Atenolol was administered to rats at a dose of 10 mg/kg intraperitoneally and B/P was determined at 30 min after the administration of the compound. Imipramine was administered at a dose of 20 mg/kg intraperitoneally and B/P ratio was determined at 3 h after administration of the compound. For the assessment of B/P ratio of atenolol and imipramine, brain and plasma samples were extracted (same as CM156 extraction procedure) with acetonitrile and analyzed using UPLC/MS/MS.

2.2.11. Identification of urinary metabolites of CM156 by tandem mass spectrometry

The metabolism of the CM156 in rats was characterized using liquid chromatography (UPLC)/tandem mass spectrometry (MS/MS) with precursor ion and constant neutral loss scans followed by product ion scans [115]. Tandem mass spectrometry is capable of rapidly identifying metabolites with characteristic sub-structures without the necessity of baseline separation of each compound. The identification of metabolites using MS/MS is based on the assumptions that most metabolites retain much of their original scaffolding and undergo the same fragmentation pattern as the parent compound. The structures of possible metabolites are characterized by interpreting their product ion spectra or comparing the product ion spectra and LC retention times with authentic standards [115, 116].

The approach can be summarized as follows:
(1) to obtain a product ion scan of a parent drug
(2) to conduct precursor ion scans of the selected product ions obtained in previous step. This step provides the molecular ion information for the metabolites;
(3) to obtain product ion scans of the identified metabolites. This step gives the information for characterization of the metabolite structures.

2.2.11.1. Liquid chromatography-triple quadrupole mass spectrometry

All samples were analyzed for the presence of phase I and phase II metabolites on an Waters Acquity UPLC™ BEH C₁₈ column (1.7 μm, 2.1 × 50 mm). Injection volume was 5 μL. The mobile phase consisted of 0.1 % formic acid in water: acetonitrile. The gradient program was 0 to 3 min at 5 to 10 % B, 3 to 10 min at 10 to 22% B, 10 to 12 min at 22 to 50% B, 12 to 14 min at 50 to 5% B and 14 to 16 min at 5% B. The MS detection was carried out on Micromass Quattro micro™ system (Waters Corp., Manchester, UK). LC/MS/MS with positive electrospray ionization in various scan modes such as precursor (parent) ion scan, constant neutral loss scan (NLS), multiple reaction monitoring (MRM) and daughter (product) ion scan were used for the identification of phase I and phase II metabolites.

2.3. Results and discussion

2.3.1. Chromatography

Because CM156 is a polar compound, it did not retain well on a traditional C₁₈ column. Consequently, based on the retention time of the compound and the separation efficiency of the column, an Acquity BEH HILIC column was selected to develop the UPLC assay. Aripiprazole was chosen as the IS due to its similar chromatographic characteristics, strong mass response in
positive ESI mode and lack of endogenous interferences at $m/z$ 448. Different concentrations of formic acid (0.05%, 0.1% and 0.2%) in aqueous phase were tested to improve the chromatographic peak shapes and increase the MS response. The results indicated that a solution of 0.1% of formic acid improved the peak shapes and MS response of CM156 and the IS, but was unable to produce baseline separation of the two compounds. To improve the separation, various amounts of ammonium formate (1, 5, 10 mM) were added to the aqueous phase. Baseline separation of analyte and IS is very important when LC/MS is used for the analysis. This avoids the interferences caused due to possible ion suppression or enhancement effects [117]. We determined that 10 mM ammonium formate produced the best separation of the two compounds. Acetonitrile was chosen as the organic phase because it produced a higher analyte response, which led to lower background noise compared to methanol. The selected mobile phase consisted of 10 mM ammonium formate buffer solution containing 0.1% formic acid and acetonitrile (10:90 v/v). This was pumped at a flow rate of 0.2 mL/min for analysis, the retention times for CM156 and IS were 2.6 and 2.1 min and a run was completed in 4 min. As protein precipitation alone did not result in the complete purification of the plasma samples, a single-step liquid–liquid extraction was adopted to achieve high recovery of the analytes with no interferences in minimal time. Chloroform was selected as the extraction solvent because it showed invariable recoveries ranging from 86.3 to 105.9% in the concentration range from 5 to 4000 ng/mL.
<table>
<thead>
<tr>
<th>Solvent</th>
<th>% Recovery of CM156</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetonitrile</td>
<td>41</td>
</tr>
<tr>
<td>Chloroform</td>
<td>96</td>
</tr>
<tr>
<td>Dichloromethane</td>
<td>34</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>54</td>
</tr>
<tr>
<td>Methyl tert-butyl ether</td>
<td>55</td>
</tr>
</tbody>
</table>

**Table 2.1:** Recovery of CM156 in various solvents

<table>
<thead>
<tr>
<th>Conc (ng/mL)</th>
<th>Recovery (%) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>94.5 ± 4.3</td>
</tr>
<tr>
<td>400</td>
<td>92.0 ± 6.1</td>
</tr>
<tr>
<td>3000</td>
<td>89.3 ± 6.3</td>
</tr>
</tbody>
</table>

**Table 2.2:** Recovery of CM156 from spiked rat plasma

2.3.2. Mass spectrometry

Electro spray ionization positive ion monitoring mode was chosen for the measurement of CM156 in rat plasma samples. Because CM156 was a basic compound, it captured the protons easily and gave maximum detector intensity in the positive ionization mode. Upon the direct injection of CM156 and the IS in to the mass spectrometer, singly protonated ions were found to be the most sensitive ions [M+H]+. Therefore the molecular ions at \( m/z \) 390.09 and \( m/z \) 448.03 were selected as the target ions in SIR for CM156 and the IS respectively. Full mass scans of CM156 and the IS are shown in Fig. 2.2.
Fig. 2.2. Full mass scan of CM156 (A) and Aripiprazole (IS, B)

2.3.3. Method validation

2.3.3.1. Linearity of calibration curve and lower limit of quantification

Calibration standards of CM156 at concentration levels of 5, 10, 50, 100, 500, 1000, 2000 and 4000 ng/mL were extracted and assayed. A typical regression equation for the calibration curve was: $y = 0.047x + 0.1997$

Where $y$ represents the peak area ratios of CM156 to the IS and $x$ represents plasma concentrations of analyte. The calibration curve was found to be linear over the concentration range from 5 to 4000 ng/mL resulting in a correlation coefficient $r^2 > 0.995$. The Lower limit of quantification for CM156 in plasma was 5.0 ng/mL with precision (R.S.D.) below 20% and
accuracy (RE) within ±20%. This quantification was found to be sensitive enough to investigate the pharmacokinetic behavior of CM156 in preclinical studies. The LOD was estimated at 2 ng/mL.

![Calibration curve of CM156](image)

**Fig. 2.3.** Calibration curve of CM156.

### 2.3.3.2. Selectivity

The selectivity of the assay was assessed by comparing the chromatograms of six different lots of blank rat plasma with the corresponding spiked plasma. The representative chromatograms of blank plasma, plasma spiked with CM156 (400 ng/mL) and IS (300 ng/mL) are presented in Fig. 2.4. No interference were observed at the retention times of the CM156 (2.6 ± 0.1 min) or the IS (2.1 ± 0.1 min).
Fig. 2.4. Representative SIR chromatograms for CM156 and Aripiprazole (IS) in rat plasma: (a) blank plasma sample; (b) blank plasma sample spiked with CM156 at LLOQ and aripiprazole (IS) at 300.0 ng/mL; (c) rat plasma sample obtained at 1.0 hr after a single intravenous injection at a dose of 5 mg/kg.
2.3.3.3. **Precision and accuracy of the assay**

The accuracy and the intra and inter-day precision of the analytical method were evaluated with six replicates at three different concentrations 10, 400 and 3000 ng/mL. The intra-day precision ranged from 3.5% to 4.4% and the inter-day precision ranged from 2.6% to 5.6%. The accuracy of the assay ranged from -6.5 to 5.0%. The data obtained was within the acceptable limits and the method was precise and accurate. Table 2.3. summarizes intra- and inter-day precision and accuracy.

<table>
<thead>
<tr>
<th>Spiked conc. (ng/mL)</th>
<th>Intra-day precision and accuracy</th>
<th>Inter-day precision and accuracy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Measured concentration (mean ±SD, ng/mL)</td>
<td>R.S.D (%)</td>
</tr>
<tr>
<td>10</td>
<td>10.5 ± 0.4</td>
<td>3.8</td>
</tr>
<tr>
<td>400</td>
<td>411.0 ± 14.5</td>
<td>3.5</td>
</tr>
<tr>
<td>3000</td>
<td>2932.4 ± 130.2</td>
<td>4.4</td>
</tr>
</tbody>
</table>

**Table 2.3** Precision and accuracy data for CM156 in rat plasma

2.3.3.4. **Recovery and matrix effect**

The extraction recoveries of CM156 from rat plasma at the concentrations of 10, 400 and 3000 ng/mL were 97.2 ± 5.6%, 100.4 ± 6.9% and 101.5 ± 7.8%, respectively. The extraction recovery of IS from rat plasma was 58.2% at a concentration of 300 ng/mL. Recovery of the internal standard was consistent and reproducible. The matrix effects of CM156 were between 90% and 105%. The matrix effect of IS was 98.5%. There is no difference between the peak areas of standards prepared in plasma and peak areas of standards prepared in acetonitrile. These results indicated that no co-eluting substances influenced the ionization of the analyte and IS.
2.3.3.5. Stability

The stability of CM156 was evaluated under different conditions at three concentrations (10, 400 and 3000 ng/mL). The results indicated that CM156 was stable in rat plasma stored at room temperature for 12 h, at -20 °C for 1 month and during three freeze/thaw cycles. The compound was also found to be stable in reconstituted samples when stored for 24 h in the auto sampler at 25 °C. The stock solutions of CM156 and IS were stable for at least 6 h at room temperature and for one week at -20 °C. Results of the stability studies are shown in Table 2.4.

<table>
<thead>
<tr>
<th>Storage condition</th>
<th>Concentration (ng/mL)</th>
<th>R.S.D. (%)</th>
<th>RE (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Spiked</td>
<td>Measured</td>
<td></td>
</tr>
<tr>
<td>Three freeze thaw cycles</td>
<td>10</td>
<td>9.2 ± 1.1</td>
<td>12.3</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>406.4 ± 8.8</td>
<td>2.2</td>
</tr>
<tr>
<td></td>
<td>3000</td>
<td>2879.4 ± 209.8</td>
<td>7.3</td>
</tr>
<tr>
<td>Long term for 30 days (-20 °C)</td>
<td>10</td>
<td>11.2 ± 0.9</td>
<td>8.6</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>394.8 ± 17.8</td>
<td>4.5</td>
</tr>
<tr>
<td></td>
<td>3000</td>
<td>2879.4 ± 243.1</td>
<td>7.8</td>
</tr>
<tr>
<td>Short term for 12hr (25 °C)</td>
<td>10</td>
<td>8.6 ± 0.7</td>
<td>8.3</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>4.3.6 ± 9.2</td>
<td>2.2</td>
</tr>
<tr>
<td></td>
<td>3000</td>
<td>2879.4 ± 145.2</td>
<td>5.0</td>
</tr>
<tr>
<td>Auto sampler for 24hr (25 °C)</td>
<td>10</td>
<td>10.0 ± 0.2</td>
<td>3.0</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>404.4 ± 4.7</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>3000</td>
<td>2901.1 ± 59.2</td>
<td>2.0</td>
</tr>
</tbody>
</table>

Table 2.4. Stability data for CM156 in rat plasma at various storage conditions

2.3.6. Physicochemical characterization

Drugs acting on the CNS must cross the Blood brain barrier and the BBB penetration of drug candidates can be assessed by determining their physicochemical properties like Log P, Log
D and pKa. The pKa of the compound CM156 was 9.2 indicating that the compound is weakly basic in nature and it exist in equilibrium between its charged and neutral states under physiological conditions. The ideal pKa for CNS compounds is between 4-10 [118].

The partition coefficient (Log P) is a constant and is defined as the ratio of concentration of a neutral compound in aqueous phase to the concentration in an organic solvent. Log D is the log distribution coefficient at a particular pH. Log D varies with pH. Log D at pH 7.4 is often given an indication of the lipophilicity of a compound at the pH of blood or plasma. The log P o/w (pH 6.4) and log D 7.4 of the compound were 0.5 and 1.6, respectively. This indicates that the compound distributes in to both octanol and water phases in appreciable amounts.

2.3.7. In vitro metabolism studies

Metabolic stability of CM156 was investigated in liver microsomes from mouse, rat, and human. Half-life was determined from the linear portion of the curve (Fig.2.5) with a mono exponential decay using GraphPad Prism 5. The compound was highly unstable in liver microsomes and degraded more quickly in mouse and rat liver microsomes compared to human liver microsomes in the presence of NADPH. The half-lives were found to be 3.3, 4.1 and 4.6 min in mouse, rat and human liver microsomes, respectively. To evaluate the metabolism by phase II conjugation, UDPGA was introduced to the microsomal incubation mixture. There was no major difference in the metabolic stability of CM156 in the presence or absence of UDPGA as evidenced by comparable half-lives during incubation with phase I and II systems (Table 2.5). These results further confirm that phase I enzymes are predominantly responsible for the in vitro metabolism of CM156. The poor metabolic stability of CM156 might be ascribed to the weak C-N bond between the benzo[\textit{d}]thiazole-2(3\textit{H})-thione ring and the cyclohehylpiperazine
moiety in addition to the high susceptibility of the sulfur atom in the thiazole-2(3H)-thione moiety to metabolism.

<table>
<thead>
<tr>
<th>Species</th>
<th>Half-life (min) Phase I</th>
<th>Half-life (min) Phase II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat</td>
<td>4.6</td>
<td>5.0</td>
</tr>
<tr>
<td>Mouse</td>
<td>3.3</td>
<td>3.9</td>
</tr>
<tr>
<td>Human</td>
<td>4.1</td>
<td>4.6</td>
</tr>
</tbody>
</table>

**Table 2.5.** Metabolic stability of CM156 in rat, mouse and human liver microsomes

![Graph](image)

**Fig. 2.5.** Plot of the incubation time (min) vs percent drug remaining

**2.3.8. Plasma protein binding**

Compound CM156 was found to be 82% bound to rat plasma proteins at three different concentrations. The protein binding did not change with increase in concentration of the compound indicating that the binding was independent of concentration. The nonspecific binding of the compound to the components of Ultracentrifree® was less than 0.2%, indicating that ultrafiltration is a suitable method for protein binding studies of CM156.
2.3.9. Pharmacokinetics of CM156 in rats

After the administration of a single i.v. dose of 5 mg/kg, the Cmax of CM156 was 1.3 ± 0.2 µg/mL. The plasma concentrations declined very quickly indicating a rapid distribution of the novel sigma receptor ligand into the tissues. The distribution of CM156 was found to be extensive (9.6 L/kg) indicating high tissue binding. This is probably due to the hydrophobic nature of the compound. The log D Oct/PBS (pH 7.4) was 1.6. Despite its high Vd, CM156 exhibited a short half-life (65 min). The rapid elimination from the systemic circulation i.e, a high clearance (6.2 L/h/kg) might be a plausible reason for the half-life. This is supported by its extremely poor microsomal metabolic stability and high hepatic intrinsic clearance. The amount of compound excreted unchanged in urine was less than 0.1% and this further suggests that high clearance is the result of extensive liver metabolism. The mean plasma concentration-time profile is shown in Fig. 2.6. The pharmacokinetic parameters are presented in Table 2.6.

<table>
<thead>
<tr>
<th>Conc of CM156 (ng/mL)</th>
<th>% Binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>300</td>
<td>82.8</td>
</tr>
<tr>
<td>1100</td>
<td>82.3</td>
</tr>
<tr>
<td>2000</td>
<td>81.6</td>
</tr>
</tbody>
</table>

**Fig. 2.6.** Mean plasma concentrations of CM156 after a single intravenous injection of to rats at a dose of 5mg/kg (n=6)
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>t_{1/2} (hr)</td>
<td>1.1 ± 0.4</td>
</tr>
<tr>
<td>Cmax (µg/mL)</td>
<td>1.3 ± 0.2</td>
</tr>
<tr>
<td>AUC_{0→∞} (µg min/mL)</td>
<td>48.1 ± 6.3</td>
</tr>
<tr>
<td>V_d (L/kg)</td>
<td>9.6 ± 1.4</td>
</tr>
<tr>
<td>CL (L/h/ kg)</td>
<td>6.2 ± 1.0</td>
</tr>
<tr>
<td>MRT (min)</td>
<td>90.1 ± 13.0</td>
</tr>
</tbody>
</table>

**Table 2.6** Pharmacokinetic parameters of CM156 in rats following a single intravenous dose of 5 mg/kg (Mean±SD, n=6).

**2.3.10. Brain to plasma ratio**

BBB separates the blood compartment from those within the brain and it is the permeability barrier for transport from and into the CNS. Adequate exposure of the drugs in the brain is essential for the treatment of CNS diseases. BBB greatly impedes entry of all molecules from blood to brain, except small and lipophilic molecules or those that enter the brain through an active transport mechanism with nutrients, and cofactors. B/P ratio is a common approach in CNS drug discovery process to estimate the ratio of drug concentration in brain and plasma. It provides a measure of partitioning of drug in to brain from blood and the results are expressed as a brain/plasma ratio [119]. Brain to plasma ratio of CM156 was determined at 10 and 20 min after the i.v. administration at a dose of 5 mg/kg. Fig 2.7 shows the calibration curve of CM156 in rat brain. The brain concentrations are 6-7 times higher than the plasma concentration. The results are in agreement with the pharmacokinetic profile, with a reported apparent volume of distribution being 9.6 ± 1.4, indicating considerable uptake of the drug into tissues. The brain to plasma (B/P) ratio of CM156 at 10 and 20 min were found to be 6.5 and 7.2, respectively. The
high B/P ratio of CM156 suggests that it is freely crossing the BBB and accumulating in the brain tissues. The B/P values at 10 and 20 min are represented in table 2.7.

**Figure 2.7:** Calibration curve of CM156 in rat brain tissues

![Calibration curve of CM156 in rat brain tissues](image)

\[
y = 29.875x + 1.7536 \\
r^2 = 0.999
\]

**Table 2.7.** Concentrations of CM156 in plasma and brain; ratio between brain and plasma concentrations (n=3)

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Time (min)</th>
<th>Concentration</th>
<th>B/P ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10</td>
<td>6.3 Brain (µg/gm)</td>
<td>0.96 Plasma (µg/mL)</td>
</tr>
<tr>
<td>5</td>
<td>20</td>
<td>3.8 Brain (µg/gm)</td>
<td>0.52 Plasma (µg/mL)</td>
</tr>
</tbody>
</table>

**2.3.11. Identification of urinary metabolites**

Compound CM156 produced a protonated molecular ion, [M+H]^+ at m/z 389, consistent with the molecular formula C_{21}H_{22}N_{3}S_{2}. The MS/MS spectrum of the m/z 389 generated a series of product ions at m/z 221, 169, 142, 124, 86, 55. The major product ion at m/z 221 was formed due to the loss of 168 (loss of cyclohexylpiperazine moiety). The product ion at m/z 169 and 86 corresponds to benzo[d]thiazole-2(3H) -thione and cyclohexyl groups, respectively. The parent compound has a labile bond between the carbon atom of the 3-butylbenzo[d]thiazole-2(3H)-
thione group (m/z 221) and the nitrogen of the cyclohexylpiperazine moiety (m/z 168). These groups with their molecular weights are represented in fig. 2.8. This C–N bond is highly susceptible to fragmentation, resulting in formation of two major fragments: a stable carbocation 4-(2-thioxobenzo[d]thiazol-3(2H)-yl)butan-1-ylium representing the major peak (m/z 221.9) in the MS/MS spectrum and a neutral fragment (m/z 168) corresponding to cyclohexylpiperazine moiety.

Fig.2.8. Product ion spectra and structure of parent compound, CM156

As the metabolites generally share a common structure with the parent compound, the initial search for the metabolites of CM156 in urine samples was conducted by a precursor ion scan using the major fragment ion of the parent compound, m/z 221. In addition to this, m/z 176 (dehydroglucuronicacid) and m/z 168, 80 were used to perform the neutral loss scan to detect the phase II metabolites; glucuronide and sulfate conjugates. The precursor ion scan of the rat urine sample yielded ions at m/z 390, the parent compound, m/z 406, 307, 321 and 375. The neutral loss scan of 176 showed a single peak with the parent ion of m/z 582.
With the aid of the precursor ion scan at m/z 221 and the neutral loss scan of 176 from the urine samples, we were able to identify the major metabolites of CM156. The interpretation of the product ion spectra of the ions with m/z 389 (CM156), 375 (M1), 307 (M2), 321 (M3), 406 (M4), 582 (M4-Glu) is explained as follows.

The protonated molecular ion of metabolite M1 showed an intense signal at m/z 374, 16 Da lower than the parent compound (CM156) suggesting the replacement of the sulfur atom with oxygen. The MS/MS spectrum of M1 (m/z 374) yielded a series of characteristic product ions at m/z 292, 263, 205, 182, 164. The product ions at m/z 292 and 205 were formed due to the loss of the cyclohexyl and cyclohexylpiperazinyl moieties, respectively. Nevertheless, the other substructures of M1 could not be identified with the product ion spectrum. M1 had similar product ions scan and it co-eluted with the authentic standard on LC. Based on its characteristic product ions and LC retention times M1 was identified and confirmed as oxidative metabolite of CM156. Fig.2.9. represents the daughter ion spectrum of urine sample (a) and synthetic standard (b).

![Product ion spectra of metabolite M1](image)

**Fig. 2.9. (a).** Product ion spectra of metabolite M1
Fig. 2.9.(b). Product ion spectra of authentic standard

The [M+H]^+ ion of M2 at m/z 307 was generated due to the loss of a cyclohexyl ring. The major fragmentation observed was probably due to the loss of the fragment 168 to form an abundant daughter ion at m/z 221. The other product ions identified were m/z 169, 124, 142, and 55. The fragment ions of M2 at m/z 221 and 169 confirm the proposed structure of M2.

Fig. 2.10. Product ion spectra and proposed structure of metabolite M2
Metabolite M3 was proposed as oxo metabolite of M2, as its molecular weight was 14 Da higher than M2. The MS/MS spectrum of M3 showed structurally significant product ions at m/z 307, 221, 169, 142, 55. The similarity in the product ions of M2 and M3 also suggests that M3 is a substructure of M2.

![Fig. 2.11. Product ion spectra and proposed structure of metabolite M3](image)

The [M+H]^+ ion at m/z 406 was 16 Da higher than that of the parent compound, suggesting that M4 was a mono hydroxylated metabolite. The MS/MS spectrum of m/z 406 yielded a series of product ions at m/z 390, 307, 221, 194, 169, 100, 55. The ion at m/z 307 was formed due to the loss of cyclohexyl ring. The ions at 221, 195 and 169 were generated due to the sequential losses of cyclohexylpiperazinyl, ethyl and butyl cyclohexylpiperazinyl moieties, respectively. Product ions at m/z 307, 221, 169 common to M2, M3 and M5 suggests that the hydroxyl group of M4 is not located on the 3-(4-(pipeazin-1-yl)butyl)benzo[d]thiazole-2(3H)-thione group (the unchanged substructure) of CM156. In addition, the specific product ions m/z 307 and 100 confirmed that hydroxyl group is present on the cyclohexyl ring, however the exact location of the on the ring could not be identified.
The metabolite M5 was 176 Da higher than that of M4. The MS/MS spectrum of M5 showed a series of fragment ion at m/z 406, 307, 221, 169, 142, 113, 55. These product ions were similar to M4, confirming that metabolite M5 was glucuronide conjugate of M4.

Fig. 2.12. Product ion spectra and proposed structure of metabolite M4

Fig. 2.13. Product ion spectra and proposed structure of metabolite M5
Metabolites were identified by LC/MS/MS operated in various scan modes and confirmed (oxidative metabolite) by comparison of their retention times on HPLC with synthetic standards. FMO’s and CYP3A4 are assumed to be involved in the metabolism of CM156. The representative chromatograms are shown in Fig. 2.14.
Fig. 2.14. Chromatograms of (A) CM156, (B) Metabolite M1, (C) Metabolite M2, (D) Metabolite M3, (E) Metabolite M4, (F) Metabolite M5

A total of 5 metabolites: 4 phase I metabolites and 1 phase II metabolites (M1, M2, M3, M4, M5) were identified in rat urine. This shows that the clearance of CM156 in rats involved both phase I and phase II pathways. The major routes of metabolism involved oxidation at sulfur (M1), loss of cyclohexyl ring attached to the piperazinyl nitrogen (M2, M3), hydroxylation and subsequent conjugation with glucuronic acid (M4, M5). The formation of M1 and also absence of aromatic hydroxylated metabolite suggests that benzothiazolethione moiety is more vulnerable to oxidation rather aromatic hydroxylation.
Based on the structures of metabolites, a plausible scheme for the biotransformation pathway of CM156 in rats is shown in Fig. 2.15.
2.4. Conclusions

A bioanalytical method was developed and validated for the determination of CM156 in rat plasma. The experimental pKa and Log D values indicates that CM156 posses desirable physicochemical properties that are required for the BBB penetration. Based on microsomal metabolic stability studies, CM156 was found to be less stable in \textit{in vitro} in rat and mouse liver microsomes compared to human liver microsomes. Intravenous pharmacokinetics in rats demonstrated its high distribution in to tissues. This could be a promising property for a compound acting on CNS. This is also further confirmed by its high b/p ratio. The amount excreted in urine was found to be less than 0.1 \%. The possible \textit{in vivo} biotransformation pathway was identified using Tandem mass spectrometer. Four phase I and one phase II metabolite of CM156 were identified using UPLC/MS/MS. The involvement of FMO was indicated through enzyme inhibition by methimazole, heat inactivation, and protection against heat inactivation by NADPH.
CHAPTER-3

DRUG METABOLISM AND PHARMACOKINETICS OF 3-(4-(4-
CYCLOHEXYLPiperazine-1-yl)PENTYL)-6-FLUOROBENZO[D]THIAZOLE-
2(3H)-ONE, A NOVEL SIGMA RECEPTOR LIGAND WITH IMPROVED
METABOLIC STABILITY

3.1. Introduction

Methamphetamine (MA) is a psychomotor stimulant, neurotoxic addictive and the second
most illicit drug abused worldwide [120-122]. It can be synthesized readily from over the
counter drugs. Current surveys estimate that around 15-16 million people have abused
methamphetamine [123]. The short-term stimulant effects of MA include euphoria,
hyperthermia, enhanced energy, increased physical activity and decrease appetite [123, 124].
Repeated MA use results in addiction, psychosis, changes in brain structure and function
involving terminals of dopamine and serotonin neurons, memory loss, neurodegeneration [121,
125, 126]. Multiple mechanisms contribute to the meth-induced neurotoxicity include oxidative
stress due to formation of oxygen and nitrogen reactive species, abnormal dopamine and
 glutamate transmission, mitochondrial dysfunctions, apoptosis, astroglial and microglial
 activations, brain hyperthermia [121, 127-129]. However, the mechanisms underlying these
neurodegenerative effects are not yet thoroughly understood. Methamphetamine enhances the
synaptic levels of norepinephrine, dopamine (DA), and serotonin (5-HT) by reversing the
transport that facilitates excess release of these mono amines and also by preventing their
reuptake [125, 130]. Currently, there is no FDA approved pharmacotherapy to treat the harmful
effects of MA [123, 131]. Various effects of methamphetamine are supposed to be related to its interaction with monoamine transporters. Nevertheless, methamphetamine also has affinity for both $\sigma_1$ and $\sigma_2$ receptors and interacts with $\sigma$ receptors at physiologically attainable concentrations suggesting their role in methamphetamine’s effects [132-135]. Sigma receptors are unique proteins discovered by Martin et al. in 1976 [136-138]. Based on the specific pharmacological and functional characteristics, sigma receptors are divided into two subtypes, $\sigma_1$ and $\sigma_2$ [139-141]. The sigma-1 receptor is a 25-29 kDa protein with 223 amino acids and was cloned in 1996 [142-144]. It is localized in various organs such as liver, heart, gastrointestinal tract and limbic system of brain is particularly rich in these receptors [140, 144]. The sigma-2 receptor is an 18-21 kDa protein, which is not yet been cloned [141, 145, 146]. Sigma receptors are distributed in the organs that mediate the actions of METH such as dopaminergic system in the brain and the activation of these receptors results in the synthesis and release of dopamine [132, 147, 148]. Sigma receptors are distributed in the organs that mediate the actions of METH such as dopaminergic system in the brain and the activation of these receptors results in the synthesis and release of dopamine [132, 147, 148].

Currently, sigma receptor antagonists are being investigated as potential agents for METH abuse and toxicity; targeting these receptors can become a promising therapeutic approach in treating addiction to METH [134, 135]. In addition, the neurotoxic and stimulant effects of METH were attenuated by sigma receptor antagonist like AC927 and CM156 [135, 149]. Even though several sigma receptors antagonists have been synthesized, a primary concern with these compounds is that they are not solely sigma selective [150]. Most of these compounds also bind to dopamine transporters, opioid receptors, or NMDA receptors.
AZ66, 3-(4-(4-cyclohexylpiperazine-1-yl)pentyl)-6-fluorobenzo[d]thiazole-2(3H)-one, is a synthetic piperazine derivative identified as a promising lead, based on its toxicological and pharmacological data. Radioligand binding studies have shown that AZ66 has high nanomolar affinity for both subtypes of sigma receptors. In addition, AZ66 exhibited an appreciably longer half-life in vitro compared to CM156. AZ66 significantly attenuated the convulsions in mice treated with toxic dose of cocaine, demonstrating its anticocaine activity [134].

3.2. Experimental

3.2.1. Chemicals and reagents

AZ66 (>99% purity, as determined using HPLC) provided by the Department of Medicinal Chemistry, The University of Mississippi (Oxford, MS, USA). The internal standard (IS), aripiprazole (99% purity), was purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetic acid was purchased from Mallinckrodt Baker Inc. (Phillipsburg, NJ, USA). Methanol, acetonitrile and water were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Ammonium acetate was purchased from Sigma-Aldrich (St. Louis, MO, USA). Rat plasma was obtained from Innovative Research (Peary Court Novi, MI, USA). All the solvents used were HPLC grade.

3.2.2. Preparation of calibration standards and quality control (QC) samples

The stock solution of AZ66 (1.0 mg/mL) was prepared by dissolving accurately weighed (Mettler Toledo, USA) amount (0.72 mg) of the compound in water (720 µL). A series (8) of working standard solutions at concentrations ranging from 50 to 35,000 ng/mL were prepared by transferring a measured quantity of stock solution in to a 1.5 mL eppendorf tube and diluting it
with required amount of methanol. The stock solution was diluted with methanol to achieve good sensitivity and response in mass spectrometry, as organic solvents give better sensitivity than water. The IS stock solution (1.0 mg/mL) was prepared by dissolving 0.54 mg of compound in 540 µL of methanol transferred using an adjustable 1 mL eppendorf pipette. The working standard solution (3 µg/mL) of IS was obtained by diluting 3 µL of the stock solution with 997 µL of methanol. All stock solutions were stored at -20 °C and brought to room temperature before use. Calibration standards were freshly prepared by diluting the blank rat plasma (100 µL) with 10 µL of working standard solutions of concentrations ranging from 10 to 35,000 ng/mL to obtain final concentrations of 1, 10, 50, 100, 500, 1000, 2000 and 3500 ng/mL. Quality control (QC) samples were prepared at three concentrations of 2, 400 and 3000 ng/mL. Blank rat plasma (100 µL) was transferred to an eppendorf tube (1.5 mL) and to this appropriate amount of working standard solutions of concentrations 20, 4000 and 30,000 ng/mL were transferred get final concentrations of 2, 400 and 3000 ng/mL.

3.2.3. Sample Preparation

A liquid liquid extraction method was applied for the extraction of AZ66 from rat plasma. Rat plasma samples (100 µL) were spiked with 10 µL measured using a pipette of the IS (3 µg/mL) and vortexed for 30 s. The samples were extracted with 800 µL of chloroform by vortex-mixing for 10 min and the resultant mixture was centrifuged (Accuspin 17R, Fisher Scientific, USA) at 10,000 x g for 10 min at 4 °C. The organic phase was then transferred using a 1 mL pipette in to a 1.5 mL eppendorf tube and dried in a vacuum oven at 25 °C. The residue was dissolved in 100 µL of methanol and vortex-mixed for 1 min and a 10 µL aliquot of sample was injected into the UPLC/MS/MS for analysis using an autosampler (Waters Corp., Milford, MA, USA).
3.2.4. Liquid chromatographic and mass spectrometric conditions

The Acquity UPLC (Waters Corp., Milford, MA, USA) system consisted of a binary solvent manager, vacuum degasser, temperature controlled column compartment and an auto-sampler. Chromatographic separation was achieved on an ACQUITY BEH RP C18 column (2.1 X 150 mm) at 25 °C. The mobile phase consisted of 10 mM ammonium acetate containing 0.1% acetic acid and methanol (30:70, v/v) and pumped at a flow rate of 0.3 mL/min. The sample injection volume was 10 µL. The total run time was 3.3 min. A Micromass Quattro micro™ system equipped with electrospray ionization source (ESI) was used for the mass spectrometric detection. The ESI was operated in the positive ionization mode. The acquisitions were performed using multiple reaction monitoring (MRM). The mass transitions chosen for quantitation were m/z 405 → m/z 181 for AZ66 and m/z 448 → m/z 285 for the IS. Fig.3.1 shows the structures of AZ66 and IS. The optimized mass spectrophotometer parameters were as follows: capillary voltage of 4.88 kV; cone voltage of 44 V; extractor voltage of 3 V; RF lens voltage of 0.5 V; source temperature of 100 °C and desolvation temperature of 250 °C. The desolvation and cone gas flows were set at 500 and 60 L/h, respectively. Argon was used as collision gas at $3.5 \times 10^{-3}$ Pirani. Collision energies were set at 30 and 21 eV for AZ66 and the IS, respectively.

![AZ66](image1.png)  
![Aripiprazole](image2.png)
**Fig.3.1.** Structures of AZ66 and Aripiprazole (IS)

### 3.2.5. Method validation

Method validation was performed according to the United States Food and Drug Administration Bioanalytical Method Validation Guidance. The assay was validated for linearity, lower limit of quantification, limit of detection, precision, accuracy, selectivity, recovery, matrix effect and stability [151].

#### 3.2.5.1. Selectivity and sensitivity

The selectivity of the assay was determined by comparing the chromatograms of blank rat plasma from six different batches with corresponding plasma samples spiked with AZ66 and the IS. Lower limit of quantification was defined as the lowest concentration that resulted in a signal to noise (S/N) ratio of 10. The acceptable criteria for LLOQ were relative standard deviation and relative error less than ±20%. The LLOQ was evaluated by analyzing samples in six replicates on three consecutive days. The limit of detection was defined as the concentration of analyte that yielded S/N ratio of 3.

#### 3.2.5.2. Linearity

Calibration standards were prepared by spiking blank rat plasma with standard working solutions of AZ66 to obtain concentrations of 1, 10, 50, 100, 500, 1000, 2000 and 3500 ng/mL. An aliquot of 10 µL of IS was also added to get a concentration of 3 µg/mL. The calibration curve was constructed by plotting the peak area ratios of analyte to the IS versus analyte concentrations. The linearity of the calibration curve was evaluated by linear regression analysis.
3.2.5.3. Recovery and matrix effect

The extraction recovery of AZ66 from rat plasma was determined at three QC concentrations (2, 400 and 3000 ng/mL). The extraction recovery was calculated by comparing the peak area ratios of blank plasma samples spiked with analyte and IS before extracting the plasma, with peak area ratios of blank plasma samples to which analyte and IS were added after the extracting the blank plasma. The matrix effect was determined at three QC levels (2, 400 and 3000 ng/mL) by comparing the analyte/IS peak area ratios of extracted plasma samples with those of corresponding AZ66 standard solutions prepared in methanol.

3.2.5.4. Precision and accuracy

The precision and accuracy of the assay were assessed at three concentrations (2, 400 and 3000 ng/mL). To determine intra-day precision and accuracy, QC samples were analyzed in six replicates at each concentration level. The inter-day precision and accuracy were determined by analyzing QC samples on three consecutive days. The precision was expressed as relative standard deviation and the accuracy as relative error. The intra-and inter-day precision and accuracy should not be more than 15% of R.S.D and 15% of RE, respectively.

3.2.5.5. Stability

To determine the stability of AZ66 in rat plasma, QC samples (six replicates) at concentrations of 10, 400 and 3000 ng/mL were exposed to various storage conditions including short-term, long-term, freeze thaw and autosampler stability studies that may be encountered during sample handling and analysis. Freeze-thaw stability was evaluated for three freeze/thaw cycles at -20 °C. Short-term temperature stability was assessed at room temperature (37 °C) for
For long-term stability, the samples were stored at -20 °C (Kenmore) for 30 days. Post-operative stability was also evaluated by analyzing the processed samples stored in the autosampler at 25 °C for 24 h. The stability of the stock solutions of analyte and IS was tested daily for a period of 1 week. After the analysis the concentrations of the stored samples for all the stability studies were compared with the freshly prepared standards.

3.2.6. Physicochemical characterization

The pKa of AZ66 was determined by potentiometric titrations [152] and ACD/Labs (Algorithm Version: v12.1.0.3346, Toronto, ON, Canada). For the potentiometric titration method, a 1 mM solution of AZ66 was prepared by dissolving 10.15 mg of compound in 25 mL of water. To this solution, 25 µL of 0.01 M sodium hydroxide solution was added using a 200 µL adjustable pipette (Fisher Scientific, NJ, USA) and the change in pH was recorded using a Mettler Toledo S20 SevenEasy pH Meter (Fisher Scientific, NJ, USA). The procedure was repeated until a constant pH was obtained. Thereafter, 25 µL of 0.01 M hydrochloric acid was added continuously and the change in pH was recorded until a constant value is reached. The titration curves were then constructed by plotting the recorded pH versus volume of base/acid added. The intersection point of the curves was noted as the pKa of the compound.

As lipophilicity of the compound influences its transport across BBB, the octanol/water and octanol/PBS pH 7.4 partition coefficients (Log P and Log D) of AZ66 were calculated. The octanol/water (pH 6.4) and octanol/PBS (pH 7.4) partition coefficients (Log P and Log D$_{7.4}$) of AZ66 were determined using the stir flask method [153]. For the determination of Log P and Log D, both phases (octanol, water/PBS) were saturated with one another for a period of 12 h before beginning the experiment. A sample of AZ66 (0.54 mg) was accurately weighed and
transferred to a clean glass vial (4 mL) and then to this 1 mL of the saturated aqueous phase (with octanol) was added using a 1 mL Eppendorf pipette. To the above solution, 1 mL of octanol (saturated with water) was added and stirred for 24 h on a VWR Dylastir magnetic stirrer (Bridgeport, NJ, USA) using a magnetic bead. A 100 µL aliquot of the aqueous phase was collected using a pipette, transferred in to a clean 1 mL plastic insert (Microsolv Technology Corp., Eatontown, NJ, USA) that was already placed in a clean HPLC vial (Waters, MA, USA) and analyzed by UPLC/MS/MS. Calibration curves prepared in phosphate buffer and water were used for the determination of the compound concentration in aqueous phase. For the preparation of calibration curve, 0.63 mg of compound was dissolved in 630 µL of acetonitrile to get a stock solution of 1 mg/mL. From this solution, calibration standards were prepared by diluting with buffer.

The solubility of AZ66 was determined in distilled water (pH 6.4) and in phosphate buffer saline (pH 7.4) using the standard shake flask method. An excess amount (22 mg) of AZ66 was added to 100 µL of the solvent in 2 mL glass vials. The vials were constantly agitated (100 rpm) at room temperature (25 °C) for 24 h in reciprocating shaking water bath (Precision Scientific, VA, USA) for uniform mixing. After 24 h, the samples were collected using a pipette and centrifuged at 10,000 x g for 5 min. The supernatant was then analyzed using UPLC/MS/MS.

3.2.7. In vitro metabolism

In vitro metabolism studies were performed on a series of seven sigma receptor ligands (CM145, CM146, CM401, AZ68, AZ57, AZ66 and AZ77) using commercially available rat liver microsomes. All the analogs were incubated with rat liver microsomes in Tris buffer (pH
7.4). The standard incubation mixture contained 0.1 M MgCl₂, 1 mM NADPH regenerating system (NADP 1 mM, glucose-6-phosphate 5 mM, and glucose-6-phosphate dehydrogenase 1 Unit/mL), 1 mg of microsomal protein and 5 µM substrate. The final volume was adjusted to 1 mL with the appropriate amount of buffer. Incubations were performed at 37 °C and 100 rpm in shaking water bath (Precision Scientific, VA, USA) for 30 min. Metabolic stability was assessed quantitatively by measuring the disappearance of the substrate. After terminating the reaction with ice-cold methanol at pre determined time points (0, 5, 10, 15, 30, 45, 60 and 90 min), the mixture was vortexed and centrifuged. The supernatant was then analyzed by UPLC/MS/MS.

3.2.8. Determination of the human CYP enzymes responsible for AZ66 metabolism

For all most all the drugs, metabolism is the major route of elimination, and human liver cytochrome P450 (CYP) enzymes play a key role in the oxidative biotransformation of many drugs and xenobiotics. Any alterations of the activity of these enzymes lead to drug-drug interactions. It is important to assess the contribution of metabolism by CYP to the overall elimination process and to identify the specific P450 isoforms responsible for oxidative reactions. Three families of P450 enzymes (CYP1, CYP2, and CYP3) are involved in the metabolism of xenobiotics in humans, and CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, and CYP3A4/5 are responsible for the metabolism of the majority of drugs [154]. Approaches used currently for the in vitro P450 reaction phenotyping typically involves, antibody inhibition using potent and inhibitory antibodies, chemical inhibition by specific chemical inhibitors, metabolism of drugs by recombinant P450 enzymes, and correlation analysis.
In our study the major CYP enzymes responsible for the formation of metabolites of AZ66 were identified using selective chemical inhibitors for CYP isoforms. Compound AZ66 was incubated in Human Liver Microsomes (HLM) in the presence/absence of selective chemical inhibitors of CYP1A1/CYP1A2 (5 µM α naphthoflavone) CYP2C8 (12 µM quercetine), CYP2C9 (3 µM fluvoxamine) CYP2D6 (5 µM quinidine) and CYP3A4/A5 (3 µM ketoconazole) [155-157]. Liver microsomes were preincubated with inhibitors for 15 min at room temperature, followed by the addition of Tris buffer (pH 7.4), NADPH regenerating system and substrate (10 µM). The final incubation volumes were 0.5 mL. All inhibitors were dissolved in methanol and the concentration of the organic solvent in the incubation system was less than 1% (v/v). The nonspecific P450 inhibitor 1-benzylimidazole (BI) (1 mM) was used to assess the P450-dependent metabolism of AZ66 [158, 159].

In addition to CYP mediated metabolism, several other enzymes, such as the flavin-containing monooxygenases (FMO), mediate the oxidative metabolism of xenobiotics and endogenous substances. Mammalian flavin-containing monooxygenase is a hepatic microsomal enzyme system that usually oxygenates drugs containing nitrogen or sulfur. The human FMO functional gene family has 5 families each with a single member. Three of the human FMO genes, FMO1, FMO2 and FMO3, are the most extensively studied among the five as they are the primary isoforms that catalyze xenobiotic metabolism and these isoforms exhibit genetic polymorphisms. FMO1 has the wide-range substrate acceptance, it can metabolize large lipophilic compounds such as imipramine or orphenadrine, whereas, FMO3 selectively metabolizes small amine-containing compounds such as trimethyl-amine [160]. To investigate the potential role of FMO’s in the metabolism of AZ66, thermal inactivation that selectively diminish FMO activity (FMO’s are thermally labile) [161] but not the P450 activity was done by
preheating HLM at 45 °C for 5 min (without NADPH). HLM pre-incubated at 37°C for 5 min was used as controls. These preheated or controls HLM were then incubated with the test compound and NADPH, to determine the contribution of FMO. Effect of FMO activity on the attenuation of AZ66 metabolism was further confirmed by the incubation of compound in HLM with methimazole (100 µM), a selective FMO inhibitor [162]. All incubations were performed as described previously.

3.2.9. Plasma protein binding

Unbound drug concentrations correlate better with the drug’s pharmacological response and toxicity than the total drug concentration. Also, plasma protein binding can be a large factor in the distribution of drugs in the body. Therefore, binding of drugs to plasma proteins is an important factor in determining the pharmacokinetics and pharmacological effects. There are numerous methods for the determination of in vitro protein binding, such as equilibrium dialysis, ultrafiltration and ultracentrifugation [86]. Plasma protein binding of AZ66 was determined by ultrafiltration method using Centrifree® devices [163]. The unbound concentration of AZ66 was determined by spiking blank rat plasma with different concentrations of the test compound. Four different concentrations were chosen including one below the Cmax, one at the Cmax, two above the Cmax [164]. The plasma was then transferred to Centrifree® ultrafiltration device, incubated for 15 min and then centrifuged at 1000 g for 30 min. To the resultant ultra filtrate, 10 µL of internal standard (aripiprazole) was added and analyzed using UPLC/MS/MS. Ultrafiltration method is not suitable for protein binding studies if the compound binds to the equipment to a greater extent. The non specific binding should be less than 0.5 %. A control experiment was done to estimate the nonspecific binding of the substrate to the equipment, which was incorporated in the calculation of plasma protein binding [165].
3.2.10. Pharmacokinetics studies

All the experimental procedures were approved and performed in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) of the University of Mississippi. Male Sprague Dawley rats (180-200 g) were obtained from Harlan Company (Indianapolis, IN). The rats were purchased with pre-existing polyethylene cannula inserted into their right jugular vein. The rats were quarantined for 72 h and fasted just 12 h before the experiment. They were maintained in 12 h light and dark cycle for 72 h. They had access to water and food during quarantine. During the experiment the rats were housed in metabolic cages (Techniplast, USA) and allowed free movement and access to water.

The i.v formulation was prepared by dissolving 20.0 mg (accurately weighed) of AZ66 in 4 mL of saline. This solution was administered (0.5 mL) to rats (n=6) through the right jugular vein cannula using a syringe at a dose of 5 mg/kg. For oral administration, AZ66 was accurately weighed (40.2 mg) and dissolved in normal saline (4 mL). The solution was administered (1 mL) to rats at a dose of 20 mg/kg using oral gavage. The formulations were prepared on the day of dosing and filtered through 0.2 µ filter (Fisher Scientific, Pittsburgh, PA, USA) before administration. Blood samples were collected from each rat’s indwelling cannula at intervals of 0, 2, 15, 30, 60, 120, 240, 360, 480 min, 12, 24, 30, 36 and 42 h after i.v injection, whereas for the oral study the sampling was done at 0, 0.25, 0.5, 1, 1.5, 2, 3, 4, 8, 12, 18, 24, 30 36, 42 h after dosing. To clear the heparinised saline from the cannula, a blood volume of 0.05 mL was withdrawn and a clean syringe was then used to withdraw a 0.15 mL sample of blood. After each blood sampling, the cannula was flushed with 0.2 mL of a heparin-saline (10 I.U./mL) solution. Blood samples were transferred into heparinized micro centrifuge tubes and centrifuged immediately at 10,000 × g for 10 min at 4 °C using an Accuspin Micro 17R centrifuge (Fisher
Scientific, Pittsburgh, PA, USA). The plasma was separated from the blood cells, transferred to clean eppendorf tube and was frozen at -20 °C (Kenmore) until analysis. The pharmacokinetic parameters were calculated by non-compartmental analysis using WinNonlin 5.2 (Pharsight, Mountain View, CA, USA).

**Analysis of pharmacokinetic parameters**

The pharmacokinetic parameters were calculated from the corresponding plasma concentration-time curves using noncompartmental analysis (WinNonlin 4.0, Pharsight, Mountain View, CA). The area under the plasma concentration–time curve from time 0 to infinity (AUC_{int}) was calculated by the trapezoidal rule with extrapolation to time infinity. The terminal $t_{1/2}$ was calculated as $0.693/\lambda_z$, where $\lambda_z$ is the terminal phase rate constant. The plasma clearance (CL) was calculated using the equation,

$$\text{CL} = \frac{\text{Dose i.v}}{\text{AUC i.v}}$$

$$\text{CL/F} = \frac{\text{Dose oral}}{\text{AUC oral}}$$

where Dose_{i.v} and AUC_{i.v} are the i.v dose and corresponding area under the plasma concentration-time curve from time 0 to infinity, and Dose oral and AUC oral are oral dose and area under the plasma concentration-time curve from time 0 to infinity, respectively. Volume of distribution based on terminal phase ($V_z$) was calculated using

$$V_z = \frac{\text{Dose i.v}}{(\lambda_z \times \text{AUC i.v})}$$

$$V_z/F = \frac{\text{Dose oral}}{(\lambda_z \times \text{AUC oral})}$$

Absolute bioavailability (F) was calculated using the following equation

$$F = \frac{\text{AUC oral} \times \text{Dose i.v}}{\text{AUC i.v} \times \text{Dose oral}}$$
AUC i.v and AUC oral are the areas under the plasma concentration-time curves of AZ66 after i.v. and oral administration, respectively.

3.2.11. Urine sampling

Urine was collected in to BD vacutainer® containers at specified time intervals after both oral (0-4, 4-8, 8-12 and 12-24 h) and i.v (0-2, 2-4, 4-8 h) administration of the compound. Blank urine (urine before administering the test compound) that was collected from the graduated urine collection tube of the metabolic cage was also transferred to a BD vacutainer®. The volume of urine collected in graduated containers was noted during each time interval and kept frozen (Kenmore) at -20 °C until analyzed. Urine samples were analyzed by UPLC/MS/MS for the quantification of AZ66 in urine.

3.2.12. Multiple dose pharmacokinetics of AZ66 after oral administration

Rats were treated with AZ66 at dose of 30 mg/kg provided in an oral solution containing 0.5 mg of AZ66 per 0.5 mL of water (pH 6.4). The compound was administered at every half-life (4 h) for 5 half-lives in order to attain steady state. Venous blood samples (0.15 mL) were collected using a 1 mL syringe via the jugular vein cannula just before and 0.5, 1, 2 h after the first, second, third and fourth doses. After the fifth dose (last), the samples were collected at 0.5, 1 and 2 h. The blood was transferred in to a heparinized micro centrifuge tube and centrifuged at 4 °C using an Accuspin Micro 17R centrifuge. The plasma (100 µL) was collected using a pipette and transferred in to a clean eppendorf tube and stored at -20 °C until analyzed.

3.2.12.1. Tissue distribution and brain to plasma ratio studies
At the end of the repeated dose study the animals were anesthetized, the body was cut open and transcardially perfused with buffer until all the blood was removed from the organs. After the perfusion, the tissues including brain, lung, liver, kidney and heart were excised and washed with buffer. Tissues were weighed and quickly frozen at -20 °C. All tissues were homogenized in 1 mL of water and the homogenates were processed and analyzed by the developed and validated UPLC/MS/MS method described in section 3.2.3.

For the quantification of AZ66, all the tissues (lung, liver, kidney and heart) were thawed to room temperature and homogenized using a tissue homogenizer, (Tekmark, OH, USA). To an aliquot of 100 µL of the homogenate, 10 µL of IS was added and vortexed for 30 s. The mixture was extracted with chloroform (800 µL) by vortexing for 5 min and then centrifuged at 10,000 x g for 7 min. A fixed aliquot (725 µL) of the organic layer was collected after centrifugation and subjected to evaporation in a vacuum oven (Precision Scientific, Winchester, VA, USA). The residue was then reconstituted in 100 µL of water and an aliquot of 5 µL was injected using an autosampler (Waters Corp., Milford, MA, USA) into the UPLCMS/MS for the quantification of AZ66.

For the determination of brain to plasma ratio of AZ66, brains were thawed at room temperature (25 °C) and transferred to a 20 mL glass vial. To this glass vial 3 mL of water was added using a pipette and the brains were then homogenized using a tissue homogenizer, (Tekmark, OH, USA). The homogenate was then transferred using a pipette in to a fresh eppendorf tube (1.5 mL) and centrifuged at 10,000 x g for 10 min to remove the air that was entrapped during homogenization. An aliquot (100 µL) of the homogenate was transferred using a pipette into a clean eppendorf tube. To this homogenate 10 µL of IS (3 µg/mL) was added using a pipette and vortexed for 30 s. The mixture was then extracted with chloroform (800 µL)
by vortexing for 10 min and centrifuged using an Accuspin Micro 17R centrifuge at 10,000 x g for 7 min. A fixed aliquot (725 µL) of the lower organic layer was collected after centrifugation and subjected to evaporation in a vacuum oven (Precision Scientific, Winchester, VA, USA). The residue was reconstituted in 100 µL of water and aliquot of 5 µL was injected into the UPLC/MS/MS for the quantification of AZ66 in brain tissues. The plasma samples were processed as described in the section 3.2.3 and analyzed using the developed and validated UPLC/MS/MS for the quantification of AZ66 in plasma.

3.3. Results and Discussion

3.3.1. Method development

AZ66 being a basic compound exhibited a higher mass spectrometric response in ESI positive mode compared to negative mode and [M+H]+ was found to be the most intense signal with m/z at 406 [151]. Several fragment ions were observed in the product ion spectra, but the most abundant product ions were at m/z 181 and 285 for AZ66 and the IS, respectively. The mass spectrometric parameters such as collision energy, source temperature, capillary voltage, gas flow were optimized to enhance the signal intensity. Fig.3.2. shows the product ion scans of [M+H]+ of AZ66 and IS.

The chromatographic conditions were optimized through several trials to achieve good chromatographic behavior and better ionization of AZ66 and IS. At first columns such as C18 and Atlantis dC18 were tried. These two columns resulted in good peak separation but the peaks were broader with 0.4-0.5 min width. Tailing was also observed for the analyte and IS. To reduce the tailing and get narrow peaks we tried columns like HILIC and ACQUITY UPLC BEH Shield RP 18 and BEH Shield RP 18 column resulted in the best performance with a peak width of 0.2-
0.25 min for both analyte and IS. Buffers, such as ammonium acetate, formic acid and acetic acid were used alone or in combination at various concentrations for better peak shapes and higher ionization. As the compound is basic in nature, acidic modifiers (such as acetic acid) ionize the compound very well and result in much better sensitivity than the basic modifiers such as ammonium hydroxide so we selected acidic modifiers for the assay [151]. Acetic acid resulted in good sensitivity however the peak was not sharp. To improve the peak shape we added ammonium acetate. Ultimately, a 0.1% acetic acid solution with 10 mM ammonium acetate in water and methanol (30:70, v/v) was chosen as the mobile phase. This mobile phase resulted in good peak shapes and high sensitivity (LOD 0.5 ng/mL). The retention times of AZ66 and the IS were 1.3 and 1.0 min, respectively, with a total run time of 3.3 min.

Fig. 3.2. Protonated molecular ion mass spectra of AZ66 (A), aripiprazole (B)
3.3.2. Method validation

3.3.2.1. Selectivity and sensitivity

Fig. 3.3. represents typical chromatograms obtained from the analysis of blank plasma, blank plasma spiked with AZ66 and the IS, and rat plasma sample obtained 1 h after the oral administration of AZ66. No obvious interferences were observed at the retention times of AZ66 and the IS. The LLOQ for AZ66 in plasma was 1 ng/mL with precision (R.S.D.) below 20% (1.7 %) and accuracy (RE) within ±20% (2.5 %). A corresponding chromatogram is shown in Fig. 2b. The limit of detection (LOD) was defined at a signal-to-noise (S/N) ratio of 3:1 [151] and it was found to be 0.5 ng/mL. This method was found to be sensitive enough to investigate the pharmacokinetic behavior of AZ66 in rats.

![Chromatogram](image.png)
**Fig. 3.3.** Representative MRM chromatograms for AZ66 and Aripiprazole (IS) in rat plasma: (A) Total ion chromatogram of AZ66 and IS; (B) blank plasma sample; (C) blank plasma sample spiked with AZ66 at 400 ng/mL and aripiprazole (IS) at 3 µg/mL; (D) rat plasma sample obtained at 1.0 h after oral administration at a dose of 20 mg/kg.

### 3.3.2.2. Linearity of calibration curve

Fig. 3.4 shows the calibration curve of AZ66 in rat plasma. The calibration curves for AZ66 were linear over the concentration range of 1 to 3500 ng/mL ($r^2 > 0.99$). The representative regression equation for the calibration curve was:

$$y = 0.0511x + 0.1682$$

Where $y$ represents the peak area ratios of AZ66 to the IS and $x$ represents plasma concentrations of analyte. The R.S.D. of the slope was 0.00018 and the R.S.D. of the intercept was 0.0031.

**Fig. 3.4.** AZ66 calibration curve in rat plasma

### 3.3.2.3. Precision and accuracy of the assay
The results of the intra- and inter-day precision and accuracy are presented in Table 3.1. The intra-day precision for the three QC levels of AZ66 was 2.0%, 0.1% and 0.1% and the inter-day precision were 3.0%, 0.4% and 0.1%. The intra- and inter-day accuracy of the method ranged from 0.3% to 6.3%. The data indicates that the precision and accuracy were within the acceptable limits specified by US-FDA where the precision (R.S.D.) determined should not exceed 15% and accuracy (RE) should be within±15% of the actual value. These results suggest that the present method is consistent and reproducible for the quantitation of AZ66 in rat plasma.

<table>
<thead>
<tr>
<th>Spiked concentration (ng/mL)</th>
<th>Intra-day precision and accuracy (n=6)</th>
<th>Inter-day precision and accuracy (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Measured concentration</td>
<td>R.S.D. (%)</td>
</tr>
<tr>
<td></td>
<td>(mean ±SD, ng/mL)</td>
<td>R.E (%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>2.0 ± 0.02</td>
<td>2.0</td>
</tr>
<tr>
<td>400</td>
<td>401.4 ± 0.5</td>
<td>0.1</td>
</tr>
<tr>
<td>3000</td>
<td>3008.1 ± 3.7</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Measured concentration</td>
<td>R.S.D. (%)</td>
</tr>
<tr>
<td></td>
<td>(mean ±SD, ng/mL)</td>
<td>R.E (%)</td>
</tr>
<tr>
<td>2</td>
<td>2.1 ± 0.03</td>
<td>3.0</td>
</tr>
<tr>
<td>400</td>
<td>402.8 ± 1.6</td>
<td>0.4</td>
</tr>
<tr>
<td>3000</td>
<td>3009.7 ± 4.3</td>
<td>0.3</td>
</tr>
</tbody>
</table>

Table 3.1. Summary of the accuracy and precision of AZ66 in rat plasma

3.3.2.4. Recovery and matrix effect

According to US-FDA guidelines the recovery of analyte and IS needs to be invariable and reproducible and need not be 100%. The extraction recoveries of AZ66 were 97.8 ± 3.3%, 101.12 ± 1.5% and 99.4 ± 4.7% for QC samples at the concentrations of 2, 400 and 3000 ng/mL, respectively. These results suggested that the recovery of AZ66 was precise and reproducible at all the three concentrations. The extraction recovery of the IS was consistent and found to be 69.4 ± 3.5%.

The matrix effect of AZ66 was evaluated by analyzing QC samples at three concentration levels of 2, 400 and 3000 ng/mL. The mean matrix effect values ranged from 96.5 to 101.4%.
The matrix effect of the IS was 95.4%. This indicated that the ion suppression from plasma matrix for AZ66 and IS was negligible.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>%Recovery of AZ66 in QC’s</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetonitrile</td>
<td>45</td>
</tr>
<tr>
<td>Methanol</td>
<td>68</td>
</tr>
<tr>
<td>Chloroform</td>
<td>98</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>63</td>
</tr>
<tr>
<td>Methyl tert-butyl ether</td>
<td>78</td>
</tr>
</tbody>
</table>

**Table 3.2.** Recovery of AZ66 in various solvents

<table>
<thead>
<tr>
<th>Conc (ng/mL)</th>
<th>Recovery (%) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>97.8 ± 3.3</td>
</tr>
<tr>
<td>400</td>
<td>101.1 ± 1.5</td>
</tr>
<tr>
<td>3000</td>
<td>99.4 ± 4.7</td>
</tr>
</tbody>
</table>

**Table 3.3.** Recovery of AZ66 from spiked rat plasma

3.3.2.5. Stability

To investigate the stability of AZ66 in rat plasma, QC samples at three concentrations of 10, 400 and 3000 ng/mL, were stored under various conditions which may be encountered during routine sample handling, processing and analysis. Table 3.4 summarizes the results of the freeze–thaw, short-term, long-term, and postoperative stabilities of AZ66 in rat plasma. The stability of AZ66 was evaluated under different conditions at three concentrations (10, 400 and 3000 ng/mL).
### Table 3.4. Results of the stability studies of AZ66 at different storage conditions

<table>
<thead>
<tr>
<th>Storage condition</th>
<th>Concentration (ng/mL)</th>
<th>R.S.D. (%)</th>
<th>RE (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Spiked</td>
<td>Measured</td>
<td></td>
</tr>
<tr>
<td>Three freeze thaw cycles</td>
<td>10</td>
<td>10.2 ± 0.7</td>
<td>6.9</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>396.1 ±12.7</td>
<td>3.2</td>
</tr>
<tr>
<td></td>
<td>3000</td>
<td>2990.6 ±42.9</td>
<td>1.4</td>
</tr>
<tr>
<td>Long term for 30 days (-20 °C)</td>
<td>10</td>
<td>10.2 ± 0.4</td>
<td>3.9</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>402.4 ± 7.1</td>
<td>1.8</td>
</tr>
<tr>
<td></td>
<td>3000</td>
<td>3028.2 ±55.0</td>
<td>1.8</td>
</tr>
<tr>
<td>Short term for 12h (25 °C)</td>
<td>10</td>
<td>10.2 ± 0.2</td>
<td>2.4</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>401.4 ± 3.5</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td>3000</td>
<td>3015.4 ±30.6</td>
<td>0.9</td>
</tr>
<tr>
<td>Auto sampler for 24h (25 °C)</td>
<td>10</td>
<td>10.1 ± 0.2</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>403.2 ±18.6</td>
<td>4.6</td>
</tr>
<tr>
<td></td>
<td>3000</td>
<td>3222.1 ±79.4</td>
<td>2.5</td>
</tr>
</tbody>
</table>

The results indicated that AZ66 was found to be stable after three freeze/thaw cycles, at room temperature for 12 h, at -20 °C for 30 days. The compound was also stable in reconstituted samples stored in the auto-sampler at 25 °C for 24 h. The stock solutions of AZ66 and the IS were stable for at least 12 h at room temperature and for one week at -20 °C. No loss of compound was observed at all the storage condition examined, suggesting that the plasma samples can be stored at -20 °C for a period of 30 days after the pharmacokinetic study.

### 3.3.3. Determination of pKa, lipophilicity and solubility

Physicochemical properties commonly affect the transport of drugs passively across the membranes [166]. Various physicochemical properties including pKa, lipophilicity and solubility were determined for AZ66 using different in vitro methods described earlier. The pKa was found to be 8.4 indicating that the compound is basic in nature. The estimated limits for pKa of CNS
drugs for greater penetration are between 4 and 10. The lipophilicity of a compound is one of the main components that affect BBB permeability. The Log P and Log D of the compound AZ66 were 0.59 and 2.4, respectively. The optimal Log D 7.4 for CNS drugs is around 2. The acid ionization constant and lipophilicity values indicate that AZ66 can cross the blood brain barrier to an appreciable extent [167]. The solubility of AZ66 in water and phosphate buffer saline (pH 7.4) was found to be 50 and 23 mg/mL, respectively.

3.3.4. In vitro metabolism

The half-lives of the compounds ranged from 15 min to 115 min. The in vitro half-lives and respective intrinsic clearance values for the series of sigma ligands along with their chemical structures are presented in table 3.5. The rank order of metabolic stability was found to be AZ66 > AZ57 > AZ68 > CM145 > AZ77 > CM156. The microsomal metabolism of two analogs CM156, AZ77 was rapid and extensive with > 90% loss of the parent compound in 30 min and the half-life \((t_{1/2})\) was less than 15 min. AZ68, AZ57, CM145 exhibited moderate stability with 30-40% parent compound remaining after 60 min. The results demonstrated that the metabolic stability greatly increased with the insertion of stable groups like methyl and fluorine in the weakest spots. Also it was observed that replacing thiazole-2(3H)-thione in CM156 with thiazole-2(3H)-one in AZ66 resulted in higher metabolic stability in \textit{in vitro}. The in vitro half-life of AZ66 was 115 min with only 20% of the parent compound disappearing after a 60 min incubation period.

The C-N linkage and the thiazole-2(3H)-thione moiety were identified as the most labile sites (i.e., soft spots) to metabolism from the metabolite identification of CM156 in urine and liver microsomes. The next generation of sigma compounds (AZ66, AZ57, AZ68, CM145, and
AZ77) were then designed with an aim to improve the metabolic stability of the compounds by blocking these soft spots. All the analog compounds possessed the replacement of the thiazole-2(3H)-thione with thiazole-2(3H)-one except for AZ77. In addition, these compounds were made to modify the link between the cyclohexylpiperazine and benzothiazole ring. They also had a fluorine group attached to C-6 on the aromatic ring. Fluorine, because of its inductive effect, would be expected to affect the electrophilic oxidation of the substituted carbon by oxidative enzymes like cytochrome P450. These (AZ66, AZ57 and AZ68) compounds showed enhanced metabolic stability than all the other compounds (CM156, CM145, AZ77). However, the insertion of a fluorine group without replacing the sulfur atom with oxygen as in compound AZ77, failed to increase the metabolic stability in rat liver microsomes. This might indicate that sulfur oxidation is the major metabolic pathway. This was also further supported by the greater metabolic stability of the other analogs (AZ57) with modifications in the butyl chain and thiazole-2(3H)-thione ring without the fluorine group.

A dramatic increase in microsomal metabolic stability was observed with AZ66, 3-(4-(4-cyclohexylpiperazin-1-yl)pentyl)-6-fluorobenz[d]thiazol-2(3H)-one. This compound showed a 30-fold longer stability than our 1st generation compound, CM156, in rat liver microsomes. AZ68 and AZ57 also exhibited a 20-fold increase in metabolic stability. Compounds that contained sulfur (CM156, AZ77) showed poor metabolic stability, which might be due to the high susceptibility of sulfur atom to metabolism. Thus, AZ66 was identified as the promising compound that demonstrated high metabolic stability and affinity to sigma receptors and was selected for pharmacokinetic studies.
<table>
<thead>
<tr>
<th>Substrate</th>
<th>Structure</th>
<th>Half-life (min)</th>
<th>Intrinsic clearance (ml/min/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CM156</td>
<td><img src="image1" alt="Structure CM156" /></td>
<td>4.6</td>
<td>270</td>
</tr>
<tr>
<td>CM145</td>
<td><img src="image2" alt="Structure CM145" /></td>
<td>31.0</td>
<td>40.2</td>
</tr>
<tr>
<td>AZ77</td>
<td><img src="image3" alt="Structure AZ77" /></td>
<td>14.8</td>
<td>84.3</td>
</tr>
<tr>
<td>AZ57</td>
<td><img src="image4" alt="Structure AZ57" /></td>
<td>68.0</td>
<td>18.3</td>
</tr>
<tr>
<td>AZ68</td>
<td><img src="image5" alt="Structure AZ68" /></td>
<td>47.1</td>
<td>26.5</td>
</tr>
<tr>
<td>AZ66</td>
<td><img src="image6" alt="Structure AZ66" /></td>
<td>115.6</td>
<td>10.8</td>
</tr>
</tbody>
</table>

Table 3.5. Structures, in vitro half-life and Intrinsic clearance values of sigma receptor ligands.
3.3.5. CYP reaction phenotyping

Metabolism of xenobiotics can be affected by many parameters, including genetic polymorphisms, high inter-individual variability, and gender differences. Because variable expressions of individual isozymes can affect the metabolic fate of a drug candidate, it is important to determine which P450 enzymes contribute to the metabolic process. Chemical inhibitors and thermal inactivation were used to estimate the relative contribution of P450 and FMO isoforms involved in AZ66 metabolism in human liver microsomes. The findings are described below.

AZ66 metabolism was markedly decreased in HLM in the presence of a combination of CYP-specific inhibitors and a non-specific CYP inhibitor, confirming that AZ66 is most likely metabolized by diverse CYP isoforms. The individual isozymes responsible for the biotransformation of AZ66 were determined to be CYP3A4 (an important enzyme in xenobiotic metabolism) with a small contribution from CYP2C8 and CYP2D6. The overall metabolism of AZ66 was significantly inhibited by 3 µM ketoconazole (CYP3A4; 30.1%). Some inhibition was observed with 5 µM quercetin (CYP2C8; 12.7%) and 3 µM Quinidine (CYP2D6; 4.5%). α-naphthoflavone has shown negligible effect at 5 µM but inhibited up to 30% at a concentration of 100 µM. Nonspecific P450 inhibitor BI, potently inhibited parent compound consumption to a greater extent (40%). This further confirmed the substantial contribution of P450-dependent enzymes to the metabolism of AZ66. A role of flavin-containing mono-oxygenases was anticipated based on their known ability to catalyze N-oxidation reactions. The potential contribution of FMO to the metabolism of AZ66 was investigated using chemical inhibitor Methimazole and the heat lability of FMO’s in human liver microsomes. It was found that the metabolism of AZ66 was not markedly (4%) inhibited by FMO’s. This suggested that FMO’s
does not contribute appreciably to metabolism of AZ66 in human liver microsomes. Table 3.6 represents the percent inhibition of metabolism of AZ66 in the presence of various inhibitors for CYP450 and FMO. Fig.3.5 shows the effect of chemical inhibitors on the metabolism of AZ66 (10 µM).

Overall, CYP3A4, CYP2C8 and CYP1A1 (100 µM) enzymes were considered as major enzymes catalyzing AZ66 metabolism with minor contributions from CYP2D6. From the phenotyping results, we may conclude that, even if co-administered drugs inhibit one of the identified drug-metabolizing enzymes, the pharmacokinetics of AZ66 is unlikely to be noticeably affected due to probable metabolic compensatory mechanisms produced by other CYP isoforms.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Inhibitor</th>
<th>% Inhibition</th>
<th>% Remaining</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Control</td>
<td>-----</td>
<td>51</td>
</tr>
<tr>
<td>CYP1A1</td>
<td>α-naphthofalvone</td>
<td>2</td>
<td>53</td>
</tr>
<tr>
<td>CYP2D6</td>
<td>Quinidine</td>
<td>5</td>
<td>56</td>
</tr>
<tr>
<td>CYP2C8</td>
<td>Quercetine</td>
<td>13</td>
<td>64</td>
</tr>
<tr>
<td>CYP2C9</td>
<td>Fluvoxamine</td>
<td>2</td>
<td>53</td>
</tr>
<tr>
<td>CYP3A4</td>
<td>Ketoconazole</td>
<td>30</td>
<td>80</td>
</tr>
<tr>
<td>CYP (Non specific)</td>
<td>1-benzylimidazole</td>
<td>37</td>
<td>88</td>
</tr>
<tr>
<td>FMO (Non specific)</td>
<td>Methimazole</td>
<td>4</td>
<td>60</td>
</tr>
<tr>
<td>FMO Inhibition</td>
<td>Heat inactivation</td>
<td>5</td>
<td>56</td>
</tr>
</tbody>
</table>

Table 3.6. Percent inhibition of metabolism of AZ66 in the presence of various selective and non-selective inhibitors for CYP450 and FMO.
Fig. 3.5. Effect of chemical inhibitors on the metabolism of AZ66 (10 µM)

3.3.6. In vivo pharmacokinetics

Figures 3.6 and 3.8 represent the mean plasma concentration-time profiles of AZ66 after i.v and oral routes of administration. The pharmacokinetic parameters are summarized in Tables 3.7 (intravenous) and 3.8 (oral). After an i.v administration, V_d was extensive (12.6 L/kg) indicating a high tissue distribution. The plasma clearance and the half-lives were 4.1 L/h/kg and 4.2 h, respectively. After oral administration, the plasma kinetics shows that AZ66 was absorbed rapidly and had a Tmax of 2.0 h. The Cmax was found to be 0.3 µg/mL that was maintained at the same level for 4 h. The longer-lasting terminal phase (3–14 h) might have contributed to the high MRT (8.8 h). The compound exhibited moderate absolute bioavailability (58.2%) indicating a low first-pass metabolism in rats. To confirm the half-life observed after oral administration the study was again repeated in 6 animals. The half-life correlated with the previous study. The new parameters and the plasma profile were shown in table 3.9 and figure 3.10, respectively.
difference between the half lives after i.v (3.9 h) and oral (8.7 h) administration was very high. The i.v study was conducted for 8 h and it is only twice the $t_{1/2}$ observed after i.v administration. To get a correct estimate of the half-life the time period of the study needs to be at least 4-5 times of the observed $t_{1/2}$. In the present case the study period was 8 h and it might have resulted in wrong estimation of the $t_{1/2}$ as the terminal elimination was missing in the study. To estimate the correct $t_{1/2}$ the i.v study was again conducted for 42 h which would be equal to 5 times of the half-life observed after oral administration. The new $t_{1/2}$ observed after the 42 h study was 7.9 h which is very close to the oral $t_{1/2}$. The new Cmax and the CL were found to be 1.21 µg/mL and 2.63 L/h/kg. The new PK parameters and the plasma profile after i.v administration were represented in table 3.10 and figure 3.12, respectively. To further confirm the $t_{1/2}$ observed after oral administration, oral PK study was also repeated again in 2 rats and the $t_{1/2}$ (8.25 h) was found to be similar to two previous studies. The new PK parameters and the plasma profile after oral administration were represented in table 3.11 and figure 3.13, respectively.

Fig.3.6 Pharmacokinetic profile of AZ66 after intravenous administration to rats at 5 mg/kg
**Table 3.7** Pharmacokinetic parameters of AZ66 in rats following a single i.v dose of 5 mg/kg (Mean±SD, n = 5).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>$t_{1/2}$ (h)</td>
<td>3.9 ± 0.4</td>
</tr>
<tr>
<td>Cmax (µg/mL)</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td>AUC$_{0→∞}$ (µg h/mL)</td>
<td>63.2 ± 6.1</td>
</tr>
<tr>
<td>$V_d$ (L/kg)</td>
<td>12.6 ± 0.6</td>
</tr>
<tr>
<td>CL (L/h/ kg)</td>
<td>4.1 ± 0.2</td>
</tr>
<tr>
<td>MRT (h)</td>
<td>4.1 ± 3.3</td>
</tr>
</tbody>
</table>

**Fig. 3.7.** Log plasma concentration time profile after single intravenous dosing of AZ66

**Fig. 3.8.** Pharmacokinetic profile of AZ 66 after Oral administration to rats at 20 mg/kg (n=6)
Fig. 3.9. Log plasma concentration time profile after oral dosing of AZ66

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>$t_{1/2}$ (h)</td>
<td>8.8 ± 0.3</td>
</tr>
<tr>
<td>Cmax (µg/mL)</td>
<td>0.3 ± 0.02</td>
</tr>
<tr>
<td>AUC$_{0→∞}$ (µg h/mL)</td>
<td>158.2 ± 2.8</td>
</tr>
<tr>
<td>$V_d$ (L/kg)</td>
<td>78.1 ± 0.9</td>
</tr>
<tr>
<td>CL/F (L/h/ kg)</td>
<td>6.15 ± 0.1</td>
</tr>
<tr>
<td>MRT (h)</td>
<td>8.8 ± 0.2</td>
</tr>
</tbody>
</table>

Table 3.8 Pharmacokinetic parameters of AZ66 in rats following a single intravenous dose of 5 mg/kg (Mean±SD, n = 5).

Fig. 3.10. Pharmacokinetic profile of AZ 66 after Oral administration to rats at 20 mg/kg (n=6)
Fig. 3.11. Log plasma concentration time profile after oral dosing of AZ66

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>$t_{1/2}$ (h)</td>
<td>8.9 ± 0.6</td>
</tr>
<tr>
<td>Cmax (µg/mL)</td>
<td>0.3 ± 0.04</td>
</tr>
<tr>
<td>AUC$_{0→∞}$ (µg h/mL)</td>
<td>148.2 ± 2.8</td>
</tr>
<tr>
<td>$V_d$ (L/kg)</td>
<td>74.1 ± 0.2</td>
</tr>
<tr>
<td>CL/F (L/h/kg)</td>
<td>6.1 ± 0.4</td>
</tr>
<tr>
<td>MRT (h)</td>
<td>9.1 ± 0.3</td>
</tr>
</tbody>
</table>

Table 3.9. Pharmacokinetic parameters of AZ66 in rats following a single oral dose of 20 mg/kg (Mean±SD, $n=6$).

Fig. 3.12. Plasma concentration vs time profile after i.v administration of AZ66 at a dose of 5 mg/kg
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>$t_{1/2}$ (h)</td>
<td>7.9 ± 0.3</td>
</tr>
<tr>
<td>Cmax (µg/mL)</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td>$AUC_{0→\infty}$ (µg h/mL)</td>
<td>73.2 ± 8.1</td>
</tr>
<tr>
<td>$V_d$ (L/kg)</td>
<td>30.1 ± 3.6</td>
</tr>
<tr>
<td>CL (L/h/ kg)</td>
<td>2.6 ± 0.4</td>
</tr>
</tbody>
</table>

Table 3.10. PK parameters of AZ66 in rats following i.v dose of 5 mg/kg (Mean±SD, n=6).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>$t_{1/2}$ (h)</td>
<td>8.2 ± 0.3</td>
</tr>
<tr>
<td>Cmax (µg/mL)</td>
<td>0.3 ± 0.01</td>
</tr>
<tr>
<td>$AUC_{0→\infty}$ (µg h/mL)</td>
<td>178.2 ± 7.8</td>
</tr>
<tr>
<td>$V_d/F$ (L/kg)</td>
<td>64.1 ± 1.6</td>
</tr>
<tr>
<td>CL/F (L/h/ kg)</td>
<td>6.4 ± 0.1</td>
</tr>
<tr>
<td>MRT (h)</td>
<td>9.9 ± 0.1</td>
</tr>
</tbody>
</table>

Table 3.11. PK parameters of AZ66 in rats after single oral dose of 20 mg/kg (Mean±SD, n=2).

Fig. 3.13. PK profile of AZ 66 after oral administration to rats at 20 mg/kg (n=2)
3.3.7. Plasma protein binding

Protein binding of AZ66 was determined by the ultrafiltration method using Centrifree® devices [163]. Four different physiologically relevant concentrations of the test compound were used to assess the extent of protein binding. AZ66 bound moderately to rat plasma proteins with 71.3% binding. Protein binding did not increase with increase in concentration of the compound in the plasma and thus was independent of concentration. Table 3.12 represents the protein binding of AZ66 in rat plasma. The nonspecific binding of the compound to the components of Ultracentrifree® was less than 0.2%.

<table>
<thead>
<tr>
<th>Conc of AZ66 (ng/mL)</th>
<th>% Binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>700</td>
<td>72.1</td>
</tr>
<tr>
<td>1000</td>
<td>69.5</td>
</tr>
<tr>
<td>2000</td>
<td>71.6</td>
</tr>
<tr>
<td>3000</td>
<td>72.0</td>
</tr>
</tbody>
</table>

Table 3.12. Plasma protein binding of AZ66 at different concentrations

3.3.8. Multiple dose pharmacokinetic studies

Fig. 3.14. shows the log plasma concentration time profile after multiple oral dosing of AZ66. Peak plasma levels achieved after the last dose was found to be 0.49 µg/mL. Trough levels just before the next dose were consistently in the range of 0.13 to 0.3 µg/mL. The mean plasma and tissue levels of AZ66 after administration of 30 mg/kg per every 4 h are summarized in Fig.3.14 and Table 3.13. C$_{1\text{min}}$, the trough concentration after first dosing was 0.13 µg/mL. Peak concentration in plasma after the first and last dose (C$_{\text{max}}$) was found to be 0.25 and 0.49 µg/mL, respectively. The time to attain C$_{\text{max}}$ (T$_{\text{max}}$) was 2 h and it did not change during repeated doses. The accumulation factor (R) was 2.37, calculated as the ratio of C$_{\text{ss, min}}$ to C$_{1\text{min}}$. 
The area under the plasma concentration-time curve of a dosing interval after the first and last dose (AUC₀⁻⁴), determined by trapezoidal summation were found to be 37.1 and 100.5 µg-min/mL, respectively. After 5 half-lives, AZ66 was present in brain and peripheral tissues in measurable amounts. The concentration was highest in lungs (13.3 µg/gm) compared to all other tissues. The mean brain/plasma ratio for AZ66 was 7.54±0.46. The results indicate that AZ66 readily absorbed and distributed in brain and other tissues and accumulated after the multiple dosing.

**Fig.3.14.** Log plasma concentration time profile after multiple oral dosing of AZ66.

**Fig.3.15.** Mean plasma and tissue levels after administration 30 mg/kg per every 4 h.
Table 3.13. The mean plasma and tissue levels after administration 30 mg/kg per every 4 h.

3.3.8.1. Brain to plasma ratio

The extent of CNS penetration of the compound AZ66 was determined using Brain to Plasma ratio study. The compound was administered orally at a dose of 30 mg/kg for five half-lives to attain steady state. At the end of the study, the brains were harvested and analyzed to determine the B/P ratio. The results showed that AZ66 crossed the BBB and penetrated into the brain tissue to a greater extent. The B/P ratio of the compound AZ66 was found to be 7.54 ± 0.46. Table 3.14 shows the B/P ratio of AZ66 in rats after repeated oral dosing. The brain concentration was 7.5 times greater than the plasma. The high volume of distribution observed during the single dose studies further confirms its high tissue distribution.

<table>
<thead>
<tr>
<th>Tissues</th>
<th>Conc. (µg/gm) in Tissues after 5 doses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td>0.51</td>
</tr>
<tr>
<td>Brain</td>
<td>3.87</td>
</tr>
<tr>
<td>Heart</td>
<td>1.86</td>
</tr>
<tr>
<td>Liver</td>
<td>7.68</td>
</tr>
<tr>
<td>Kidney</td>
<td>9.68</td>
</tr>
<tr>
<td>Lung</td>
<td>13.33</td>
</tr>
</tbody>
</table>

Table 3.13. The mean plasma and tissue levels after administration 30 mg/kg per every 4 h.

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>No of Doses</th>
<th>Concentration</th>
<th>B/P ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Brain (µg/gm)</td>
<td>Plasma (µg/mL)</td>
</tr>
<tr>
<td>30</td>
<td>5</td>
<td>3.87</td>
<td>0.51</td>
</tr>
</tbody>
</table>
Table 3.14. Brain to plasma ratio of AZ66 in rats after repeated oral dosing

3.4. Conclusions

Though AZ66 is a structural analogue of CM156, its pharmacokinetic behavior was relatively different. CM156 has a plasma $t_{1/2}$ of 1 h after i.v. administration where as for AZ66 it is 7.9 h which is very much greater than that of CM156. More rapid clearance from plasma and low Vd of CM156 (6.2 mL/min/kg, 9.6 L/Kg) compared to AZ66 (2.6 mL/min/kg, 30.6 L/Kg) might have contributed to this difference. The modification in chemical structure resulted in altered physicochemical parameters i.e. the replacement of sulfur atom in CM156 with oxygen in AZ66 decreased the pKa and log D. This may be explained as one of the possible reason for the higher metabolic stability of AZ66 compared to CM156. The low pKa reduced the lipophilicity of AZ66, as more amount of the compound remains in the neutral form and thus it exhibited comparatively less protein binding and high stability. A bioanalytical method was developed and validated for the determination of AZ66 in rat plasma and has been applied for pharmacokinetic studies in rats. The preclinical pharmacokinetic studies demonstrated that AZ66 has excellent oral bioavailability, a long half-life and low clearance. The results indicate that AZ66 readily absorbed and distributed in to brain and other tissues and accumulated after the multiple dosing. Together, all these studies show that first AZ66 has good efficacy and ADME properties, justifying further development of this compound towards clinical candidate status.
BIBLIOGRAPHY


VITA

Seshulatha Jamalapuram was born on March 5, 1984 in Khammam, AP, India. She pursued her Bachelors in Pharmacy from Sarojini Naidu Vanitha Pharmacy Mahavidyalaya, Osmania University, Hyderabad, India and graduated with University rank in June 2005. She secured 92.08 Percentile in GATE-2005, Graduate Aptitude Test in Engineering and Pharmaceutical Sciences. She joined the Master’s program in Pharmacy with a specialization in Pharmaceutics at the University College of Pharmaceutical Sciences, Kakatiya University, Warangal, India and graduated with a first class in August 2008. She was a recipient of All India Council for Technical Education and Training (AICTE) Research Fellowship during Master’s education. She did her maters project work at Dr. Reddy’s Laboratories, Hyderabad, India. Seshulatha joined the Department of Pharmaceutics, The University of Mississippi as a graduate student in September 2008. Seshulatha pursued her doctoral program in bioanalytical and preclinical DMPK under the guidance of Dr. Bonnie A. Avery. During doctoral program, she has published 3 research papers in peer reviewed international journals and presented many research posters in various regional and international conferences. She received “Graduate Student Council Research Award” for one of her project in 2011. She is an active member of American Association of Pharmaceutical Scientists (AAPS), served as a reviewer for abstracts. She won AAPS student travelship awards consecutively for 3 years in APQ and FDD sections. She also received Best poster presentation award, Southern Regional Discussion Group, AAPS, 2011. She was also a recipient of Natural product neuroscience fellowship, CORE-NPN, 2010-2011. She was inducted by Phi Kappa Phi and Rho-Chi, The academic Honor societies in Pharmacy in
2010. During her graduate studies she served as a Graduate Teaching Assistant for Physical Pharmacy and Pharmaceutical Compounding labs, Pharmacokinetics course. Seshulatha received the Doctor of Philosophy degree in Pharmaceutics in April 2013.