METABOLIC INTERACTIONS BETWEEN OVER-THE-COUNTER AND ILLICIT DRUGS AT CYTOCHROME P450

By

KARA KELLEY CROSSWY

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University of Louisville

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Thesis Approved:

Dr. David R Wallace

Thesis Advisor

Dr. Robert W. Allen

Dr. R. Tom Glass

Dr. A. Gordon Emslie

Dean of the Graduate College

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LIST OF SYMBOLS

AHMC	Fluorescent metabolite, 3-[2-(N, N-diethyl-N-methylamino) ethyl]-7-methoxy-4-methylcoumarin
AMMC	Nonfluorescent probe, 3-[2-(N, N-diethylamino) ethyl]-7-hydroxy-4-methylcoumarin hydrochloride
AMP	Amphetamine
ANOVA	Analysis of Variance
BSA	Bovine Serum Albumin
CMT	Cimetidine
CNS	Central Nervous System
DA	Dopamine
DEA	Drug Enforcement Agency
DEX	Dextromethorphan
DOR	Dextrorphan
DMSO	Dimethyl Sulfoxide
GABA	γ- Amino Butyric Acid
GC	Gas Chromatography
HBr	Hydrogen Bromide
HCl	Hydrogen Chloride
HHA	3, 4-dihydroxyamphetamine
HHMA	3, 4-dihydroxymethamphetamie

HMA	4-hydroxy-3-methoxyamphetamine
HPLC	High Performance Liquid Chromatography
HTS	High Throughput Inhibitor Screening
IC_{50}	50% Inhibitory Concentration
i.p.	Intraperitoneal
i.v.	Intravenous
K_m	Substrate concentration as reaction is 50% of V_{max}
LOD	Limit of Detection
LOL	Limit of Linearity
MA	Methamphetamine
MDA	Methylenedioxyamphetamine
MDMA	3, 4-methylenedioxymethamphetamine
MS	Mass Spectrometry
m/z	Mass to Charge Ratio
NE	Norepinephrine
NMDA	N-methyl-$_D$-aspartate
OTC	Over The Counter
PCP	Phencyclidine
p-OH	*p*-hydroxymethamphetamine
RH	Organic Substrate
SEM	Standard Error Mean
SPE	Solid Phase Extraction
VC	Vehicle Control

V_d	Volume of Distribution
V_{max}	Maximum Velocity of reaction
5-HT	Serotonin

CHAPTER I

INTRODUCTION

Methamphetamine (MA) and 3, 4-methylenedioxymethamphetamine (MDMA) are abused drugs gaining popularity for recreational use. Co- administering one of these drugs with an over the counter drug (OTC), polydrug use, is a growing concern. This concern is due to possible drug- drug interactions that can affect the metabolism of the scheduled drugs resulting in toxic effects. Another concern is how these interactions affect toxicology analysis. One type of interaction can cause a scheduled drug to accumulate at higher concentrations which can lead to toxicity. This increase could cause higher detection of the parent drug thus possibly causing the results to be misinterpreted. Determining possible drug interactions between OTC drugs and scheduled drugs are therefore important for interpreting adverse effects. This study will look at two common OTC drugs, two popular scheduled drugs, and the possible interactions that can occur between them. The question posed by this study is: **Does exposure to OTC drugs, cimetidine and dextromethorphan, alter the metabolism and subsequent clearance of the scheduled drugs MA and MDMA?**

Cimetidine (CMT) and MDMA are inhibitors of CYP2D6 (Van et al., 2006) whereas dextromethorphan (DEX) and MA are reported substrates for the CYP2D6 isozyme (Brown, 2001). Inhibitors, lead to increased substrate drug effect

caused by an increase in drug concentration (Brown, 2001; Cupp et al., 1998; Badyal et al., 2001). Clemens et al. (2005) confirm an increase in adverse effects when MDMA and MA are taken concurrently. These studies illustrate the problems associated with co-administering drugs and their potential drug–drug interactions. Drugs and Human Performance Fact sheets from the National Highway Traffic Safety Administration (2005) also report "potential inhibitors of the CYP2D6 isozyme could decrease the rate of methamphetamine elimination if administered concurrently, while potential inducers could increase the rate of elimination."

The main focus of this research is to determine the interactions between OTC drugs, CMT and DEX, and scheduled drugs, MA and MDMA when administered concurrently. The types of interactions possible are inhibition of metabolism of the scheduled drug or OTC drugs, stimulation of metabolism for one or all of the drugs, or no interaction between the drugs administered together. This is significant because drug interactions may lead to the misinterpretation of toxicology results. The skewed results could be due to the combination of MA with another drug that inhibits or competes for an enzyme. These results mislead the analyst and cause problems in the accurate interpretation of the data.

Three assumptions are made in this study. One assumption is there are interactions when the OTC drugs are co- administered with MA or MDMA. Another assumption is that the primary metabolism of the drugs occurs via the P450 CYP2D6 enzyme. The third assumption is the metabolism of the scheduled drugs decreases therefore increasing the drug concentration with one or all of the different combinations.

These assumptions are presumed since the drugs are either inhibitors or substrates of CYP2D6.

The limitation of this study is the non-use of human subjects. Rats are a convenient and inexpensive model system for studying drug kinetics. Initial studies utilize commercially available kits containing human CYP2D6 isozyme. Rat studies examine CYP2D2, the rat isozyme for the human CYP2D6 isoform.

CHAPTER II

REVIEW OF LITERATURE

II.A. Cytochrome P450- CYP2D6

The cytochrome P450 (CYP) enzyme system is a superfamily of hemoproteins that catalyze the metabolism of numerous compounds (Badyal et al., 2001). The enzymes mainly involved with metabolism are located in the liver, primarily the endoplasmic reticulum of hepatocytes (Brown, 2001). But the enzymes are also found in other areas like the small intestine, kidney, lung and brain. More than thirty CYP human isozymes have been identified. The most common type of catalyst reaction (Figure 1) is the monooxygenase reaction, where one atom of oxygen is inserted into an organic substrate (RH), and the other oxygen atom is reduced to water:

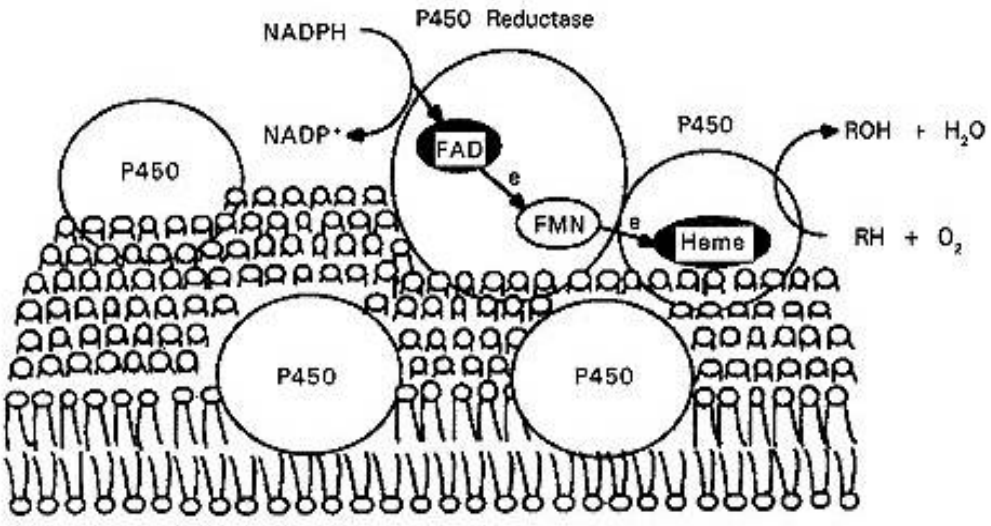

Figure 1. The CYP monooxygenase reaction. Source: Ohkawa et al., 2002.

The P450 name refers to the "pigment at 450 nm" which comes from the characteristic Soret peak formed by absorbance of light at wavelengths near 450 nm when the heme iron is reduced and complexed to carbon monoxide. The naming for the specific gene includes the CYP root symbol, an Arabic numeral to denote the family, letters (A, B, C) to indicate the subfamily, and another Arabic numeral to specify the gene (Badyal et al., 2001).

Nomenclature

- Root: CYP
- Family: CYP2
- Subfamily: CYP2D
- Gene: CYP2D6

Metabolism is characterized by two phases that increase substrate solubility allowing clearance from the body (Figure 2). Cytochrome P450 is an important part of the phase I metabolism of drugs, which alters a molecule by transforming the nature of a functional group or adding a functional group to introduce or unmask polar bodies. This includes: hydroxylation, oxidation, desulfuration, sulfoxide formation, deacetylation, deamination, nitro reduction, reduction, and dealkylation routes of metabolism. Phase II metabolism is a conjunctive process that includes adding a gluconuride ester or sulfate to a functional group (Liska, 1998).

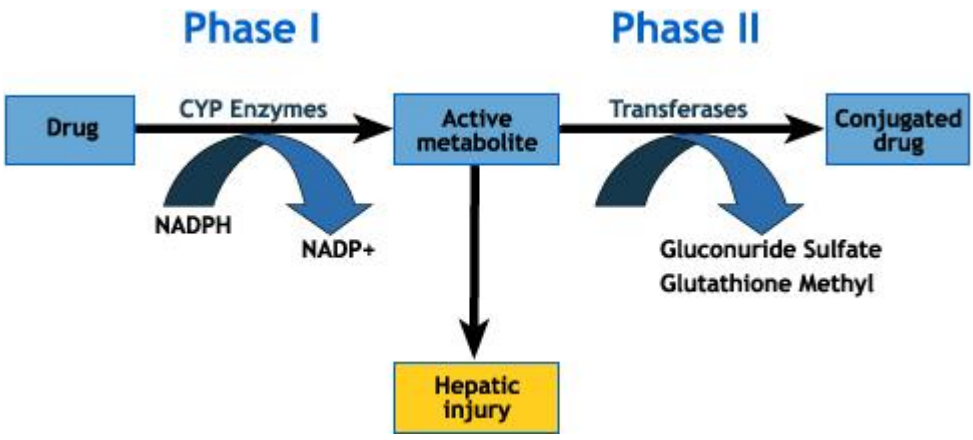

Figure 2. Phase I and II metabolism. Source: DNA direct
http://www.dnadirect.com/professionals/tests/tamoxifen/cytochrome.jsp

The CYP2D6 allele is located on chromosome 22 and comprises <5% of the total CYP proteins (Badyal et al., 2001). This isozyme is the second largest metabolizer of drugs in the body at 19% (Figure 3). It has been studied extensively due to its genetic polymorphisms and its large number of substrates (Badyal et al., 2001). Due to the genetic polymorphism there are classifications for an individual to be either a poor

metabolizer, normal metabolizer, or an extensive metabolizer. Poor metabolizers cannot metabolize certain drugs due to lacking of a gene for the isozyme. Normal metabolizers can metabolize drugs since they have the appropriate gene (Brown, 2001). Extensive metabolizers can metabolize drugs quicker than the other two classifications. Poor metabolizers may not obtain the prescribed effect from a specific drug, or they may reach toxic drug concentrations when prescribed usual doses due to their inability to metabolize the drug.

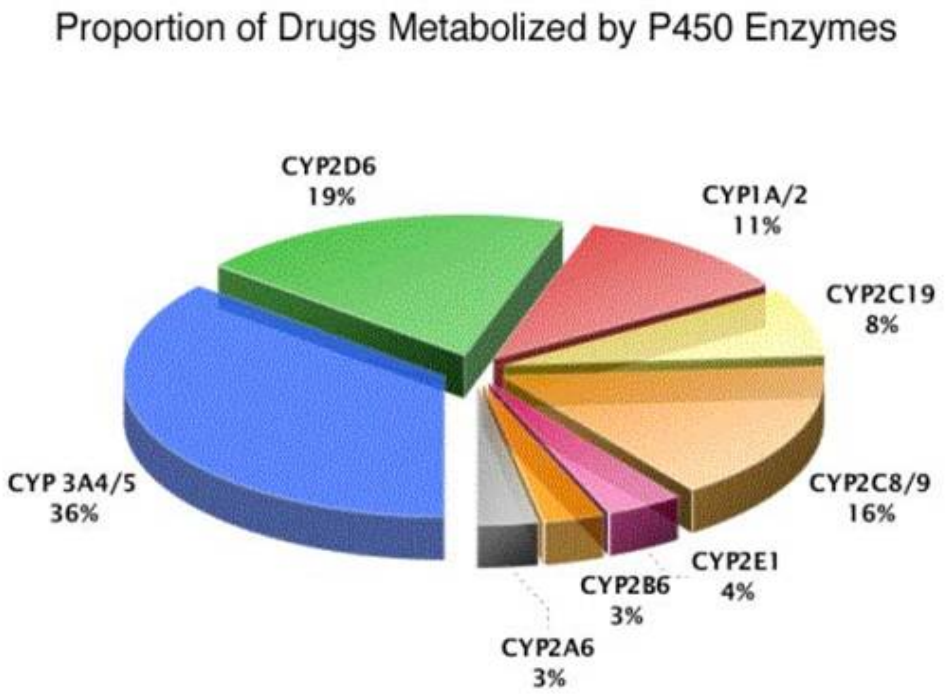

Figure 3. The proportion of drugs metabolized by the different P450 enzymes. Source: Wrighton et al., 1992.

P450 isoforms can indirectly be induced or inhibited which can cause drug interactions to occur. Enzyme induction occurs when a drug stimulates the synthesis of enzyme protein production, thus increasing the enzyme's metabolizing capacity (Cupp et

al., 1998). Enzyme inhibition occurs when there is competition with another drug for the binding site (Cupp et al., 1998) or decrease in enzyme protein. Inhibition magnitude is a function of the inhibiting agent concentration and the affinity of the agent for CYP2D6 (Brown, 2001). CYP2D6 inducers include phenobarbital, and rifampicin; inhibitors include tricyclic antidepressants (Zoloft), selective serotonin reuptake inhibitors (Prozac), CMT, MDMA, and quinidine; substrates include tricyclic antidepressants (Elavil), MA, and DEX (Brown, 2001).

II.B. Methamphetamine

N, α-dimethylphenethylamine, methamphetamine or MA, is a derivative of amphetamine (AMP) and belongs to the AMP class (Logan, 2002). MA is commonly accepted as more addictive and favored by drug addicts (Shoblock et al., 2003) and has greater central nervous system (CNS) efficacy than AMP. This efficacy is most likely due to MA's greater ability to penetrate the CNS. Amphetamines represent a class of compounds, phenethylamines, which have varying degrees of sympathomimetic activity (Shoblock et al., 2003). The sympathetic nervous system is stimulated by endogenous neurotransmitters [norepinephrine (NE), dopamine (DA), serotonin (5- HT)], and sympathomimetic drugs imitate the neurotransmitters' actions (Logan, 2002). Amphetamines are stimulant drugs that affect the CNS by causing the neurotransmitter DA to be displaced from its storage vesicles in the nerve terminal. This displacement results in the synaptic dopaminergic receptor to be hyperstimulated (Logan, 2002). At higher doses of MA, DA and 5-HT, concentrations are decreased in the brain, due to a

reduction in the enzyme activity responsible for their synthesis, tyrosine hydroxylase and tryptophan hydroxylase, respectively (Logan, 2002).

There are two forms of MA: the *d*-isomer and the *l*-isomer. The *d*-isomer is the preferred form due to greater CNS stimulant effects (Logan, 2002). MA is N-demethylated to p-hydroxymethamphetamine (~15%) and the active metabolite AMP (4-7%) via the CYP2D6 isozyme. Then AMP is metabolized to *p*-hydroxyamphetamine and other metabolites (Logan, 2002; Moore, 2003). Figure 4 illustrates the metabolic pathway for MA and AMP (Feldman et al., 1999). MA is well absorbed orally and highly lipid soluble. It has a volume of distribution (V_d) of 3-7 L/ kg and a bioavailability of ~67%. In humans, peak plasma concentration of 0.020 mg/ L at 2.6 h can be reached with a single oral dose of 0.125 mg and a half-life of 7-10 h (Logan, 2002; Moore, 2003). MA is excreted in the urine 30- 54% unchanged, while 10% of the dose is excreted as AMP (Logan, 2002). Elimination of MA and AMP are dependent on urinary pH; urinary acidification decreases the half-life and alkalization increases the half- life. Table 1 summarizes the pharmacokinetics.

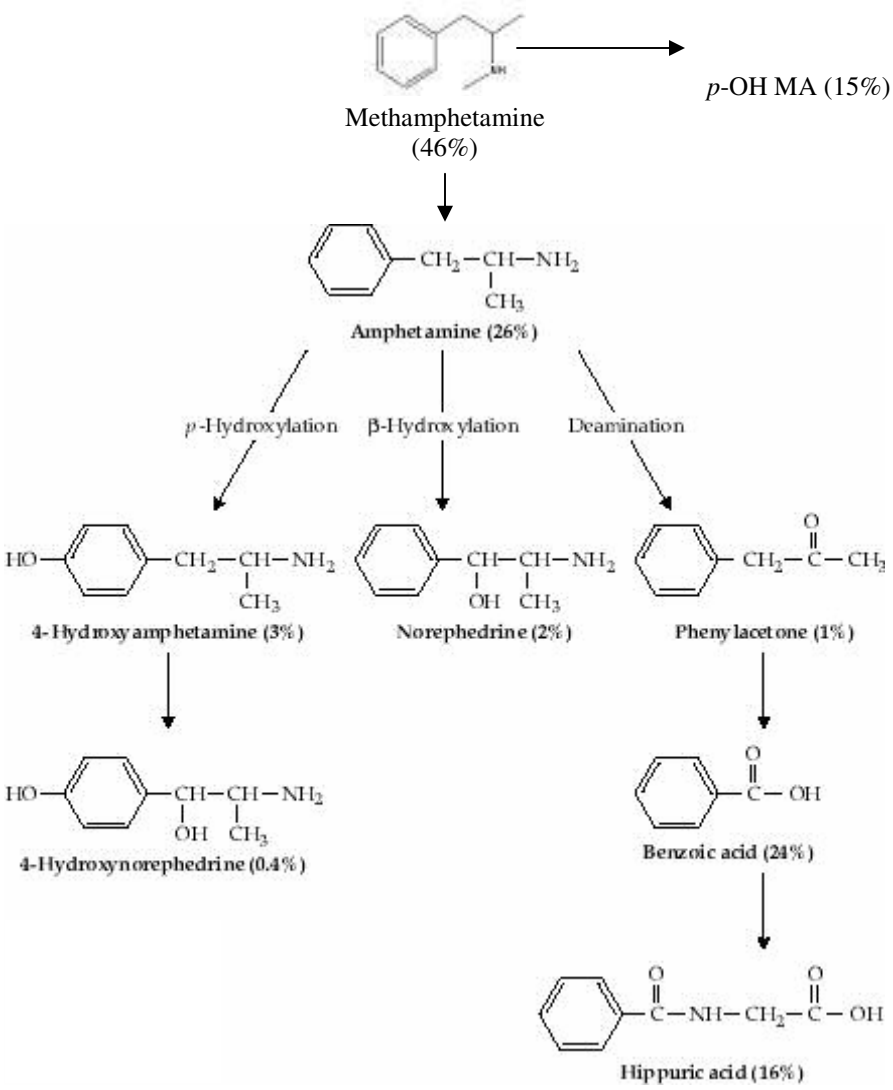

Figure 4. MA and AMP metabolism schematic. Reproduced with modifications from Feldman et al., 1999.

Table 1. Pharmacokinetics summary for each drug

Drug	Primary Active Metabolite	Primary Metabolizing Enzyme	Dose (in humans)	Plasma Levels	Half life	Volume of Distribution
MA	AMP (~7%)	CYP2D6	0.125 mg	0.02 mg/ L @ 3.6 h	7- 10 h	3-7 L/ kg
MDMA	MDA	CYP2D6	1.5 mg	0.33 mg/ L @ 2.0 h	~7 h	~4.9 L/ kg
CMT	S-oxide (19%)	CYP2D6	75-117 mg	0.5-1.1 mg/ L @ 1 h	2 h	0.8-1.39 L/ kg
DEX	Dextrorphan (20%)	CYP2D6	30 mg	<5 ng/ mL @ 4 h	2- 4 h	5.0-6.4 L/ kg (for dogs)

AMP was first synthesized in 1887. In the 1920s it was used as a nasal decongestant to replace ephedrine and marketed in the 1930s as a Benzedrine inhaler. Its ability to treat narcolepsy, hyperactivity in children and as a stimulant was quickly recognized. In the late 1940s and 1950s AMP and related drug use reached epidemic quantities due to its use by soldiers, factory workers, and prisoners of war. After World War II, Japanese marketed AMP and other related drugs like MA without a prescription thus increasing use and abuse. MA abuse became a social problem in the subsequent decades after WWII. The Controlled Substances Act with the five levels of scheduling was enacted in 1986. Due to increasing abuse, MA/ AMP's highly addictive potential, and some medicinal use led to the classifications of MA and AMP as DEA schedule II controlled substances.

Currently MA is an easily obtainable drug and is relatively easy to make due to the availability of the ingredients. Many of the ingredients are found in OTC drugs containing ephedrine or pseudoephedrine and household products like hydrochloric acid, drain cleaner, battery acid, lye, lantern fuel, antifreeze, and red phosphorous

(http://www.kci.org/meth_info/faq_meth.htm). Also the internet provides access to recipes to make MA at home. Therefore most street MA is made in clandestine laboratories greatly increasing its availability. This availability is a main reason why MA has become the drug of choice for stimulant abusers.

Table 2. Physiological and psychological effects of MA

Physiological	Psychological
• increased blood pressure • increased respiration rate • elevated temperature • palpitations • irregular heartbeat • bronchial muscle dilation • vasoconstriction • bladder contraction • dry mouth • diarrhea • nausea • abdominal cramps • loss of appetite • twitching • pallor • dilated pupils • horizontal gaze nystagmus at high doses • faster reaction time • increased strength • tooth decay • increased blood glucose levels	• euphoria • intensified emotions • increased feeling of self-esteem and well being • excitation • exhilaration • rapid flight of ideas • increased libido • rapid speech • motor restlessness • hallucinations • delusions • psychosis • insomnia • reduced fatigue or drowsiness • increased alertness • sensations of extreme physical and mental power • anxiety • exhaustion • paranoia • agitation • poor impulse control

(Moore, 2003; Logan, 2002; www.nhtsa.dot.gov/people/injury/research/job185drugs/technical-page.htm).

II.C. Methylenedioxymethamphetamine

3, 4-Methylenedioxymethamphetamine, MDMA or ecstasy, is a recreational illicit drug that is very popular at all-night parties or raves. MDMA is an analog of MA formed by methylenedioxy substitution (Logan et al., 2003), and belongs to the AMP class. MDMA is also structurally similar to mescaline (Oesterheld et al., 2004) and methylenedioxyamphetamine, MDA, (Logan et al., 2003). MDMA affects several neurotransmitter systems including NE, DA, 5-HT, and the neurotransmitter γ- amino butyric acid, GABA, (Logan et al., 2003). MDMA is a strong and selective 5- HT neurotoxin in numerous animal species, including non- human primates (Casco et al., 2005). In rats, MDMA stimulates 5-HT release which causes 5- HT synaptic concentrations to increase and depletion of 5- HT presynaptic stores (Logan et al., 2003). This process is believed to result from MDMA's effect in reversing the 5-HT uptake transporter (Logan et al., 2003).

There are two forms of MDMA: the R (-)-isomer and the more potent neurotoxin, the S (+)-isomer. MDMA is O-demethylated to 3, 4-dihydroxymethamphetamine, HHMA, via CYP2D6 (Escobedo et al., 2004; Oesterheld et al., 2004) and N-demethylated to the active metabolite MDA via CYP1A2 and CYP2D6 (Logan et al., 2003). It is a potent mechanism based (irreversible inhibitor) of CYP2D6 (Van et al., 2006). Figure 5 illustrates the metabolism schematic for MDMA (Escobedo et al., 2004). MDMA is well absorbed orally (Spiller, 2004) and soluble in water with a V_d of ~4.9 L/ kg (De Letter et al., 2002). In humans, it reaches peak plasma concentrations of 0.33 mg/ L at 2 h with an oral dose of 1.5 mg and a half-life of ~7 h (Logan et al., 2003). MDA plasma concentrations peak later at 4-6 h and do not exceed 5% of the concentration of

the parent compound (Logan et al., 2003). MDMA is excreted in the urine 65% unchanged (Spiller, 2004). Table 1 summarizes the pharmacokinetics.

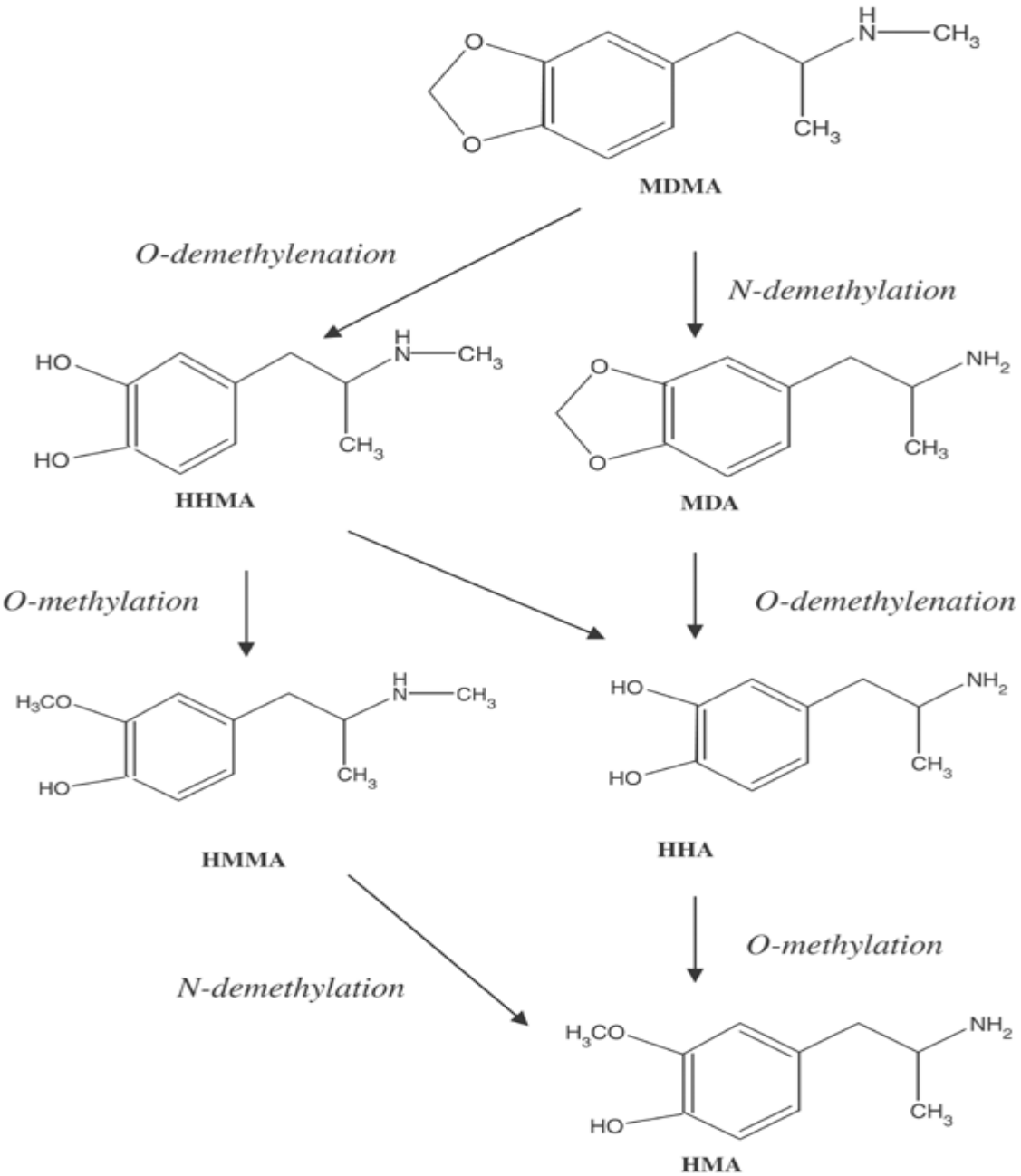

Figure 5. MDMA metabolism schematic shown in part; HHMA: 3, 4-dihydroxymethamphetamine, HMA: 4-hydroxy-3-methoxyamphetamine, HHA: 3, 4-dihydroxyamphetamine (reproduced from Escobedo et al., 2004).

MDMA was first synthesized as an appetite suppressant in 1914 by Merck, but never marketed as such. During the 1970s and 1980s, MDMA became popular as an adjunct for psychotherapy, but was never approved by the FDA. MDA was originally termed as ecstasy (the active metabolite) and is related to saffrole. It is used as a psychoactive agent in mace. It was first synthesized in 1910 and has been used as an anorexiant, antitussive, and ataractic or tranquilizers. It was abused in the 1960s and 1970s for its psychoactive and hallucinogenic properties and therefore, it and MDMA (due to structural similarity) were classified as DEA schedule I controlled substances with no medicinal uses.

Casco et al. (2005) provided evidence to support long-term electrophysical abnormality in MDMA users and suggested that typical recreational doses of MDMA are enough to cause long-term altered cortical activity in humans. MDMA and MA when taken concurrently can produce greater adverse effects, dependent on the order of administration. If MA is administered after MDMA, this can lead to greater hyperthermia and greater depletion of 5- HT compared to MDMA administered after MA (Clemens et al., 2005). Clemens et al. (2005) also report an increase in adverse effects when MDMA and MA are taken concurrently. This information is important to drug users who co- administer the two.

Table 3. Physiological, psychological and positive effects of MDMA

Physiological	Psychological	Positive Effects
• increased blood pressure • increased heart rate • peripheral bronchodilation • vasoconstriction • nausea • dehydration • jaw clenching • blurred vision • vomiting • tachycardia • dry mouth • bruxism • muscle tension • increased sweating • ataxia • pupillary dilation • nystagmus • appetite suppression • urinary urgency • double vision • hyperthermia • hyponatremia • convulsions • catatonic stupor • motor ticks	• hallucinations • enhanced mood • emotional sensitivity • euphoria • confusion • depression • desire to be in motion • insomnia • panic attacks • paranoia • irritability • difficulty concentrating • exhaustion • irritability • fatigue • anxiety	• changes in feelings and emotion • enhanced communication • empathy • changes in cognitive or mental associations • euphoria or elation • changes in perception, including hallucinations

(Logan et al., 2003; NIDA Info facts: MDMA 2006)

II.D. Cimetidine

Cimetidine (CMT) is used as a treatment for acid reflux disease, heartburn, and ulcers. CMT is available over the counter only as Tagamet®. CMT blocks the H2 histamine receptors in the parietal cells of the stomach (Jantratid et al., 2006), thus reducing the amount of stomach acid. CMT is a compound that can directly bind to the

cytochrome P450 heme iron reactive site thus inhibiting all cytochrome-dependent phase I enzyme activity (Liska, 1998). Therefore, CMT has been associated with many drug-drug interactions involving the inhibition of this isozyme (Park et al., 2005; Madeira et al., 2004). One study shows CMT combined with MA causes "increased levels of methamphetamine and amphetamine in the brain of rats" (Suzuki et al., 1987). This study suggests that increased levels of MA and AMP are due to inhibition of CYP2D6 by CMT.

CMT is metabolized by the P450 enzymes, and its major metabolite is an S-oxide (Lu et al., 1998). Figure 6 illustrates the metabolism schematic of CMT (Rendic, 1999). CMT is slightly soluble in water, 11.4 mg/ mL at 37°C with a pH of 9.3 (Jantratid et al., 2006). The V_d is ~0.8-1.39 L/ kg and bioavailability is between 56-68% (Jantratid et al., 2006). Tagamet® product information (2005) report peak blood levels in humans of 0.5-1.1 mg/ L with doses of 75-117 mg. The half-life of CMT is 2.0 h and 1.7 h for the metabolite, S-oxide (Larsson et al., 1982). CMT is excreted 70% unchanged and ~19% as S-oxide (Tagamet® product information 2005). Table 1 summarizes the pharmacokinetics.

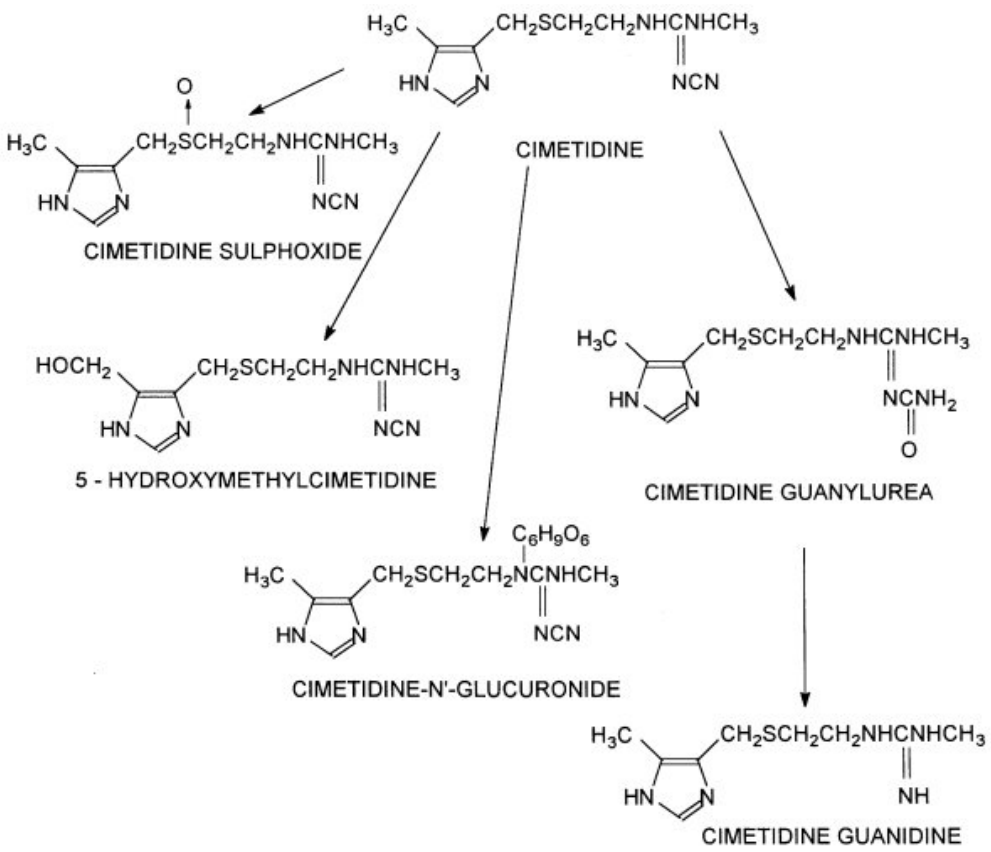

Figure 6. CMT metabolism schematic reproduced from Rendic, 1999.

Table 4. Physiological and psychological effects of CMT

Physiological	Psychological
• constipation • fatigue • headaches • diarrhea • nausea • vomiting • insomnia • dizziness • drowsiness • tiredness • rash • muscle pain • enlargement of the breasts • impotence (usually seen in patients on high doses for prolonged periods) • decreased white blood cell counts • irregular heartbeat • skin reactions • visual changes • allergic reactions • hepatitis	• confusion • hallucinations (usually in elderly or critically ill patients)

(Tagamet® Product Information 2005; www.medicinenet.com/cimetidine/article 2006)

II.E. Dextromethorphan

Dextromethorphan (DEX) is an antitussive used in cold and cough medications to relieve nonproductive coughs (Abdul Manap et al., 1999). DEX is considered a dextrorotary morphinan because it does not bind to opioid receptors, thus it has no analgesic activities; however, it does bind to a site associated with sigma-site ligands and also to the phencyclidine (PCP) N-methyl-$_D$-aspartate (NMDA) glutamate receptor channel site (Nicholson et al., 1999). DEX has been used at higher doses as a recreational drug that produces dissociative effects similar to PCP and ketamine

(Nicholson et al., 1999; Lotrich et al., 2005). Also at higher concentrations, DEX is a N-methyl-$_D$-aspartate (NMDA) antagonist like PCP and ketamine (Lotrich et al., 2005). Since 1975 the popularity and abuse of DEX has been recognized but it has not been placed on the Controlled Substances Act (Center for Substance Abuse Research, 2005).

DEX is a substrate for CYP2D6 and may cause drug-drug interactions when taken in combination with other CYP2D6 substrates or inhibitors. It can also be used to determine what type of metabolizer an individual is with regards to CYP2D6 activity. This is measured by how much of the active metabolite, dextrorphan (DOR), is formed (Magarey, 1997). One study showed DEX and DOR decreased MA self- administration at doses <30 mg/ kg in rats (Glick et al., 2001). This study suggests that taking DEX with MA decreases levels of MA. Another study showed an interaction between DEX and an inhibitor, quinidine, of CYP2D6. Quinidine inhibited the metabolism of DEX, thus increasing the parent compound to metabolite ratio (Abdul Manap et al., 1999).

DEX is O-demethylated to DOR, 20%, (Witherow et al., 1999) via CYP2D6 (Abdul Manap et al., 1999). Figure 7 illustrates the metabolism schematic for DEX (Min et al., 1999). DEX is soluble in water (1.5 g/ 100 ml at 25°C) with a V_d (for dogs) of 5.0-6.4 L/ kg (no data available for human V_d; Magarey, 1996). In humans, peak plasma levels <5 ng/ mL at 4 h are reached with a dose of 30 mg and a half- life of 2-4 h (Magarey, 1996). DEX is excreted in the urine ~11% unchanged (Magarey, 1996). Table 1 summarizes the pharmacokinetics.

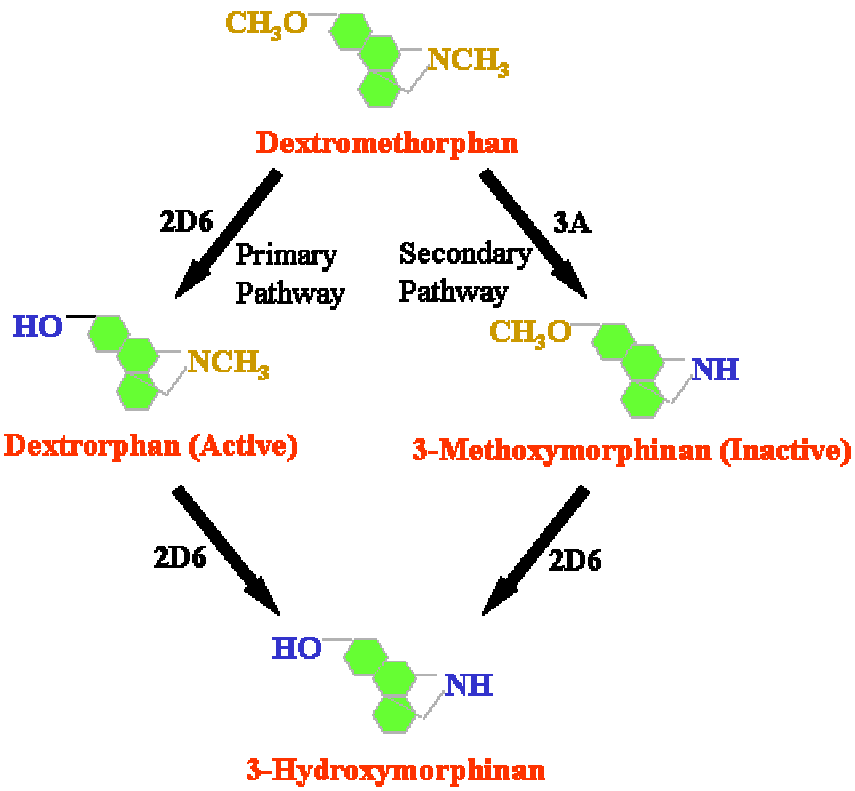

Figure 7. DEX metabolism schematic reproduced from University of Colorado Center for Health Sciences 1999 **http://www.uchsc.edu/sm/psych/ppfr/cyp_metabolism.htm**.

Table 5. Physiological and psychological effects of DEX

Physiological	Psychological
• blurred vision • difficulty urinating • drowsiness • dizziness • nausea • vomiting • shakiness and unsteady walk • slowed breathing • constipation • headache • stomach pain • ataxia • respiratory depression • tachycardia • dry mouth • seizures	• euphoria • hallucinations • confusion • unusual excitement • nervousness • restlessness • irritability • confusion • CNS stimulation • lethargy

(Magarey, 1996; Nicholson et al., 1999; Medline plus Drug Info, 2003)

II.F. Summary

MA and MDMA are becoming increasingly popular drugs, thus polydrug use is a growing concern especially with OTC drugs like CMT and DEX. The concern is due to the types of interactions these combinations would have on the user and the problems for toxicology testing. Determining the effects between OTC drugs and scheduled drugs are important in preventing adverse effects. The purpose of this study is to determine if interactions occur between two over the counter drugs, cimetidine and dextromethorphan, and two scheduled drugs, methamphetamine and 3, 4-methylenedioxymethamphetamine, and if interactions do occur, then what are those interactions?

II.F.i. Hypothesis

The hypothesis for this study is that two OTC drugs, CMT and DEX, will alter the metabolism of two abused drugs, MA and MDMA resulting in increased plasma levels of MA and MDMA.

II.F.ii. Aims

The aims for this study include:

- Potential interactions of MA or MDMA and OTC drugs using purified human CYP2D6
- *In vivo* studies will examine the effects of the combinations of the OTC and scheduled drugs: 1) MA on CYP2D2 activity and 2) MA concentration following pretreatment with CMT, DEX, or CMT/ DEX.

CHAPTER III

METHODOLOGY

III.A. Materials

Methamphetamine HCl, 3, 4-methylenedioxymethamphetamine HCl, cimetidine, dextromethorphan HBr, acetonitrile, dimethyl sulfoxide (DMSO), 3-[2-(N, N-diethyl-N-methylamino) ethyl]-7-methoxy-4-methylcoumarin (AMMC) were purchased from Sigma Chemical Company (St. Louis, MO). The high throughput inhibitor screening kit CYP2D6/ AMMC was purchased from BD Biosciences Gentest™ (Woburn, MA). The P450 HTS kit supplies the required components including: insect cell microsomes (Supersomes®) made from human baculovirus insect cell expressing human CYP2D6 (CYP2D6*1 + P450 reductase), nonfluorescent substrate, fluorescent metabolite, control insect cell membrane protein, cofactors (1.3 mM NAPD+, 66 mM $MgCl_2$, and 66 mM glucose 6-phosphate), glucose 6-phosphate dehydrogenase (40 Units/ ml in 5 mM sodium citrate buffer (pH 7.5)), reaction buffer solution (0.5 M potassium phosphate, pH 7.4, filter sterilized), stop solution reagent (0.5 M Tris base reagent, filter sterilized), CYP2D6 positive control inhibitor (quinidine), and NAPDH regenerating system.

III.A.i. HTS Kit Substrates

AMMC, 3-[2-(N, N-diethyl-N-methylamino) ethyl]-7-methoxy-4-methylcoumarin, (Figure 6) is a nonfluorescent substrate that demethylates to the fluorescent metabolite AHMC, 3-[2-(N, N-diethylamino) ethyl]-7-hydroxy-4-methylcoumarin hydrochloride, (Figure 7) which was used with the human baculovirus cell expressing CYP2D6 Supersomes®.

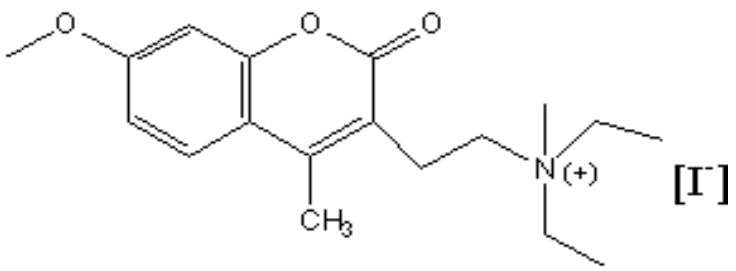

Figure 8. AMMC structure. AMMC is demethylated to AHMC, the fluorescent product.

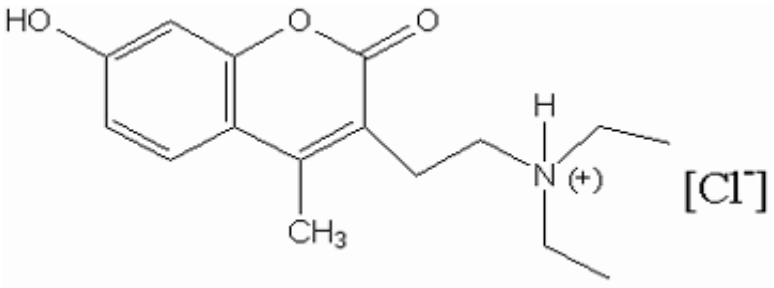

Figure 9. AHMC structure. The fluorescent product, AHMC, is produced by the demethylation of AMMC.

III.B. *In vitro* Inhibition Studies

CYP2D6/ AMMC high throughput assay kits screen for potential inhibitors of CYP2D6 catalytic activity. Quinidine is a known potent inhibitor of CYP2D6 and used as the control compound to determine intra- and inter- assay variability. Each test compound and quinidine was assayed to determine the inhibition of the enzyme for that compound. These compounds were serially diluted (1:3) from highest concentrations (20 μM) to lowest concentrations (3 nM) and pre-incubated at 37^{o}C for 10 min. The enzyme/ substrate mix contains AMMC, the nonfluorescent substrate that produces the fluorescent metabolite AHMC. The enzyme/ substrate mix was added to quinidine and each test compound well, and then incubated at 37^{o}C for 30 min. During incubation AMMC demethylates to AHMC via CYP2D6. The AHMC fluorescence was measured using a plate reader and inhibition of the enzyme was calculated from the fluorescent values (Figure 10). The fluorescence in each well was determined using a Synergy HT Multi Detection microplate reader (Bio-TEK® Instruments, Inc., Winkooski, VT) with KC4 software (Bio-TEK® Instruments, Inc., Winkooski, VT). AHMC was measured at excitation/emission wavelengths of 360 nm/ 460 nm. The CYP2D6 assay was run four times in duplicate; statistics were run on all converging data.

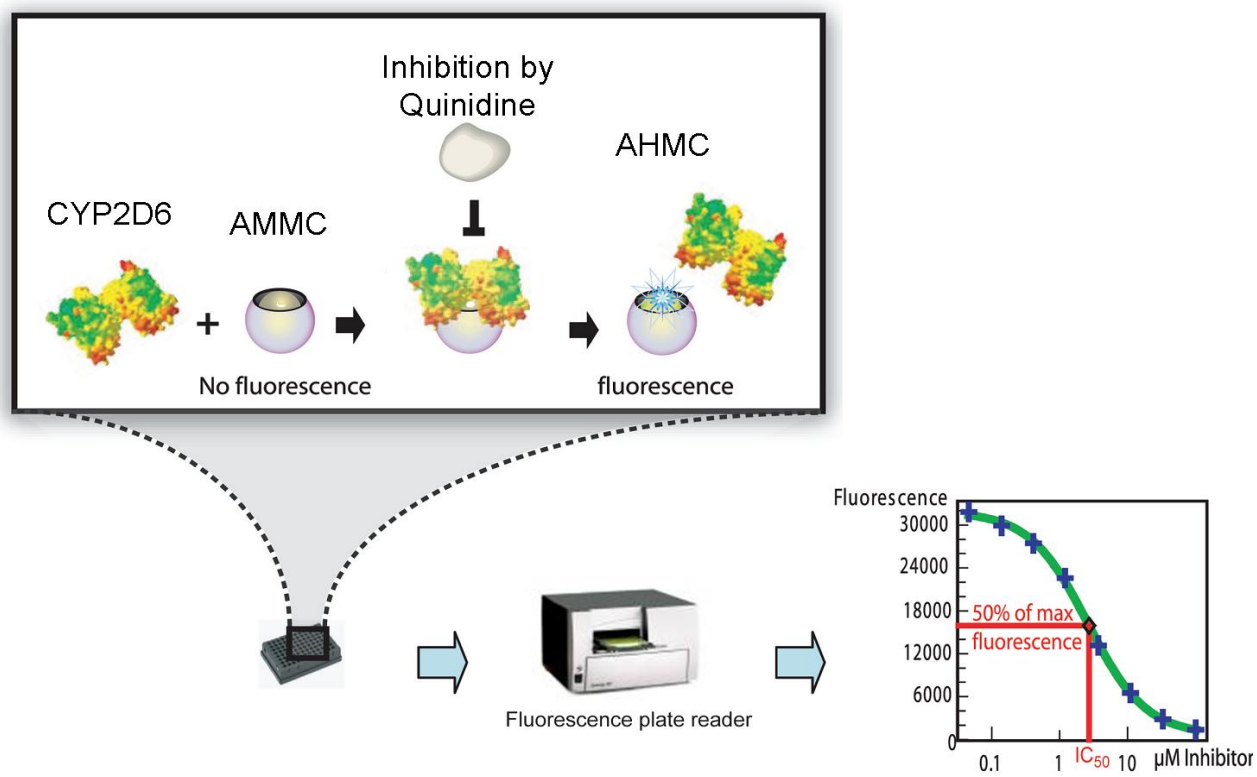

Figure 10. CYP2D6 assay schematic: AMMC is metabolized by recombinant human CYP2D6 to AHMC. IC_{50} values were calculated from the reduced AHMC fluorescence upon addition of increasing concentrations of quinidine or a test compound. IC_{50} is the inhibitor concentration that causes a 50% decrease in enzyme activity. (Adapted from Krippendorff et al., 2007)

Stock solutions (1 mM) of CMT, DEX, MA and MDMA were each prepared in HPLC-grade acetonitrile prior to being serially diluted for CYP2D6 assay. All fluorescent assays were performed in black 96-well reading plates with flat clear bottoms to prevent fluorescence bleed over to adjoining wells and contamination of first readings (BD Falcon™ Assay plates, Franklin Lakes, NJ). Test groups for this assay were: CMT, DEX, MA, MDMA, CMT/ MA, CMT/ MDMA, DEX/ MA, DEX/ MDMA, CMT/ DEX/ MA, and CMT/ DEX/ MDMA.

Prior to initiation of the assay, NAPDH-cofactor mix was prepared by adding 1.5 ml of cofactors, 1.2 ml of glucose 6-phosphate dehydrogenase and 0.8 ml of control

insect cell membrane protein to 117 ml of water (total volume 120 ml). The NAPDH-cofactor mix (144 µl) was pipetted into well 1 (Table 6) of each test compound and quinidine row. Cofactor/ acetonitrile mix was prepared by using 96 ml of the already prepared NAPDH-cofactor mix and adding 4 ml of acetonitrile (total volume 100 ml). The cofactor/ acetonitrile mix (100 µl) was added to the remaining wells 2-12. Six microliters of quinidine (CYP2D6 selective inhibition control inhibitor, 25 µM) or an individual test compound (CMT, DEX, MA, and MDMA) were added to well 1 of each row (150 µl total volume in well 1). Test compounds of two or more drugs were added equally to well 1 (example: CMT/ MA, 3 µl of CMT and 3 µl of MA was added to well 1). Rows 1 and 2 were duplicates and contained quinidine for each plate assayed. Rows 3-8 contained one of the test compounds in duplicate (Table 6). Then each row that contained either a test compound or quinidine was serially diluted (1:3) beginning with well 1 (50 µl) in succession to well 8. In order to have a consistent 100 µl (total volume) in each well, the excess 50 µl from well 8 was discarded. No test compounds or quinidine were added to wells 9 through 12. Wells 9-10 were the AHMC control fluorescence, and wells 11- 12 served as the blanks.

Table 6. Sample 96-well plate

Serial Dilution of test compounds and quinidine (columns 1-8) in duplication									No inhibitor		Blanks	
A	500 nM	167 nM	55.6 nM	18.5 nM	6.17 nM	2.06 nM	0.69 nM	0.23 nM				
B	Well 1	Well 2	Well 3	Well 4	Well 5	Well 6	Well 7	Well 8	Well 9	Well 10	Well 11	Well 12
C	20.0 µM	6.67 µM	2.22 µM	0.74 µM	0.25 µM	0.08 µM	0.27 µM	0.009 µM				
D												
E	10.0 µM	3.33 µM	1.11 µM	0.37 µM	0.12 µM	0.04 µM	0.01 µM	0.004 µM				
F												
G	6.67 µM	2.22 µM	0.74 µM	0.25 µM	0.08 µM	0.27 µM	0.009 µM	0.003 µM				
H												

Rows A and B illustrate concentration values serially diluted ranging from 500 nM to 0.23 nM for quinidine in duplicate. C and D illustrate concentration values serially diluted ranging from 20 µM to 9 nM for tests compounds of one drug (CMT, DEX, MA, or MDMA) in duplicate. E and F illustrate concentration values of each drug serially diluted ranging from 10.0 µM to 4 nM for test compounds with two drugs (CMT/ MA, CMT/ MDMA, DEX/ MA, or DEX/ MDMA) in duplicate. G and H illustrate concentration values of each drug serially diluted ranging from 6.67 µM to 3 nM for test compounds with three drugs (CMT/ DEX/ MA or CMT/ DEX/ MDMA) in duplicate. The dilution factor was 1:3.

The plates were covered with the clear plate lid and pre-incubated for 10 min at 37^{o}C. After preincubation, the plates were removed and 100 µl of the enzyme/ substrate mix was added to wells 1 through 10. The enzyme/ substrate mix was prepared by adding 95 ml of 37^{o}C water, 900 µl of CYP2D6*1 + P450 reductase (enzyme), and 36 µl of 10 mM AMMC (substrate) to the prewarmed buffer solution. Plates were incubated for 30 min at 37^{o}C, and the reaction was stopped by the addition of 75 µl stop reagent solution to all wells (total volume 275 µl). Enzyme/ substrate mix was not added to

wells 11- 12, and fluorescence from these wells (11- 12) constituted background fluorescence. Background values were subtracted from the treatment wells.

III.C. Rats

Male Sprague-Dawley rats (6 months 375-425 g, Harlan Sprague-Dawley Laboratories, Indianapolis, IN) were randomly selected for the process, group housed, and allowed access to food and water ad libitum in a temperature controlled room (23$\pm$ 2^{o}C) and 12-hour cycle for light and dark. All animals experienced the same environmental conditions over the course of the experiment. Twenty rats were needed for this experiment, n= 4 for each group.

III.D. Drug Administration and Treatment

Rats were randomly assigned to one of the 5 treatment groups: The first group was the control group (naive); the second group was treated with the vehicle (VC; 0.9% saline and DMSO); the third group was treated with CMT; fourth group was treated with DEX; the fifth group was treated with CMT and DEX. Each group, except for naive, contained 100 µl/ ml of DMSO to promote solubility, 4% DMSO was injected into each rat. Rats were lightly anesthetized in a carbon dioxide gas chamber. All compounds were administered via intraperitoneal (i.p.) injections. On Day 8 the drug- treated rats were challenged with 5 mg/kg (i.p.) injections of MA. Table 7 summarizes the concentrations and groupings used for each treatment.

After i.p. MA injection (7 h post injection), the rats were lightly anesthetized using carbon dioxide gas, and sacrificed by decapitation. The median and the left lateral

lobes, the largest lobes of the liver, were harvested from each rat and frozen in liquid nitrogen. Samples were stored at -80^{o}C until ready for use.

Table 7. *In vivo* groupings and concentrations for drug treatments

Treatment	Day 1-7							Day 8 5mg/ kg MA i.p.
Naive	-	-	-	-	-	-	-	-
Saline (VC)	x	x	x	x	x	x	x	x
CMT (10.0 mg/kg For 7 days)	x	x	x	x	x	x	x	x
DEX (10.0 mg/kg For 7 days)	x	x	x	x	x	x	x	x
CMT/DEX (10.0 mg/kg For 7 days; 10.0 mg/kg For 7 days)	x	x	x	x	x	x	x	x

Naive group not challenged with MA. Saline, CMT, DEX, CMT/ DEX groups were challenged with MA at the end of the seven day treatment period.

III.E. Microsome preparation

The microsomal fractions were prepared from stored liver tissue with slight modifications as described by Nelson et al., 2001. The frozen (-80°C) rat livers were thawed and minced in homogenizing buffer 2- 4 ml (0.1 M potassium phosphate (pH 7.4) and 0.25 M sucrose), then brought to 30 ml with additional homogenizing buffer. Homogenization was completed with 10 strokes at 900 rpm using a mechanically driven Teflon pestle (GlasCol, Terre Haute, IN) in a glass homogenizer (Wheaton, USA).

Nuclei and mitochondria were removed by centrifugation at 9,000 *g* (7659 rpm) for 20 min in a Beckman Avanti J-25 centrifuge (Fullerton, CA) at 4°C using a JA-14

rotor. The resulting homogenate was then centrifuged at 100,000x *g* (24,140 rpm) for 60 min in a Beckman L7-55 ultracentrifuge (Fullerton, CA) using a Ti-45 rotor. The resulting pellet (containing microsomes) was resuspended in 20 ml of incubation buffer (0.1 M potassium phosphate (pH 7.4), 0.25 M sucrose, 1 mM EDTA, 5% glycerol) and used immediately in the assay, or stored frozen (-80°C) until ready for use. Florence et al. (1982) showed washed microsomes could be stored at -80ºC for up to 30 days without loss of activity. All stored microsomes in the present studies were used prior to the end of the 30 day period.

III.F. Protein Analysis

Commercially available Bio-Rad Protein Assay (Bio-Rad Life Science Group, Richmond, CA) is based on the method of Bradford (1976) and measures the amount of protein in a sample. It involves the addition of an acidic dye (Coomassie® Brilliant Blue G-250 dye) to the solution containing proteins, followed by measurement of absorbance at 595 nm with a microplate reader or spectrophotometer. The measured absorbance at 595 nm is directly proportional to the amount of protein concentration in the sample.

The acidic blue dye (40 µl) was added to each well of the clear 96- well plate (Falcon^{TM}, Franklin, NJ). Microsomal samples (160 µl) were mixed in duplicate with the dye. The plate also contained the standard curve (eight known concentrations of the protein standard), bovine serum albumin (BSA), and a blank sample. Comparison to the standard curve was used to interpolate the protein concentration of the test samples. The plate was incubated for five minutes at room temperature and read by the plate reader.

Based on specific CYP2D2 activity, the calculations for pmol/ mg protein/ min were determined.

III.G. *In vivo* Kinetic Studies

The *in vivo* kinetic studies used the HTS kit from the *in vitro* studies but with more modifications, described below. The HTS assay was used to quantify the CYP2D2 (rat) enzyme activity for each of the drug treatment groups following MA challenge. This quantification was done by measuring the reduction of AMMC to AHMC. Activity of CYP2D2 enzyme can be determined following seven day exposure to CMT, DEX, and CMT/ DEX, or saline using AMMC as a probe. This probe has been shown to be highly selective for rat CYP2D2 as well as the human CYP2D6 isoform (Stresser et al., 2002). The human CYP2D6 enzyme (CYP2D6*1 + P450 reductase) was replaced with microsomal fractions containing CYP2D2 in the assay protocol.

Prior to initiation of the assay, cofactor/ acetonitrile mix was prepared as before and (100 µl) was added to wells 1-7 of the 96-well plate. The enzyme mix for the treatment groups was prepared for each microsomal fraction by adding H_2O, buffer (0.5 M potassium phosphate, pH 7.4, filter sterilized), and enzyme (microsomal fraction), a 79:20:0.75 mix. The enzyme mix for well 1 (blank) was prepared by adding H_2O, buffer, and enzyme (CYP2D6 from the HTS kit), also a 79:20:0.75 mix. The blank well contained no AMMC therefore 100 µl of enzyme mix was added to that well. For the treatment groups, 99.7 µl of enzyme mix and 0.3 µl of AMMC (in varying concentrations 3.29 µM to 0.5 µM) was added (total volume 100 µl) to wells 2-7. Varying AMMC concentrations produced a concentration response curve that determined enzyme kinetics. The plate was incubated for 30 min at 37^{o}C, and the reaction was terminated with the

addition of stop reagent (75 µl). The fluorescence of the blank (well 1) for each test group was subtracted from each treatment well. The fluorescence of the AHMC product was read at excitation/emission wavelengths of 360 nm/ 460 nm. This assay was performed in duplicate with a n= 4.

III.H. Solid Phase Extraction and Gas Chromatography/ Mass Spectrometry

Blood obtained from the jugular, supernatant saved from the microsomal preparation, and tissues from the excised brain were used for Solid Phase Extraction (SPE) and Gas Chromatography/ Mass Spectrometry (GC/ MS) analysis. MA was extracted using Bond Elut certify SPE columns (Varian, Palo Alto, CA) and analyses were done on an Agilent 6890 series GC System (Palo Alto, CA) interfaced with an Agilent 5973 Inert Mass Selective Detector (Palo Alto, CA).

SPE is an accepted method used to extract basic drugs like MA because of its reproducibility, minimized solvent usage, and automation. This method creates short, adsorbent, fast- flow columns that selectively bind drugs from aqueous mixes. It allows carbohydrates, proteins, and polar lipids to pass through and allows the elution of drugs with efficient solvents. SPE uses silica gel for the stationary phase.

MA extractions were prepared by mixing various amounts of blood, supernatant or brain tissue, dependent on amount collected, with 1 ml of 100 mM potassium phosphate buffer (pH 6.0) followed by filtration through a 0.45 µM syringe filter (VWR, Batavia, IL). The column was conditioned using 2 ml of methanol followed by 2 ml of 100 mM potassium phosphate buffer (pH 6.0). The sample was added at a flow rate of 2 ml/ min. The columns were washed with 6 ml of HPLC grade H_2O and 3 ml of 1 M

acetic acid immediately following the addition of the sample without letting the column dry. The column dried under vacuum for 5 min at room temperature, followed by a 6 ml methanol wash. The samples were eluted with 2 ml of methylene chloride/ isopropyl alcohol (80:20) with 2% ammonium hydroxide and evaporated to dryness in a Speed-Vac (Savant, Ramsey, MN) forming a white powder residue. The resulting residue was stored in the -80^{o}C freezer until further analysis.

The residue was reconstituted using ethyl acetate, a common organic solvent used for GC/MS. The GC/ MS was operated in electron impact mode with an ionization voltage 70 eV and all other parameters set at autotune values. A Restek Rxi^{TM}-5ms (Bellefonte, PA) capillary column (30 m X 0.25 mm ID, 0.25 μm film thickness) was used for chromatographic separation. One microliter splitless injections were made, and the purge valve had a split vent flow of 49.8 mL/ min. Inlet and thermal auxiliary temperatures were 250^{o}C and 280^{o}C, respectively. Oven temperature was held at 100^{o}C for 1.00 min, ramped to 300^{o}C at 15^{o}C/ min and held for 2.00 min. The carrier gas was 99.99% pure helium, and the gas line was equipped with moisture, hydrocarbon, and 2 oxygen traps. A column head pressure of 10.5 psi gave a helium flow rate of 1.0 mL/ min. Using this method, MA standards eluted at 4.3 min. The MS detector was operated in scan mode detecting *m/z* 58, 91 and 117 for MA.

III.I. Statistical Analyses

For the *in vitro* CYP2D6 inhibition assay, data was collected as means of fluorescence read at excitation/ emission wavelengths of 360 nm/ 460 nm. To determine the percentage of AHMC fluorescence data was calculated by subtracting blank well

values from the tested wells then the difference was divided by the highest fluorescence value (AHMC fluorescence control, which contained no test compound or quinidine) and multiplied by 100. The inhibitory potency of quinidine and each test compound IC_{50} value, inhibitor concentration that causes a decrease in enzyme activity by 50%, was determined by nonlinear regression and analyzed using one- way analysis of variance (ANOVA) followed by Bonferroni's multiple comparisons correction as appropriate. The maximum inhibition compared to maximum quinidine inhibition was calculated by dividing the lowest fluorescence value by the fluorescence control value (for quinidine and each test compound) and that resulting value (each test compound percent mean) was divided by quinidine percent mean and multiplied by 100. The quinidine percent mean was set at 100.

For the *in vivo* enzyme kinetic assay, data was collected the same as the previous study. Kinetic curve data was calculated by subtracting blank values from the tested wells and analyzed using a nonlinear fit of the data. V_{max}, maximum enzyme velocity, for each treatment group was calculated using values obtained from the kinetic curves, the AHMC standard curve, and the protein assay standard curve. Then data was analyzed using nonlinear regression and one- way ANOVA followed by Bonferroni's multiple comparisons correction as appropriate. The AHMC and protein standard curves were calculated by subtracting the blank values from each test well and analyzed using linear regression of the data. The data from the kinetic curves was transferred to the AHMC standard curve and the values interpolated using linear regression. These values were then divided by the amount of protein determined by the protein assay standard curve and then divided by the incubation time (30 min). The treatment group protein values were

determined by subtracting the blank value from each test well and interpolated those values into the protein standard curve using linear regression of the data. K_m, substrate concentration that leads to half the maximum velocity, was calculated using data obtained from the kinetic curves and analyzed using nonlinear regression and one- way ANOVA followed by Bonferroni's multiple comparisons correction as appropriate.

All statistical analyses of data were performed with Prism v5.0 GraphPAD Software (San Diego, CA) and considered significant if $p \leq 0.05$. Repetitive experiments number (n) is declared in figure captions.

CHAPTER IV

RESULTS

IV.A. *In vitro* CYP2D6 Assay

Determination of inhibitor potency To determine inhibition of CYP2D6 by quinidine and test compounds with concentrations ranging from 0.23 to 2.0 x 10^5 nM, the reduction of AHMC fluorescence was measured. Gentest's CYP2D6/ AMMC high throughput inhibitor screening assay was performed to determine the amount of reduced AHMC which is proportional to the inhibition. The inhibitor potency was calculated as the inhibition concentration that causes a 50% decrease in enzyme activity (IC_{50}). The IC_{50} values for quinidine and each test compound (CMT, DEX, MA, MDMA, CMT/ MA, CMT/ MDMA, DEX/ MA, DEX/ MDMA, CMT/ DEX/ MA, and CMT/ DEX/ MDMA) are listed in Table 8 and graphically represented in Figures 11- 12 expressed as mean $\pm$ S.E.M. The test compounds' IC_{50} values were compared to the quinidine IC_{50} value (IC_{50} value of 3.8 nM), a known potent inhibitor of CYP2D6, and one- way ANOVA reported no significant differences, $p < 0.05$. This may be because the potency for each of the drugs is similar. Crespi et al. (1997) reported an IC_{50} value for quinidine of 8.9 nM from similar high throughput studies, and Gentest, the manufacturer, reports a value of 11 nM for quinidine. Therefore the assay was performed within the manufacturer's specifications. The inhibition curves of quinidine and each test compound are

graphically represented in Figures 13-17. Quinidine, CMT, MA, MDMA, CMT/ MDMA, DEX/ MA, DEX/ MDMA, CMT/ DEX/ MA and CMT/ DEX/ MDMA were expressed as n=4 in duplicate since there were four experiment runs with statistical and graphical data. DEX and CMT/ MA were expressed as n=3 in duplicate since one experiment run did not provide statistical or graphical data.

Table 8. IC_{50} values for quinidine and each of the treatment groups' concentrations.

Treatment	Quinidine	CMT	DEX	MA	MDMA	CMT/ MA	CMT/ MDMA	DEX/ MA	DEX/ MDMA	CMT/ DEX/ MA	CMT/ DEX/ MDMA
IC_{50} (mean) nM	3.8	1139	25.2	1.2	36.3	994.3	350.6	24.7	47.9	127.0	77.7
S.E.M	1.33	1137	25.1	0.4	19.5	993.3	186.4	24.6	26.3	118.5	46.4
Conc. Range nM	0.23- 5×10^2	9.0- 2×10^4	9.0- 2×10^4	9.0- 2×10^4	9.0- 2×10^4	4.0- 1×10^4	4.0- 1×10^4	4.0- 1×10^4	4.0- 1×10^4	3.0- 6.67×10^3	3.0- 6.67×10^3

Each test compound and quinidine was assayed to determine the inhibition of the enzyme for that compound. Increasing concentrations (3 nM to 20 μM) of quinidine and each test compound were incubated with CYP2D6 supersomes for 30 min at 37ºC. The reduction of AHMC fluorescence was measured and the IC_{50} value, inhibitor concentration that causes a decrease in enzyme activity by 50%, was compared to known IC_{50} values. The IC_{50} value was determined by nonlinear regression and analyzed using one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparisons correction as appropriate. Each treatment group and quinidine was performed in duplicate and n= 3- 4.

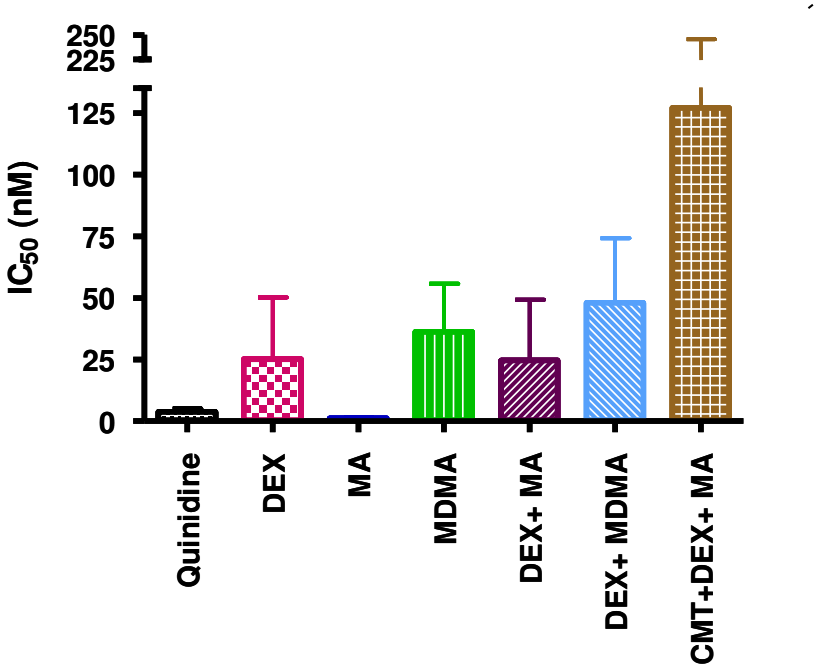

Figure 11. Each test compound and quinidine was assayed to determine the inhibition of the enzyme for that compound. Increasing concentrations (3 nM to 20 μM) of quinidine and each test compound were incubated with CYP2D6 supersomes for 30 min at 37ºC. The reduction of AHMC fluorescence was measured and the IC_{50} value, inhibitor concentration that causes a decrease in enzyme activity by 50%, was compared to known IC_{50} values. The IC_{50} value was determined by nonlinear regression and analyzed using one- way analysis of variance (ANOVA) followed by Bonferroni's multiple comparisons correction as appropriate. Results are expressed as mean $\pm$ S.E.M., *n*= 3- 4 in duplicate.

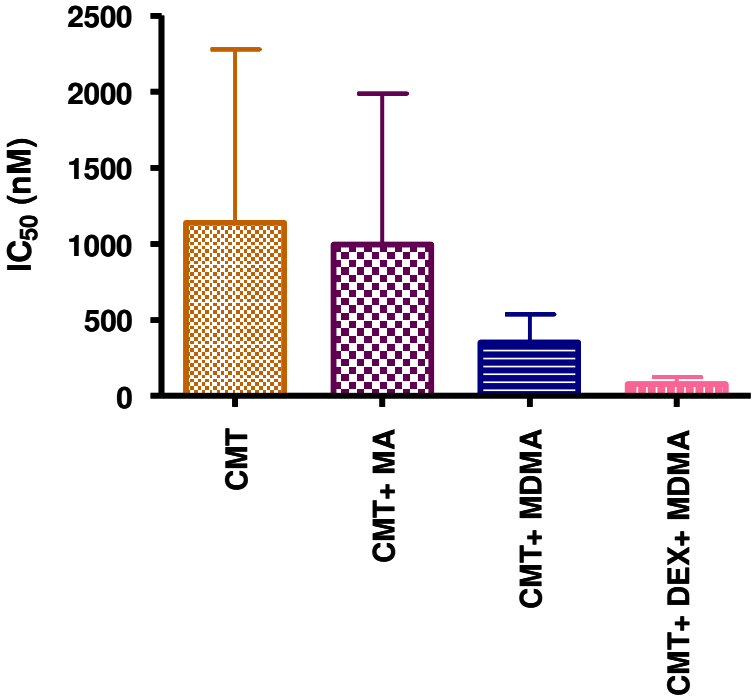

Figure 12. Each test compound and quinidine was assayed to determine the inhibition of the enzyme for that compound. Increasing concentrations (3 nM to 20 µM) of quinidine and each test compound were incubated with CYP2D6 supersomes for 30 min at 37ºC. The reduction of AHMC fluorescence was measured and the IC_{50} value, inhibitor concentration that causes a decrease in enzyme activity by 50%, was compared to known IC_{50} values. The IC_{50} value was determined by nonlinear regression and analyzed using one- way analysis of variance (ANOVA) followed by Bonferroni's multiple comparisons correction as appropriate. Results are expressed as mean $\pm$ S.E.M., *n*= 3- 4 in duplicate.

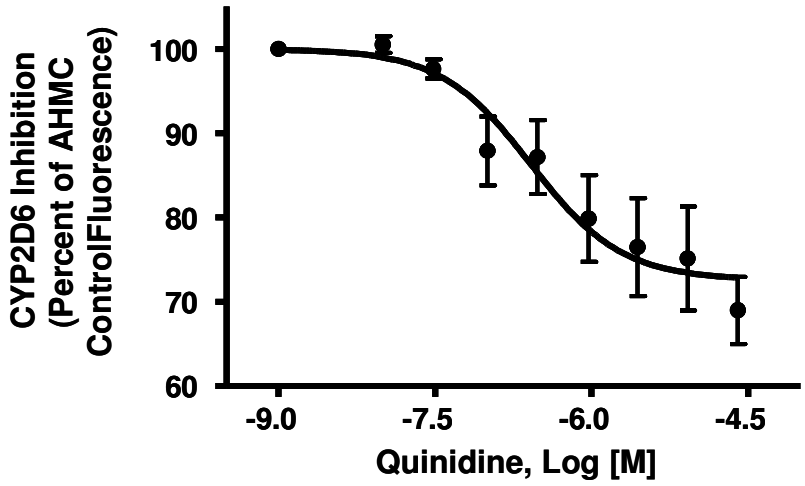

Figure 13. Quinidine percent inhibition of CYP2D6 activity. Increasing concentrations (0.23 to 5.0 x 10^2 nM) of quinidine were incubated with CYP2D6 supersomes for 30 min at 37ºC. The reduction of AHMC fluorescence was measured and the IC_{50} value was compared to known IC_{50} values. The calculated IC_{50} value for quinidine is 3.8 $\pm$ 1.3 nM. The percent of AHMC fluorescence was calculated by subtracting blank well values from the tested wells, this difference was then divided by the highest fluorescence value (AHMC fluorescence control, contained no test compound or quinidine) and multiplied by 100. Data expressed as total inhibition mean $\pm$ S.E.M., n= 4 in duplicate.

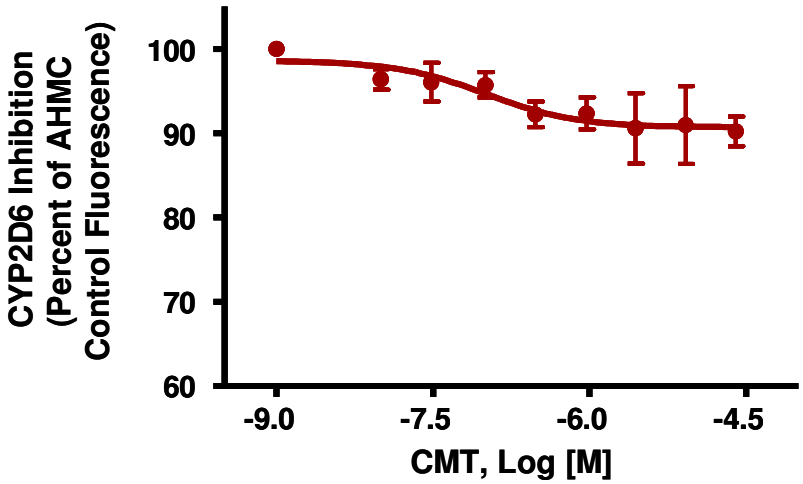

Figure 14. CMT percent inhibition of CYP2D6 activity. Increasing concentrations (9.0- 2.0 x 10^4 nM) of CMT were incubated with CYP2D6 supersomes for 30 min at 37ºC. The reduction of AHMC fluorescence was measured and the IC_{50} value was compared to the quinidine IC_{50} value. The calculated IC_{50} value for CMT is 1139 $\pm$ 1137 nM. The percent of AHMC fluorescence was calculated by subtracting blank well values from the tested wells, this difference was then divided by the highest fluorescence value (AHMC fluorescence control, contained no test compound or quinidine) and multiplied by 100. Data expressed as total inhibition mean $\pm$ S.E.M., n= 4 in duplicate.

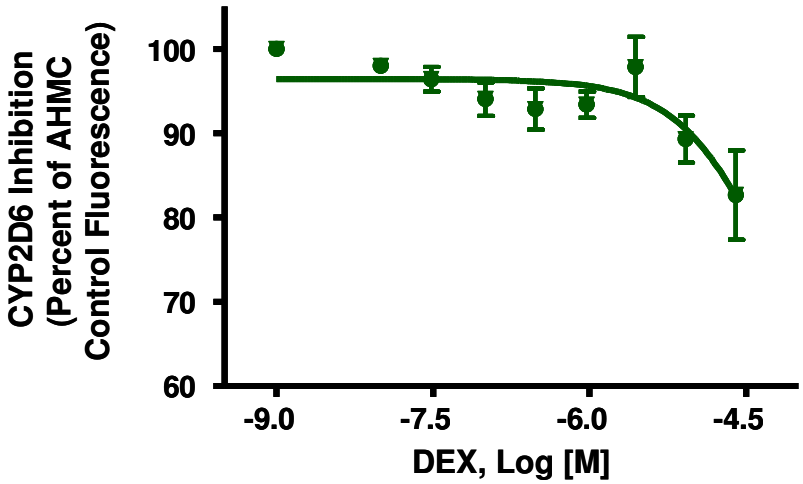

Figure 15. Representative DEX percent inhibition of CYP2D6 activity. Increasing concentrations (9.0- 2.0 x 10^4 nM) of DEX were incubated with CYP2D6 supersomes for 30 min at 37ºC. The reduction of AHMC fluorescence was measured and the IC_{50} value was compared to the quinidine IC_{50} value. The calculated IC_{50} value for DEX is 25.2 $\pm$ 25.1 nM. The percent of AHMC fluorescence was calculated by subtracting blank well values from the tested wells, this difference was then divided by the highest fluorescence value (AHMC fluorescence control, contained no test compound or quinidine) and multiplied by 100. Data expressed as total inhibition mean $\pm$ S.E.M., *n*= 3 in duplicate.

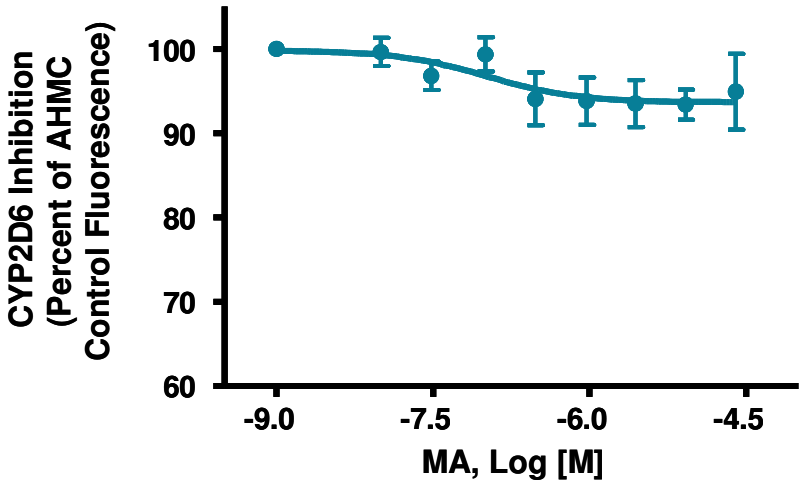

Figure 16. MA percent inhibition of CYP2D6 activity. Increasing concentrations (9.0- 2.0 x 10^4 nM) of MA were incubated with CYP2D6 supersomes for 30 min at 37ºC. The reduction of AHMC fluorescence was measured and the IC_{50} value was compared to the quinidine IC_{50} value. The calculated IC_{50} value for MA is 1.2 $\pm$ 4.0 nM. The percent of AHMC fluorescence was calculated by subtracting blank well values from the tested wells, this difference was then divided by the highest fluorescence value (AHMC fluorescence control, contained no test compound or quinidine) and multiplied by 100. Data expressed as total inhibition mean $\pm$ S.E.M., n= 4 in duplicate.

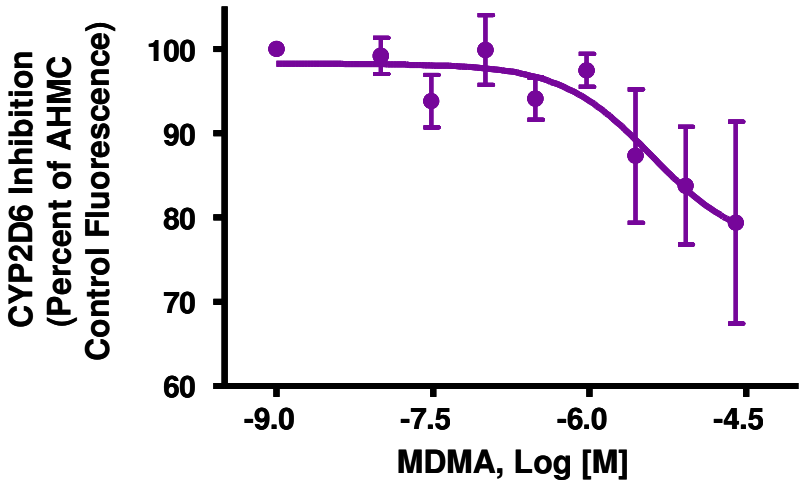

Figure 17. MDMA percent inhibition of CYP2D6 activity. Increasing concentrations (9.0- 2.0 x 10^4 nM) of MDMA were incubated with CYP2D6 supersomes for 30 min at 37ºC. The reduction of AHMC fluorescence was measured and the IC_{50} value was compared to the quinidine IC_{50} value. The calculated IC_{50} value for MDMA is 36.3 $\pm$ 19.5 nM. The percent of AHMC fluorescence was calculated by subtracting blank well values from the tested wells, this difference was then divided by the highest fluorescence value (AHMC fluorescence control, contained no test compound or quinidine) and multiplied by 100. Data expressed as total inhibition mean $\pm$ S.E.M., *n*= 4 in duplicate.

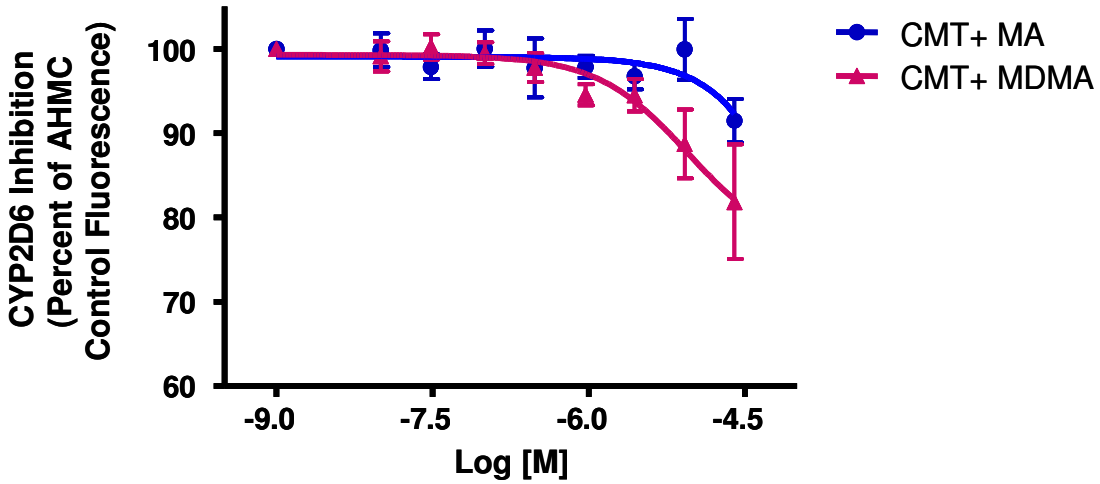

Figure 18. Representative percent inhibition of CYP2D6 activity for CMT combined with MA or MDMA. Increasing concentrations (4.0- 1.0 x 10^4 nM) of each test compound (CMT/ MA and CMT/ MDMA) were incubated with CYP2D6 supersomes for 30 min at 37ºC. The reduction of AHMC fluorescence was measured and the IC_{50} values were compared to the quinidine IC_{50} value. The calculated IC_{50} value for CMT/ MA is 994 $\pm$ 993 nM. The IC_{50} value for CMT/ MDMA is 351 $\pm$ 186 nM. The percent of AHMC fluorescence was calculated by subtracting blank well values from the tested wells, this difference was then divided by the highest fluorescence value (AHMC fluorescence control, contained no test compound or quinidine) and multiplied by 100. Data expressed as total inhibition mean $\pm$ S.E.M., *n*= 3 in duplicate.

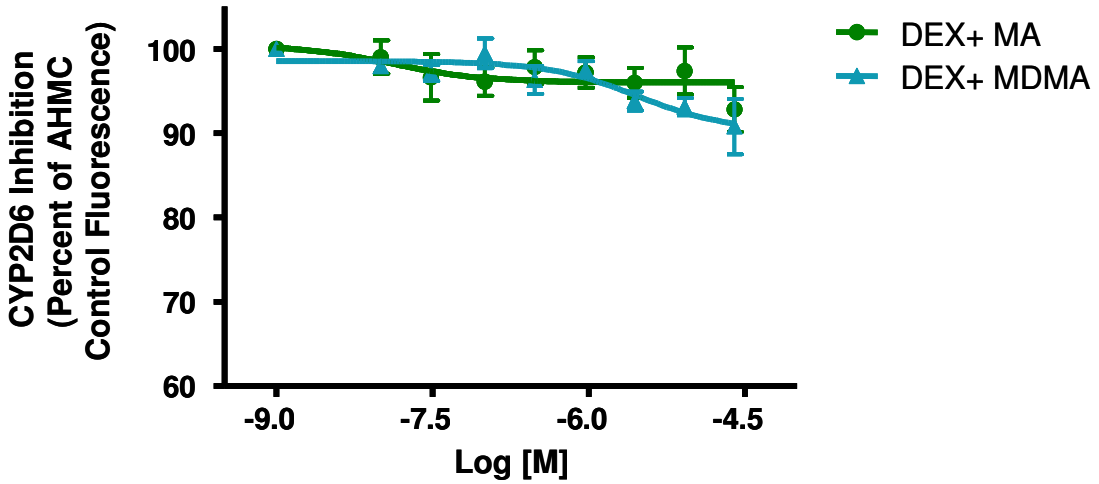

Figure 19. Percent inhibition of CYP2D6 activity for DEX combined with MA or MDMA. Increasing concentrations (4.0- 1.0 x 10^4 nM) of each test compound (DEX/ MA and DEX/ MDMA) were incubated with CYP2D6 supersomes for 30 min at 37ºC. The reduction of AHMC fluorescence was measured and the IC_{50} values were compared to the quinidine IC_{50} value. The calculated IC_{50} value for DEX/ MA is 24.7 $\pm$ 24.6 nM. The IC_{50} value for DEX/ MDMA is 47.9 $\pm$ 26.3 nM. The percent of AHMC fluorescence was calculated by subtracting blank well values from the tested wells, this difference was then divided by the highest fluorescence value (AHMC fluorescence control, contained no test compound or quinidine) and multiplied by 100. Data expressed as total inhibition mean $\pm$ S.E.M., n= 4 in duplicate.

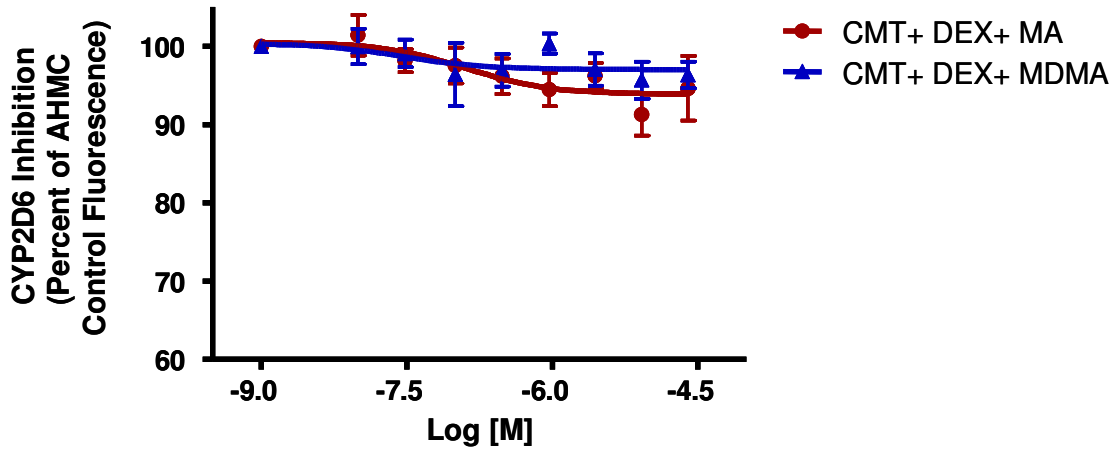

Figure 20. Percent inhibition of the effects of CMT/ DEX combined with MA and CMT/ DEX combined with MDMA on CYP2D6 activity. Increasing concentrations (3.0 to 6.67 x 10^3 nM) of each test compound (CMT/ DEX and MA or CMT/ DEX and MDMA) were incubated with CYP2D6 supersomes for 30 min at 37ºC. The reduction of AHMC fluorescence was measured and the IC_{50} values were compared to the quinidine IC_{50} value. The calculated IC_{50} value for CMT/ DEX and MA is 1270 $\pm$ 1185 nM. The IC_{50} value for CMT/ DEX and MDMA is 77.8 $\pm$ 46.4 nM. The percent of AHMC fluorescence was calculated by subtracting blank well values from the tested wells, this difference was then divided by the highest fluorescence value (AHMC fluorescence control, contained no test compound or quinidine) and multiplied by 100. Data expressed as total inhibition mean $\pm$ S.E.M., n= 4 in duplicate.

Determination of maximum inhibition compared to quinidine To compare the test compounds to maximum quinidine inhibition the data obtained from the CYP2D6 assay was used, and quinidine inhibition was set at 100 thus the test compounds became a percentage of it. Maximum inhibition of CYP2D6 activity in the presence of test compounds' [CMT, CMT/ MA, DEX/ MA, CMT/ DEX/ MA, and CMT/ DEX/ MDMA ($F_{10,\ 43}$= 4.976, p<0.05)] compared to maximum quinidine inhibition decreased significantly from quinidine and CMT/ MDMA inhibition (Figure 21). Maximum MA ($F_{10,\ 43}$= 4.976, p<0.05) inhibition significantly decreased compared to maximum quinidine inhibition (Figure 21). The test compounds maximum inhibition [CMT, MA,

CMT/ MA, DEX/ MA, CMT/ DEX/ MA, and CMT/ DEX/ MDMA] showed a 75-85% decrease compared to maximum quinidine inhibition. This suggests that the test groups inhibited CYP2D6 significantly less than quinidine. This may be because the efficacy for each of the drugs is significantly different from each other.

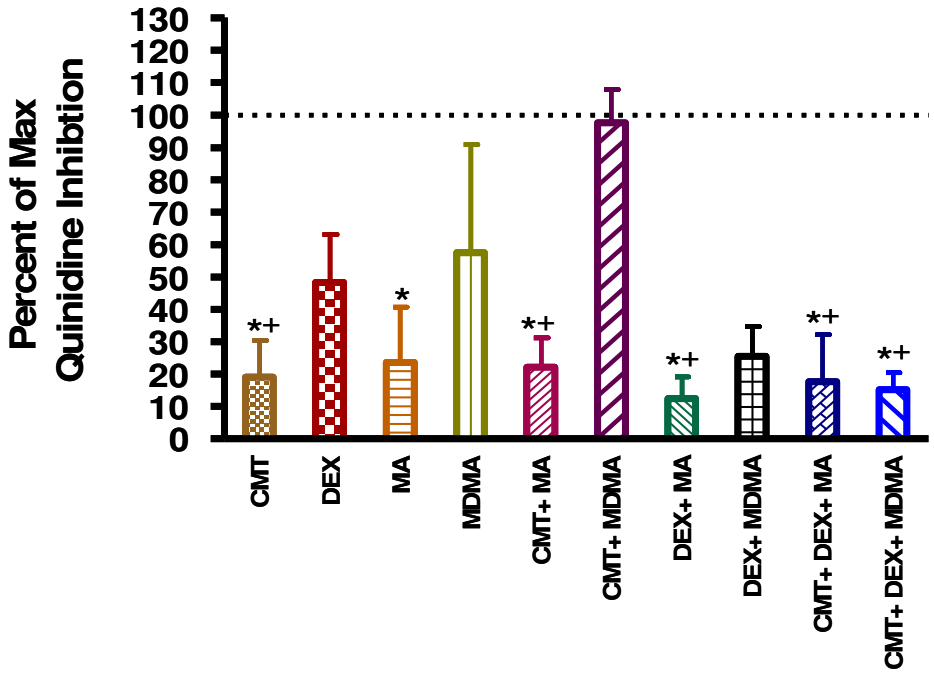

Figure 21. Maximum inhibition compared to quinidine was determined from data obtained from the CYP2D6 assay. Quinidine inhibition percentage was set at 100 thus the test compounds became a percentage of it. The dotted line represents quinidine. Maximum CMT, CMT/ MA, DEX/ MA, CMT/ DEX/ MA and CMT/ DEX/ MDMA inhibition compared to maximum quinidine inhibition decreased significantly from quinidine and CMT/ MDMA inhibition. Maximum MA inhibition decreased significantly compared to maximum quinidine inhibition. Results are expressed as a percentage of the maximum quinidine inhibition of the mean $\pm$ S.E.M. (n= 4 in duplicate for quinidine and all test compounds) *p< 0.05 when compared to quinidine, ^{+}p< 0.05 when treatments compared to CMT/ MDMA.

IV. B. *In Vivo* Assay

Determination of enzyme velocity To determine V_{max}, the maximum enzyme velocity, values obtained from the kinetic curves (Figures 23-28), the AHMC standard curve (Figure 29), and the protein assay standard curve were used (Figure 30). The V_{max} value in the CMT treated group increased significantly from naive ($F_{4, 15}$= 4.344: p< 0.05). However, all MA treated groups showed very high increases (280- 490%) from naive. The V_{max} values for all experimental groups are listed in Table 9 and Figure 22 illustrates the graphical representation. Saline, DEX, and CMT/ DEX were expressed as n= 4 in duplicate since there were four experimental runs with statistical and graphical data. Naive and CMT graphs (Figures 23, 24, and 26) are expressed as n= 5 but calculations are expressed as n= 4 in duplicate since one run provided graphically data but no statistical data.

Table 9. V_{max} values for each treatment groups.

Treatment	Naive	Saline	CMT	DEX	CMT/ DEX
V_{max} (mean) pmol/ mg protein/ min	19.92	65.86	98.28	70.46	56.38
S.E.M.	5.084	12.02	22.09	15.06	6.096

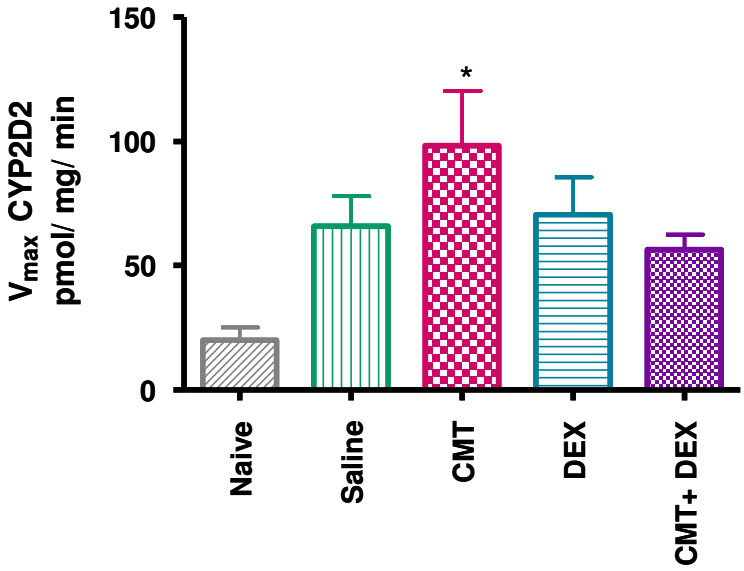

Figure 22. V_{max} values were determined from the data obtained from the kinetic curves, the AHMC standard curve, and the protein assay standard curve. Then data was analyzed using nonlinear regression and one- way ANOVA followed by Bonferroni's multiple comparisons correction as appropriate. Data from the kinetic curves was transferred to the AHMC standard curve and the values interpolated using linear regression, this determined the pmol values. These values were then divided by the amount of protein determined by the protein assay standard curve and then divided by the incubation time (30 min). The treatment group protein concentrations (mg) were determined by interpolating the values into the protein standard curve using linear regression of the data. CMT increased significantly compared to naive. Results are expressed as mean $\pm$ S.E.M., n= 4 in duplicate for all treatments groups, *p< 0.05 when compared to naive.

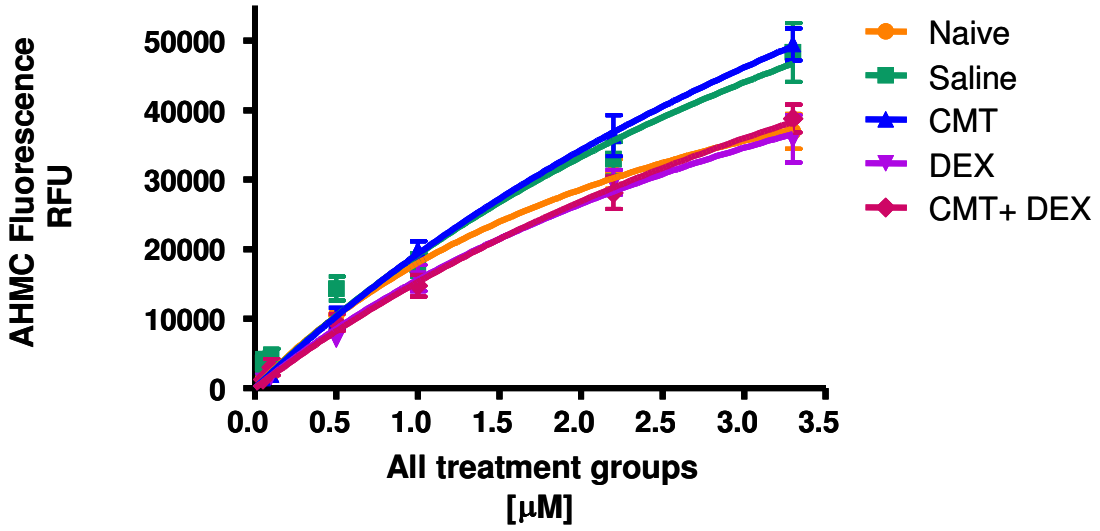

Figure 23. Kinetic analysis of CYP2D2 activity in rat microsomes. Increasing concentrations (0.05 to 3.29 μM) of AMMC were incubated with CYP2D2 from each treatment group for 30 min at 37°C. Reduction of AMMC to AHMC fluorescence was measured, K_m values obtained, and V_{max} values determined with calculations. Data expressed as mean ± S.E.M., *n*= 4 in duplicate for saline, DEX, and CMT/DEX and *n*= 5 in duplicate for naive and CMT.

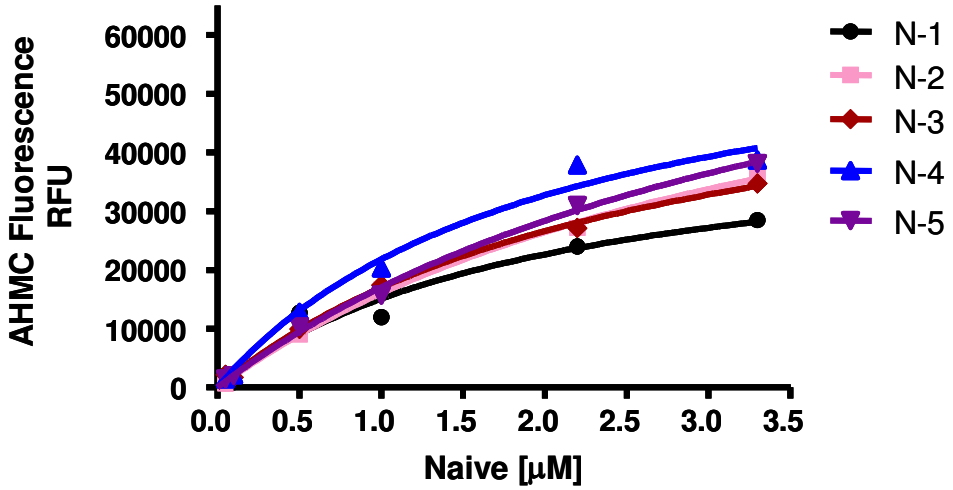

Figure 24. Naive kinetic analysis of CYP2D2 activity in rat microsomes. Increasing concentrations (0.05 to 3.29 μM) of AMMC were incubated with CYP2D2 from the naive for 30 min at 37°C. Reduction of AMMC to AHMC fluorescence was measured, K_m values obtained, and V_{max} values determined with calculations. Calculated V_{max} and K_m values for the naive group, 19.92 ± 5.084 pmol/ mg protein/ min and 3.081 ± 0.459 μM, respectively with a R^2 value of 0.9436. Data expressed as mean, *n*= 5 in duplicate for graphing purposes and *n*= 4 in duplicate for calculations.

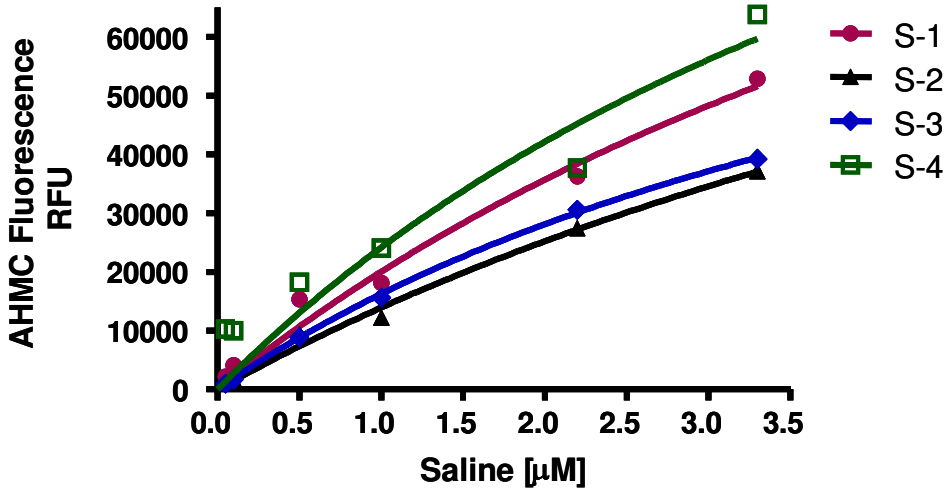

Figure 25. Saline kinetic analysis of CYP2D2 activity in rat microsomes. Increasing concentrations (0.05 to 3.29 µM) of AMMC were incubated with CYP2D2 containing saline for 30 min at 37°C. Reduction of AMMC to AHMC fluorescence was measured, K_m values obtained, and V_{max} values determined with calculations. Calculated V_{max} and K_m values for the saline group, 65.86 $\pm$ 12.02 pmol/ mg protein/ min and 6.806 $\pm$ 0.733 µM, respectively with a R^2 value of 0.9439. Data expressed as mean, *n*= 4 in duplicate.

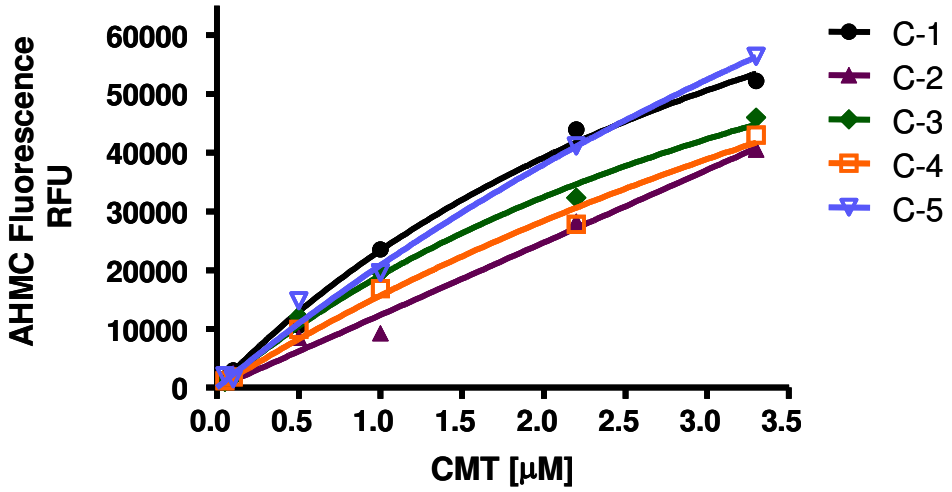

Figure 26. CMT kinetic analysis of CYP2D2 activity in rat microsomes. Increasing concentrations (0.05 to 3.29 µM) of AMMC were incubated with CYP2D2 containing CMT for 30 min at 37°C. Reduction of AMMC to AHMC fluorescence was measured, K_m values obtained, and V_{max} values determined with calculations. Calculated V_{max} and K_m values for the CMT group, 98.28 $\pm$ 22.09 pmol/ mg protein/ min and 6.728 $\pm$ 1.341 µM, respectively with a R^2 value of 0.9757. Data expressed as mean, *n*= 5 in duplicate for graphing purposes and *n*= 4 in duplicate for calculations.

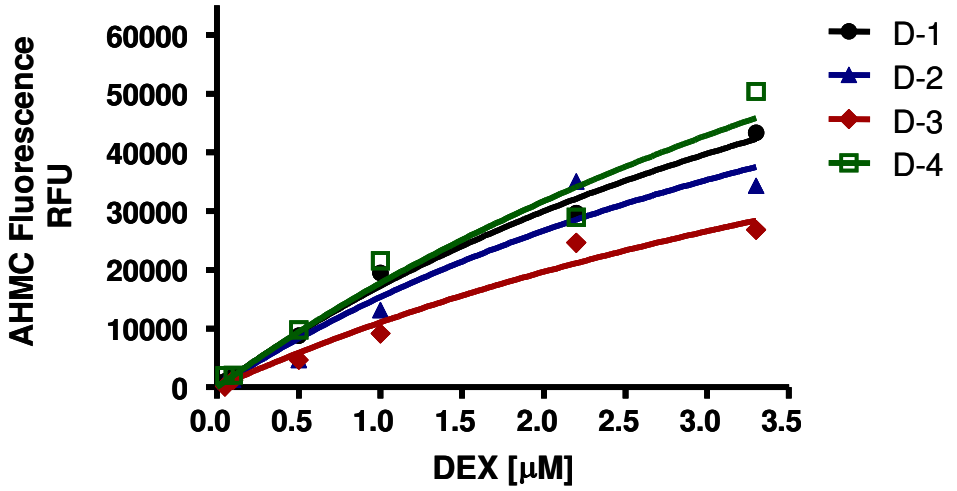

Figure 27. DEX kinetic analysis of CYP2D2 activity in rat microsomes. Increasing concentrations (0.05 to 3.29 µM) of AMMC were incubated with CYP2D2 containing DEX for 30 min at 37°C. Reduction of AMMC to AHMC fluorescence was measured, K_m values obtained, and V_{max} values determined with calculations. Calculated V_{max} and K_m values for the DEX group 70.46 $\pm$ 15.05 pmol/ mg protein/ min and 6.397 $\pm$ 0.465 µM, respectively with a R^2 value of 0.9443. Data expressed as mean, *n*= 4 in duplicate.

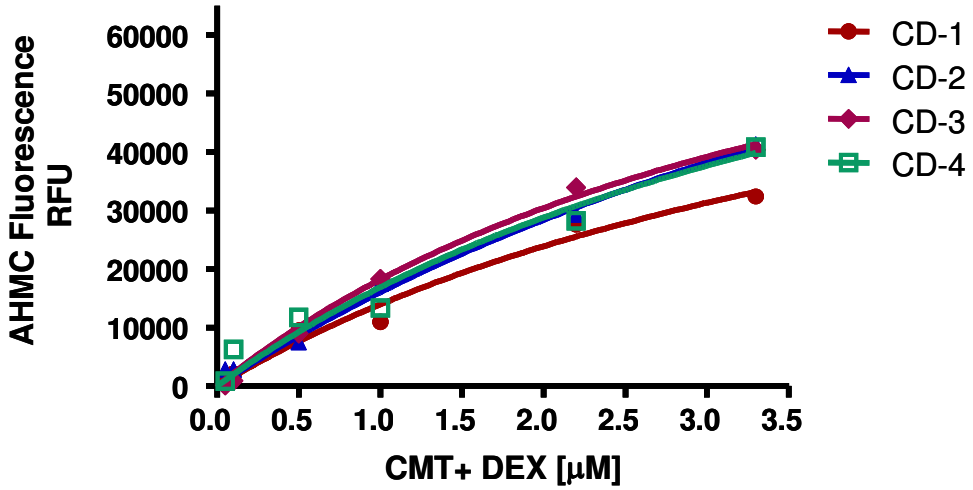

Figure 28. CMT/ DEX kinetic analysis of CYP2D2 activity in rat microsomes. Increasing concentrations (0.05 to 3.29 µM) of AMMC were incubated with CYP2D2 containing CMT/ DEX for 30 min at 37°C. Reduction of AMMC to AHMC fluorescence was measured, K_m values obtained, and V_{max} values determined with calculations. Calculated V_{max} and K_m values for the CMT/ DEX group 56.38 $\pm$ 6.096 pmol/ mg protein/ min and 5.087 $\pm$ 0.547 µM, respectively with a R^2 value of 0.9492. Data expressed as mean, *n*= 4 in duplicate.

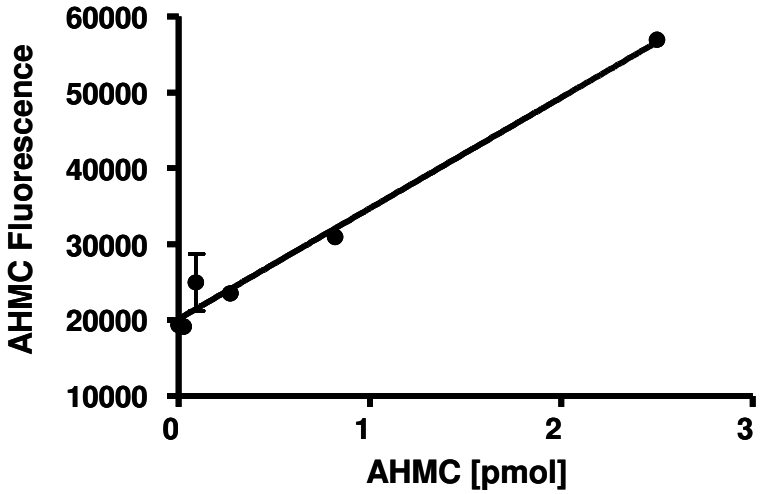

Figure 29. Standard curve of AHMC used to interpolate V_{max} values. Increasing concentrations (0.03 to 2.5 pmol) of AHMC fluorescence was measured and the blank values subtracted from each test well. The data was analyzed using linear regression. The line has a R^2 value of 0.9690.

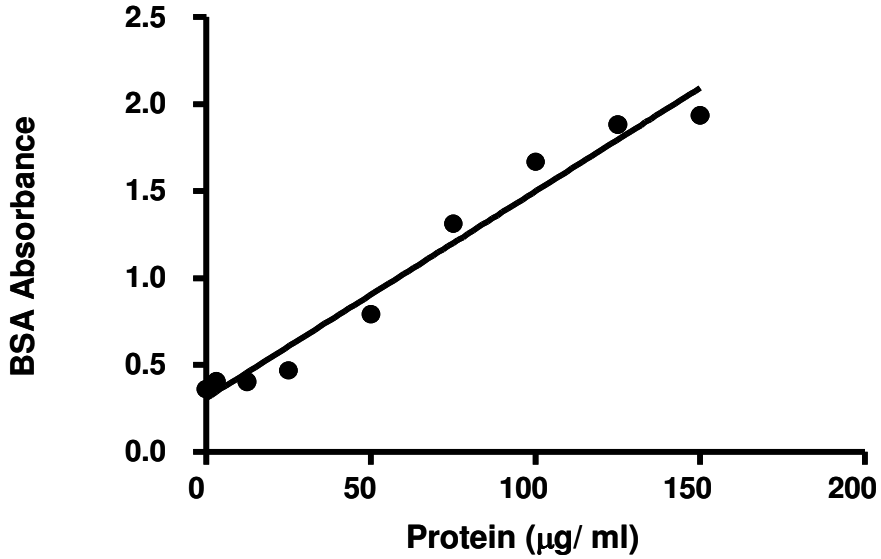

Figure 30. Standard curve for the Bio-Rad Protein assay used to interpolate V_{max} values. Increasing concentrations (3.125 to 150.00 μg/ ml) of bovine serum albumin (BSA) absorbance at 595 nm was measured. The data was analyzed using linear regression. The line has a R^2 value of 0.9562.

Determination of K_m To calculate K_m, the substrate concentration that leads to half of the maximum velocity, data obtained from the kinetic curves and the nonlinear analysis were used (Figures 23-28). The K_m value in the CMT and saline treated groups increased significantly compared to the naive group ($F_{4, 15}$= 4.071, p<0.05, Figure 31). Comparison within the treatment groups reported no significant differences compared to naive. All the MA treated groups showed very high increases (165- 220%) from naive. This suggests that MA administration resulted in an increase in K_m by 2 fold. This means that 2x the substrate amount is needed to reach a ½ max response. The K_m values are listed in Table 10. All calculations are expressed as n= 4 in duplicate.

Table 10. K_m values for each treatment groups.

Treatment	Naive	Saline	CMT	DEX	CMT/ DEX
K_m (mean) µM	3.081	6.806	6.728	6.397	5.087
S.E.M.	0.459	0.733	1.341	0.465	0.547

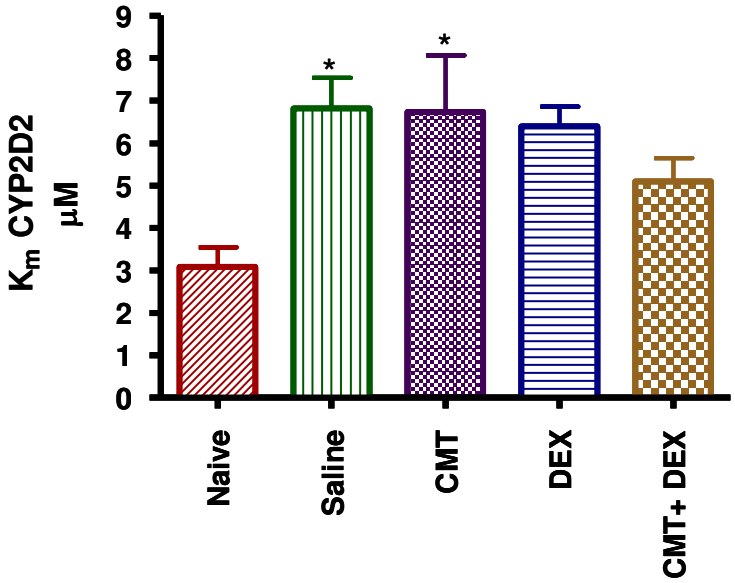

Figure 31. K_m values were determined from kinetic analysis of each treatment group. Then data was analyzed using nonlinear regression and one- way ANOVA followed by Bonferroni's multiple comparisons correction as appropriate. CMT and saline were significantly different from the naive group. Results are expressed as mean ± S.E.M., *n*= 4 in duplicate for all treatment groups, **p*< 0.05, when compared to the naive group.

IV. C. Gas Chromatography and Mass Spectrometry

Determination of MA levels *in vivo* To detect MA, source samples of blood obtained from the jugular, supernatant saved from the microsomal preparation, and brain tissue were used. MA was extracted using SPE, reconstituted in ethyl acetate, and then injected onto the GC/ MS. MA standards eluted at approximately 4.3 min (Figure 32), and MA ions were detected at 58, 91, and 119 m/z (Figure 33). Our limit of detection (LOD) was 1271.48 ± 191.69 ng, and the limit of linearity (LOL) was 5.0 x 10^5 ng. The limits were calculated from the standard curve of MA (Figure 34) and the baseline. However, none of the samples showed a MA peak. This problem was most likely due to MA in the samples below the LOD; and since MA distributes throughout the body in rats

with a V_d range of 1.26 to 9.0L/ kg (Figure 35) (Kitaichi et al., 2004; Riviere et al., 1999). To achieve levels that are above the LOD the rats should have been sacrificed at the half-life for rats $t_{1/2}$= 1 hr (Milesi- Halle et al., 2005) not the half-life for humans $t_{1/2}$= 7- 10 hrs (Logan, 2002). The percentage of MA remaining after 7 hrs was 0.78 %.

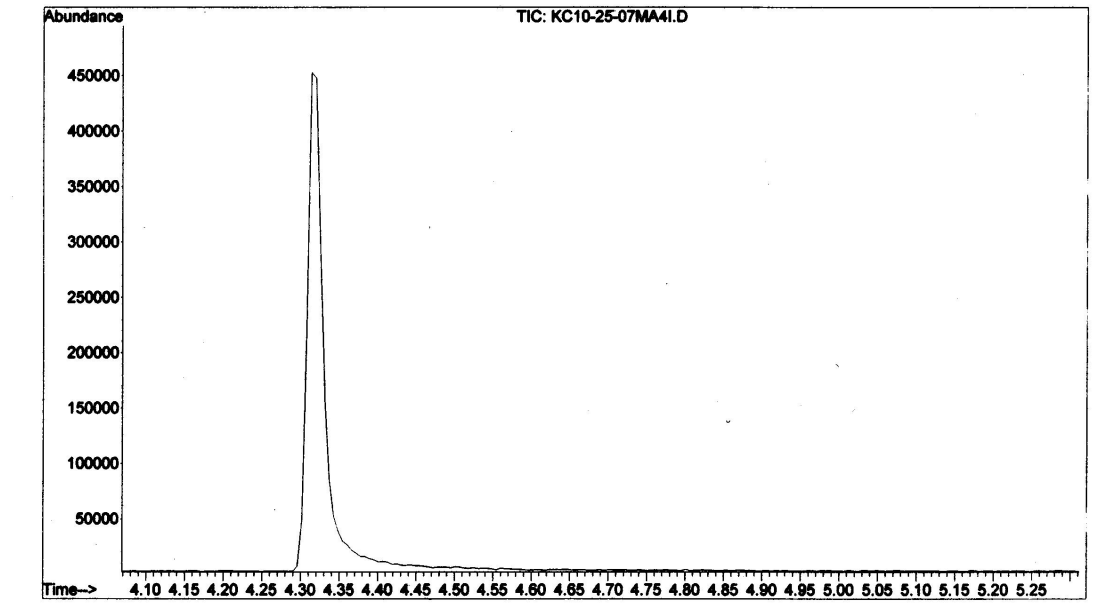

Figure 32. Representative standard MA chromatogram. MA eluted at 4.3 min with an abundance of 450,000. This correlates to 125,000 ng in the MA standard. The GC system was an Agilent 6890 series interfaced with an Agilent 5973 inert mass selective detector. The column used was a Restek Rxi^{TM}- 5ms capillary column (30m X 0.25 mm i.d., 0.25 µm film thickness). The sample was injected in the splitless mode and the oven temperature was set at 100^{o}C for 1.00 min, ramped to 300^{o}C at 15^{o}C/ min & held for 2.00 min.

Library Searched : C:\Database\NIST129K.L
Quality : 86
ID : Methamphetamine Hydrochloride

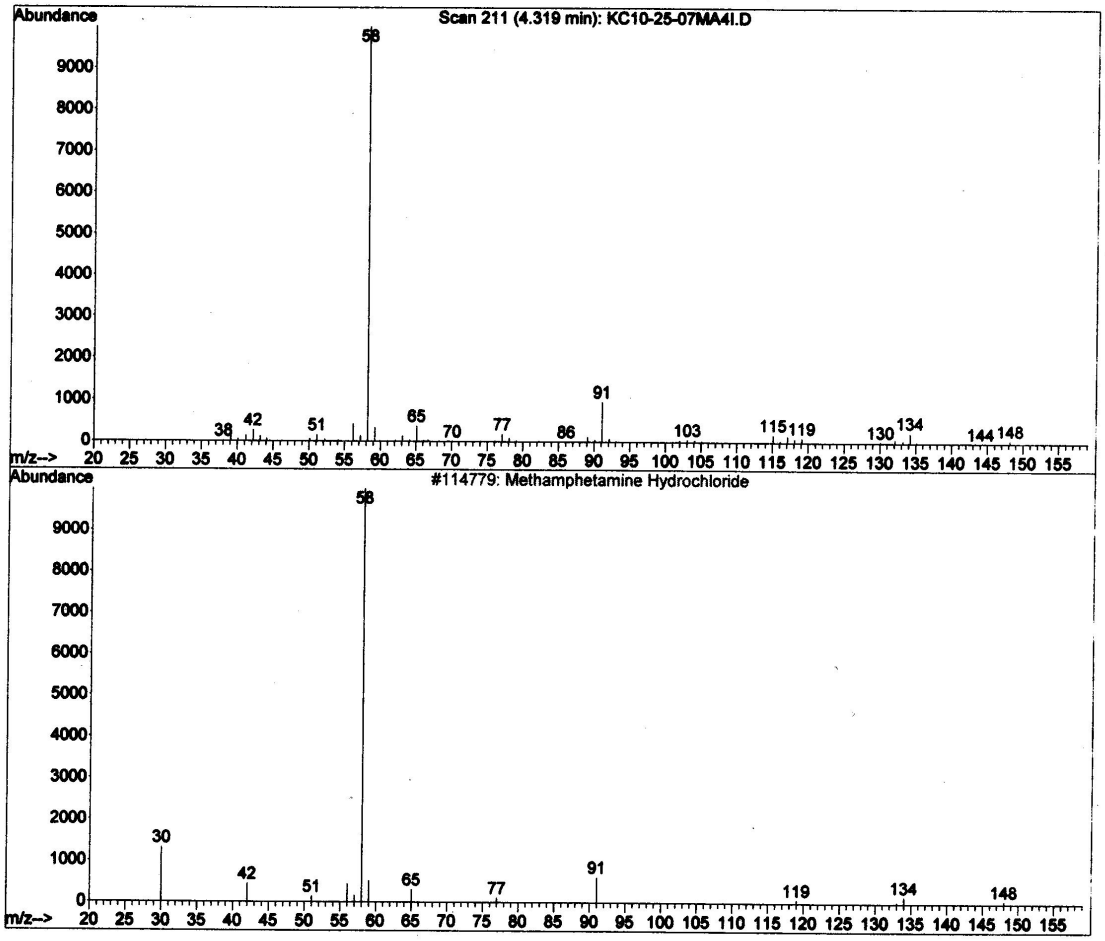

Figure 33. Representative MA spectrum (top) and corresponding library match spectrum (bottom). The top spectrum shows MA ions detected at 58, 91 and 119 m/z from the MA standard. This matches the library match spectrum used to identify the compound.

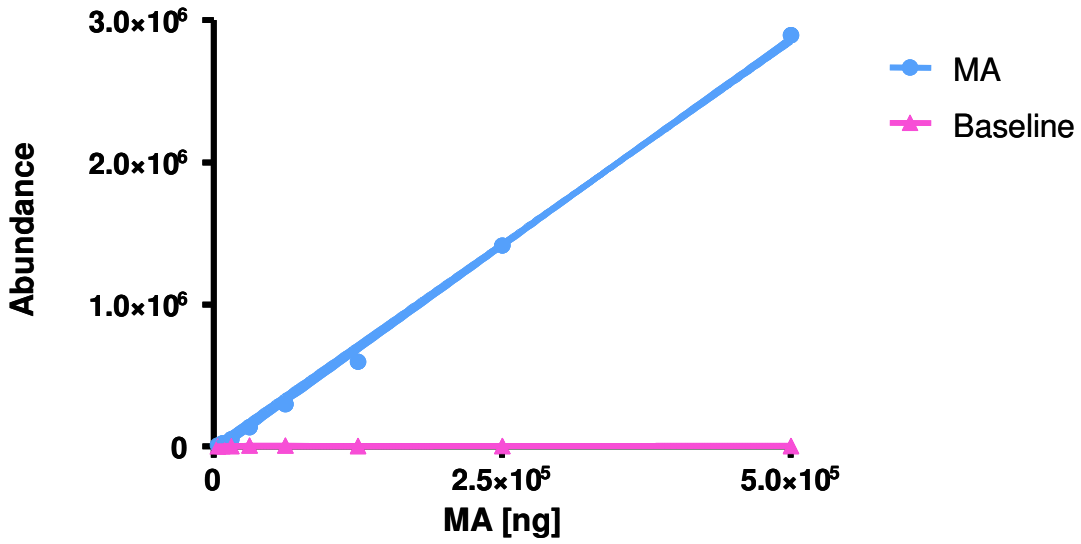

Figure 34. Standard curve of MA used to determine LOD and LOL. Increasing concentrations (3906.25 to 500,000 ng) of MA were measured. The data was analyzed using linear regression. The MA standard line has a slope value of 5.702 ± 0.0998 ng/ abundance. The baseline has a slope of 0.0117 ± 0.0053 ng/ abundance. The LOD value was determined to be 1271.48 ± 191.69 ng, and the LOL was 5.0 x 10^5 ng.

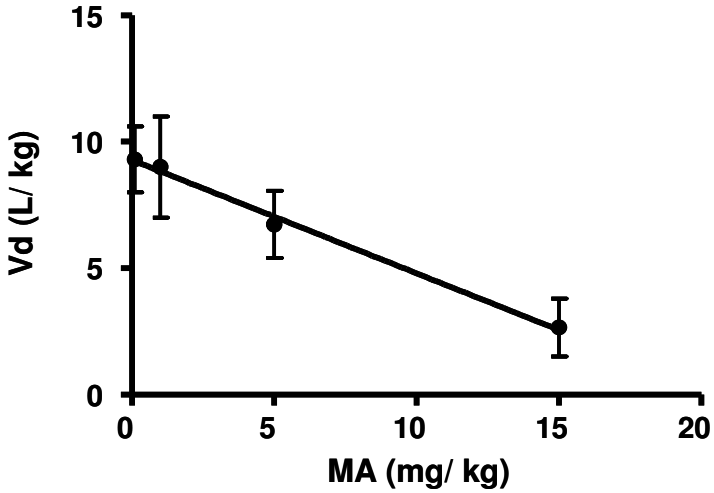

Figure 35. Volume of distribution for MA in rats has been reported to vary widely. The data was analyzed using linear regression. These values were obtained from Riviere et al., 1999 (0.1 mg/ kg has a Vd of 9.3 ± 1.3 L/ kg and 1.0 mg/ kg has a Vd of 9.0 ± 2.0 L/ kg), Kitaichi et al., 2004 (5 mg/ kg has a Vd of 6.73 ± 1.32 L/ kg), and Hutchaleelaha et al., 1996 (15 mg/ kg has a Vd of 2.64 ± 1.16 L/ kg). The slope of the line is -0.4494 ± 0.0219 and an R^2 value of 0.9952.

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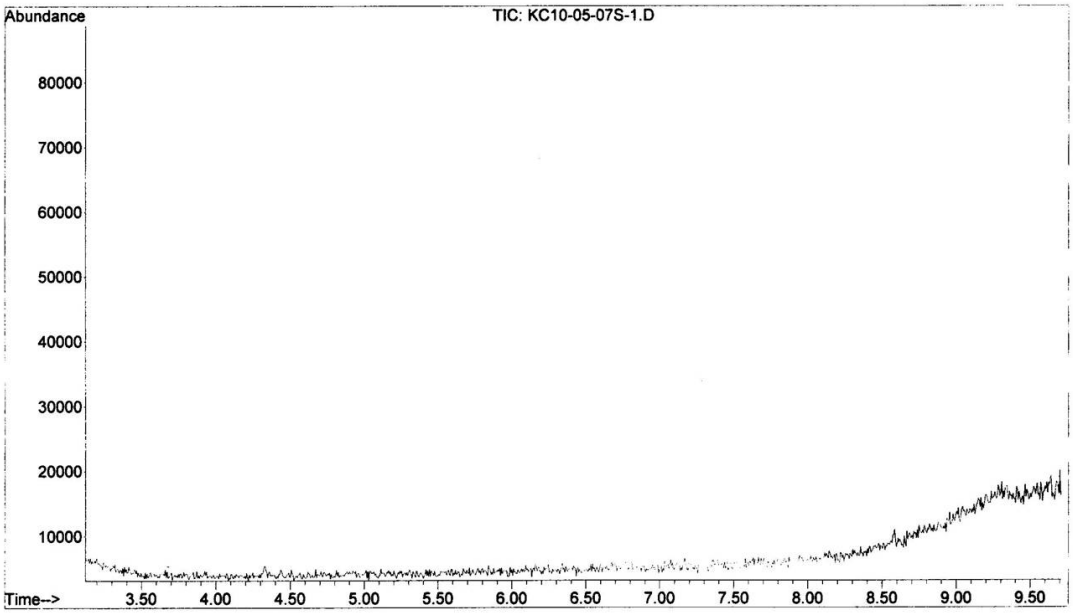

Figure 36. Representative MA sample for all source samples (brain tissue, supernatant, and blood). No MA peak was observed, since the MA concentration was below the LOD 1271.48 $\pm$ 191.69 ng. This was due to sacrificing the rats 7 hrs after the MA injection instead of 1 hr (the half- life for rats).

CHAPTER V

DISCUSSION

V. A. Introduction

The scheduled drugs MA and MDMA are addictive stimulant drugs. This addictive potential of these drugs has been reported, therefore, MA is a schedule II drug (addictive and medicinal use), and MDMA is a schedule I drug (addictive and no medicinal use). These drugs are still widely used and easily obtainable. Therefore polydrug use is a growing concern. The current study examined this concern involving the combination of the two scheduled drugs, MA and MDMA, and two common and inexpensive OTC drugs, CMT and DEX. CMT is a histamine blocker that reduces the production of stomach acid and is found only as Tagamet®. DEX is a dextrorotary morphinan since it does not bind to opioid receptors but does have a high affinity for sigma site ligand receptors and a lower affinity for the PCP receptor; it is found in most OTC cold and cough medications as an antitussive.

The purpose of this study was to determine the interactions at CYP2D6 between the OTC drugs, CMT and DEX, and two scheduled drugs, MA and MDMA when administered concurrently. The initial study examined the inhibition of the isozyme CYP2D6 activity caused by each of the drugs individually and in combination. This study determined the concentrations of drugs to be used in the *in vivo* study. The *in vivo*

study measured the kinetics of CYP2D6 V_{max} and K_m, the maximum velocity of the reaction and the substrate concentration at which the reaction velocity is 50% of the maximum velocity, respectively, by indirectly looking at the effects using the reduction of AMMC to AHMC.

Overall findings *in vitro*, calculated IC_{50} values for the test compounds were not significantly different when compared to quinidine and each other. Maximum inhibition compared to maximum quinidine inhibition showed that CMT, CMT/ MA, DEX/ MA, CMT/ DEX/ MA and CMT/ DEX/ MDMA decreased significantly from quinidine and CMT/ MDMA inhibition, a 75- 85% decrease compared to quinidine. Maximum MA inhibition decreased significantly compared to maximum quinidine inhibition. Inhibitory potential (IC_{50}) and maximum inhibition studies compared each test compound to quinidine inhibition and maximum quinidine inhibition values. This comparison indicated that each test compound inhibited CYP2D6 activity to some extent. This inhibition data gives relevance to the *in vivo* study. Overall findings *in vivo*, The V_{max} value in the CMT treated group decreased significantly when compared to naive. The K_m values in the CMT and saline treated groups decreased significantly when compared to naive. All MA challenged groups showed increases in V_{max} (280- 490%) and K_m (165- 220%) values compared to the naive group. Therefore, Ma challenge resulted in an increase in both kinetic parameters suggesting that the low affinity/ high capacity CYP2D2 isoform was upregulated.

V. B. *In Vitro* Assay

In order to determine the inhibition of CYP2D6 isozyme caused by the OTC and scheduled drugs, it was first necessary to determine concentrations that would cause inhibition of the isozyme. The initial concentrations were chosen from a review of the literature, and inhibition of the enzyme activity was determined using the CYP2D6/ AMMC high throughput inhibition screening assay.

The test groups did inhibit CYP2D6 activity but the IC_{50} values were not significantly different from quinidine. The IC_{50} value for quinidine of 0.0038 μM was consistent with the value reported by the manufacturer, Gentest. Others have shown that quinidine is an effective inhibitor with a range of 0.41 μM to 0.0089 μM dependent on substrate and product concentrations (Taavitsainen et al., 2000; Yamamoto et al., 2002; and Crespi et al., 1997). The test compound's [CMT, CMT/ MA, DEX/ MA, CMT/ DEX/ MA, and CMT/ DEX/ MDMA] maximum inhibition significantly decreased compared to maximum quinidine and CMT/ MDMA inhibition. Maximum MA inhibition significantly decreased compared to maximum quinidine inhibition. This data showed there was an effect on the catalytic activity of CYP2D6. Suggesting that all the test compounds caused some level of inhibition of CYP2D6, i.e. weak inhibition, therefore further studies are relevant. A review of literature uncovers a few studies reporting effects of MA or MDMA on CYP2D6 activity (Taavitsainen et al., 2000; Van et al., 2006; Wu et al., 1997; Heydari et al., 2004). However, MA and MDMA studies used different substrates or kinetic parameters like kinact (maximal rate of enzyme inactivation) and KI (inhibitor concentration that supports half the maximal rate of inactivation) to determine the enzyme activity. Taavitsainen et al. (2000) reported an

IC_{50} value for MA of 414 μM, but this study used DEX as the substrate probe not AMMC. Heydari et al. (2004) reported MDMA's rate of activation is decreased when quinidine is added. Studies that include CMT use different methods like Western blot analysis with serum containing anti-CYP2D6 (Orishiki et al., 1994) and use different factors to determine the kinetic parameters for CMT (Madeira et al., 2003; Martinez et al., 1999). DEX has the most studies on its effects of CYP2D6, and these studies use DEX as a probe to determine enzyme activity or phenotyping (Frank et al., 2007; Madeira et al., 2003; Martinez et al., 1999). Also DEX has been used to determine the type of metabolizer an individual is (Kerry et al., 1994). Studies on DEX inhibition report IC_{50} values for DEX of 1.89 μM to 2.0 μM (Yamamoto et al., 2002; Chauret et al., 2001) dependent on drug concentration.

V. C. *In Vivo* Assay

It is important to know how the drugs will affect CYP2D6 activity *in vivo*. Rats were injected for seven days with one of the four treatment groups. On the eighth day, rats were challenged with MA, and then sacrificed after 7 hrs. The livers harvested from the rats contain CYP2D2 isozyme. The CYP2D6 assay was modified to include the rat isozyme instead of CYP2D6. Then GC/ MS analysis was performed to determine the metabolized concentration of MA. The concentrations for the treatment groups were determined from the *in vitro* study. The concentrations for analysis CMT, DEX and CMT/ DEX were 10 mg/ kg for seven days for each drug. One i.p. injection of 5 mg/ kg was used for MA.

The CYP2D6 assay was modified by replacing human CYP2D6 with rat CYP2D2 microsomes. The reduction of AMMC to AHMC was measured at varying concentrations to determine the V_{max} and K_m values. The V_{max} value in the CMT treated group increased significantly from naive, however, all MA treated groups showed an increase in V_{max} values (280- 490%). The K_m values in the CMT and saline treated groups increased significantly when compared to naive, but again all groups treated with MA showed an increase in K_m values when compared to naïve (165- 220%). This suggests an overall trend, MA caused the increase in CYP2D6 activity and no effect was seen due to the OTC drugs. This data implies that MA may be an inducer via CYP2D2. Dostalek et al. (2005) suggests that MA may be an inducer of DEX metabolism via CYP2D2; therefore the co-administration of MA with DEX may result in decreased drug plasma levels thus a decrease in drug effects. The K_m value in the naive group was consistent with the K_m value determined by Gentest (1 μM). This suggests that the results from both assays are relevant to each other and can be compared. Most studies that measure enzyme activity of CYP2D6 or CYP2D2 use DEX as the substrate (Van et al., 2006), but this study uses the reduction of AMMC to AHMC to determine the enzyme activity. A few studies report the kinetic parameters for MA, CMT and DEX, but most use different methods or *in vitro* instead of *in vivo* (Lin et al., 1997; Madeira et al., 2004). Lin et al. (1997) report V_{max} and K_m values for MA, but the values are for both isomers of MA and both types of reactions (4-hydroxylation and N-demethylation) whereas this study did not differentiate between the two isomers or the two types of reactions. Madeira et al. (2004) report V_{max} and K_m values for CMT but the study is done *in vitro* instead of *in vivo* and with DEX as the probe.

V. C. i. *In vivo* GC/MS

The GC/ MS analysis is used to identify chemical compounds. Source samples of blood obtained from the jugular, supernatant saved from the microsome preparation, and excised brain tissue were used for analysis by the GC/ MS. Unfortunately; no MA was detected in the source samples. This problem was most likely due to MA in the samples below the LOD because the rats were sacrificed 7 half-lives after the MA challenge. The LOD was determined to be 1271.48 $\pm$ 191.69 ng and the limit of linearity (LOL) was 5.0 x 10^5 ng. Rats have a short MA half-life ($t_{1/2}$= 1 hr) compared to humans ($t_{1/2}$= 7-10 hrs) for that reason the rats should have been sacrificed an hour after injection (Milesi- Halle et al., 2005). Therefore, after 7 hrs, MA in rats is not detectable. MA distributes throughout the body in rats with a wide range of V_d values (Segal et al., 2005). These values range from 1.26 to 9.0 L/ kg (Kitaichi et al., 2004; Riviere et al., 1999). Peak plasma levels in rats after 1 hr have been reported an average of 362 ng/ mL after a 5 mg/ kg i.v. (Kitaichi et al., 2004; Fujimoto et al., 2007). After 3 mg/ kg i.p. administration, plasma levels have been reported of 200 ng/ mL (Okuda et al., 2004: Table 11).

Table 11. Plasma levels for MA in rats after one hour.

MA dose	Route	Plasma level	Reference
5 mg/kg	i.v.	375 ng/ mL	Kitaichi et al., 2004
5 mg/ kg	i.v.	350 ng/ mL	Fujimoto et al., 2007
3 mg/kg	i.p.	200 ng/ mL	Okuda et al., 2004

V. D. Summary

CYP2D6 is an important area of research due to the large role it plays in metabolism, its genetic polymorphisms among humans, and its large number of substrates. The chances of a drug- drug interaction are possible. People need to be aware of what can happen when drugs are co-administered, especially when taking scheduled drugs. Since MA and MDMA are popular and easily obtainable, drug- drug interactions are probable. This study determined that the inhibitor potency of all test compounds and quinidine were relatively the same. It was determined that some maximum test compounds inhibition decreased significantly compared to maximum quinidine and CMT/ MDMA inhibition. This suggests that all the test compounds inhibited CYP2D6 activity; one or all of the drugs may not be metabolized as quickly resulting in toxicity of those drugs. The quinidine IC_{50} value was consistent with reported values. This indicates that the CYP2D6 was performed in accordance to the manufacturer's specifications. The V_{max} value in the CMT treated group increased significantly compared to naive. The K_m values in the CMT and saline treated group increased significantly compared to naive. Both kinetic parameters showed there was an increase after the MA challenge but no effects due to the OTC drugs. This suggests that the low affinity/ high capacity CYP2D2 isoform was upregulated caused by the excess drugs meaning that more enzyme was present to help metabolize the drugs. This implies that MA is an inducer via CYP2D2. This is important to know if MA is an inducer via CYP2D2 the rat isoform or is this also true for CYP2D6 the human isoform, especially when DEX is co- administered. This information is imperative since DEX can be used as a recreational drug at high concentrations, thus if the two are combined DEX metabolism

may be increased causing a decrease in the effects of the drug, therefore causing an increase in use and abuse. No data was obtained from the GC/ MS analysis, due to the LOD 1271.48 $\pm$ 191.69 ng.

V. E. Future Studies

Further studies need to be conducted to determine how these drugs interact at higher concentrations of MA, CMT or DEX. It would be interesting to know what happens *in vivo* with more than one MA injection. Another study to be conducted will examine if MA is an inducer of DEX via CYP2D6. This information is imperative since DEX metabolism may be increased causing a decrease in the effects of the drug, and possibly resulting in an increase in use and abuse. The *in vivo* study could be replicated using MDMA instead of MA to determine if and how CMT and DEX affects MDMA. Also to achieve GC/ MS results the rats should be sacrificed after 1 hr of being injected with MA.

Another aspect that should be examined is the effects of co-administering OTC drugs on the metabolism of MA in poor metabolizers vs. extensive metabolizers. The effects of the combination of the two OTC drugs and MA would be interesting, since both OTC drugs are inexpensive and MA is easy to obtain.

Another area of study that would be interesting is to examine if co-administration of antidepressants and scheduled drugs affect the metabolism of each other. If there is an effect what type of effect, or drug- drug interactions occur. This information is a significant area of study, since users may want to try to increase the effects of one of the drugs.

V. F. Summary of Conclusions

- CYP2D6 *in vitro* assay studies indicated there were no differences in test group IC_{50} values of CYP2D6 activity. The IC_{50} value for quinidine was consistent with reported values meaning that the assay is performed to the manufacturer's specifications. The test compounds [CMT, CMT/ MA, DEX/ MA, CMT/ DEX/ MA, and CMT/ DEX/ MDMA] maximum inhibition decreased significantly from maximum quinidine and CMT/ MDMA inhibition, a 75-85% decrease compare to maximum quinidine inhibition. Maximum MA inhibition decreased significantly from maximum quinidine inhibition. This data suggests that all the test compounds inhibited CYP2D6 activity; one or all of the drugs may not be metabolized as quickly resulting in toxicity of those drugs.
- CYP2D2 *in vivo* assay studies indicated the V_{max} value in the CMT treated group and the K_m values in the saline and CMT treated groups increased significantly compared to naive. However, all MA treated groups showed an increase compared to naive. This increase in both parameters suggests that there was an overall effect after the MA challenge. Thus, no effect was caused by the OTC drugs. There seems to be a low affinity/ high capacity enzyme contributing, suggesting that more enzyme was present to metabolize the excess drugs after MA was administered. This suggests that MA is an inducer via CYP2D2.
- GC/ MS indicated the MA extracted from the blood, supernatant, and brain tissue samples were below the LOD, therefore no MA was detectable after 7 hrs. This lack of detection is due to the half-life of MA for rats ($t_{1/2}$= 1 hr). The LOD was determined to be 1271.48 $\pm$ 191.69 ng and the LOL was 5.0×10^5 ng.

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VITA

Kara Kelley Crosswy

Candidate for the Degree of

Master of Science

Thesis: METABOLIC INTERACTIONS BETWEEN OVER-THE-COUNTER AND ILLICIT DRUGS AT CYTOCHROME P450

Major Field: Forensic Science

Biographical:

Education: High School Diploma-Honors
Bixby High School, Bixby, OK
August 1995- May 1999

Bachelor of Science in Chemistry
Minor in Anthropology
University of Louisville, Louisville, KY
Aug 1999- May 2005

Master of Science in Forensic Science, emphasis in Toxicology
Oklahoma State University- Center for Health Sciences, Tulsa, OK
August 2005- December 2007

Experience: Co-operative Internship at Colgate Palmolive
Quality Assurance Department,
Microbiology and Analytical Chemistry
Octorber 2004- July 2005

Name: Kara Kelley Crosswy Date of Degree: December, 2007

Institution: Oklahoma State University Location: Tulsa, Oklahoma

Title of Study: METABOLIC INTERACTIONS BETWEEN OVER- THE- COUNTER AND ILLICIT DRUGS AT CYTOCHROME P450

Pages in Study: 78 Candidate for the Degree of Master of Science

Major Field: Forensic Science

Scope and Method of Study: Drug- drug interactions between over- the- counter (OTC) and scheduled drugs may occur at cytochrome P450, which can lead to toxicity and possibly death. This study examined the effects of two OTC drugs, cimetidine (CMT) and dextromethorphan (DEX), and two scheduled drugs, methamphetamine (MA) and 3, 4-methylenedioxymethamphetamine (MDMA) at CYP2D6. Purified human CYP2D6 was used to determine the inhibitory potential (IC_{50}) of the drugs *in vitro*. This assay examined the conversion of AMMC to its fluorescent metabolite product, AHMC. Enzyme kinetics was conducted to determine V_{max} and K_m values *in vivo* using rat microsome CYP2D2 isozyme. Solid phase extraction was used to extract MA from liver supernatant using Varian Bond Elut columns. GC/ MS was performed on the extracted MA samples to examine changes in MA metabolism following exposure to CMT or DEX.

Findings and Conclusions: *In vitro*, the IC_{50} values for the test compounds and CYP2D6 activity were not different compared to quinidine IC_{50} value. Maximum inhibition of CYP2D6 activity in the presence of test compounds [CMT, CMT/ MA, DEX/ MA, DEX/ MDMA, CMT/ DEX/ MA and CMT/ DEX/ MDMA] compared to maximum quinidine inhibition decreased significantly from quinidine and CMT/ MDMA inhibition, a 75-85% decrease compared to quinidine. Maximum MA inhibition was significantly decreased compared to maximum quinidine inhibition. This data suggests that all the test compounds inhibited CYP2D6 activity; one or all of the drugs may not be metabolized as quickly resulting in toxicity of those drugs. *In vivo* CYP2D2 studies showed that the V_{max} value in the CMT treated group (98.28 + 22.09 pmol/ mg protein/ min) increased significantly compared to naive (19.92 + 5.084 pmol/ mg protein/ min). The K_m value in the saline (6.806 + 0.73 μM) and CMT (6.728 + 1.341 μM) treated groups increased significantly compared to naive (3.081 + 0.46 μM). All MA challenged groups showed increases in V_{max} (280- 490%) and K_m (165- 220%) values compared to the naive group. Therefore, MA challenge resulted in an increase in both kinetic parameters (V_{max} and K_m) suggesting that the low affinity/ high capacity CYP2D2 isoform was upregulated. This data suggests that MA is an inducer via CYP2D2, which will lead to altered drug metabolism and an alteration of the drug's effects.

ADVISOR'S APPROVAL: ~~Dr. David R Wallace~~