METHOD DEVELOPMENT FOR THE DETECTION OF SYNTHETIC CATHINONES AND INVESTIGATION OF THEIR METABOLISM USING HUMAN MICROSOMES

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Abstract: Abuse of designer drugs such as synthetic cathinones presents a challenge to both medical and forensic experts. Detecting cathinone exposure in humans requires a sensitive, reliable method and treatment involves an understanding of the physiological response of the body to these novel compounds. This study focused on developing a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for the detection and quantitation of sixteen popular synthetic cathinone analogues. The method was found to be linear from a lower limit of 1ng/ml-10ng/ml, depending on the drug, to an upper limit of 25ng/ml for all tested drugs. This LC-MS/MS method was then employed to study the interaction of specific bath salts, mephedrone (MEPH) and buphedrone (BUPH), with cytochrome p450 (CYP) enzyme systems. The IC50s of MEPH and BUPH were determined to be 10.1 μM and 61.7 μM, respectively, using a fluorescence-based CYP2D6 inhibitor screening kit, demonstrating an inhibiting interaction with CYP2D6. A human liver microsomal preparation consisting of 20 Phase I metabolic enzymes was then tested with MEPH and BUPH, which demonstrated no significant change in parent compound concentration over the course of an hour. These findings suggest that MEPH and BUPH act as a CYP2D6 inhibitors, but are not metabolized as a substrate by the enzymes in the test system.

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CHAPTER I

INTRODUCTION

According to a 2011 survey performed by the Substance Abuse and Mental Health Service Administration, 22.5 million Americans self-reported as current illicit drug users.[1] Despite these reported illicit drug users, the Federal Bureau of Investigation's Uniform Crime Reporting (UCR) Program found that only 1.5 million were arrested that year.[2] Additionally, 25% of the 5.1 million patients treated for drug-related injuries in 2011 were being treated for symptoms brought on by an recreational drug use.[3] These data demonstrate that drug abuse presents a significant threat to both the health of individuals and to the public health. The first step in treating or prosecuting illegal drug exposure is the detection of the consumed drug. Synthetic cathinones, colloquially known as "bath salts" or "plant food", [4] can pose a significant problem as many forensic and clinical toxicology laboratories do not have methods to detect this class of novel synthetic drugs.

A hallmark of the synthetic drug market is its malleability; a manufacturer can alter a drug's structure in an attempt to avoid detection and prosecution. An analogue is a slight variation in the chemical structure of a drug (such as the transfer of a methyl group to a nearby carbon), while the resultant chemical remains in the same class of compounds. As of 2011, 30 synthetic cathinones were documented;[8] it is likely that there are more undocumented synthetic cathinone analogues which avoid detection. Each analogue will

interact differently with a given method of detection; as new drugs are made, the method of detecting these drugs must be updated to include the most recent analogues. In addition, cathinone analogues may interact with the body differently and have varying effects on enzymes, which may result in toxicity in situations that can cause life-threatening injury if their physiological effects are not anticipated, such as when a patient abuses cathinones when taking other, prescribed medications or is being treated for cathinone exposure.

The ability to detect synthetic cathinones in exposed individuals is important for a number of reasons. First, when prosecuting an individual for illicit drug consumption or manufacturing, the forensic investigator's case is greatly strengthened if the investigators are able to determine the identity of the compound in question and whether or not the concentration in the body is capable of causing a physiological effect. Second, effective medical treatment of illicit drug exposure often involves identifying the specific drug the individual has consumed because different drugs will bring about different physiological effects. Treatment and addiction rehabilitation may vary from one drug to the next and a medical professional will be able to more effectively treat their patient with all of the necessary information, including an understanding of cathinone metabolism. Third, cathinone use and abuse result in fatalities, and the detection of a broad array of these compounds will be of benefit to death investigators. Finally, this study would also potentially improve cathinone detection *in vivo* as novel, long-lasting metabolic products are identified that might be the targets of new analytical method.

Several studies in the literature identify methods for determining the concentration of synthetic cathinones in various biological specimens. This endeavor often employs analytical instrumentation that consists of a chromatographic separation followed by analysis of the drug via mass spectrometry. Of the methods available, liquid chromatography has experienced the greatest success and is the most common method employed for synthetic cathinone detection by researchers and forensic experts.[5-7] While methods for the detection and quantitation of synthetic cathinones exist, the rapidly changing designer drug landscape requires researchers to constantly develop new methods.

One of the challenges involved in synthetic cathinone detection is the ability to acquire calibrated drug standards. Synthetic cathinones are Schedule I drugs, and only licensed institutions are permitted to acquire drug standards for the study of these compounds. The Oklahoma State University Forensic Toxicology and Trace Laboratory (FTTL) is an institution permitted to study synthetic cathinones, thus offering an exceptional opportunity to add to the literature on synthetic cathinone detection and address the largely unexplored area of cathinone metabolism.

The purpose of this research is to develop a method for the detection and quantitation of synthetic cathinones and a subsequent investigation of the metabolism of synthetic cathinones using commercially available, human-derived hepatic enzymes. The cathinone analogues in this study were chosen based on high popularity and structural diversity, and the results of this study were then statistically evaluated for significance. The resulting LC-MS/MS detection method could be employed by law enforcement agencies to quantify these compounds in biological samples, allowing these agencies to prosecute synthetic cathinone drug users and dealers. The metabolism investigation can

help physicians make more informed treatment decisions, provide information to the death investigation process, and lead to better detection as metabolites are identified as analytical targets.

CHAPTER II

LITERATURE REVIEW

2.1. Introduction

Illegal drug use is a growing problem worldwide. According to the Federal Bureau of Investigation's Uniform Crime Reporting (UCR) Program, the highest number of arrests in the year 2011 was for drug abuse violations with over 1.5 million of the 12.4 million arrests made that year.[2] In the same year, an estimated 22.5 million Americans 12 years of age or older self-reported as current illicit drug users (defined by the study as having used illicit drugs within a month prior to being interviewed).[1] The nature of a self-reported survey tends to lean toward an underestimation of the true number, in reality the number of drug abusers in the United States is likely much higher. While the number of arrests for drug abuse violations is incredibly high, the number of unconvicted illicit drug users is substantially higher. The sheer volume of arrests for drug abuse violations and the number of drug abusers present a substantial need for a method that allows for the accurate detection and quantitation of the drugs an individual may be taking. Detection and quantitation is the first step in combatting the illicit drug epidemic.

In addition to the legal ramifications, illicit drug exposure is a danger to the health of those exposed. Drug-related injuries are becoming more commonplace and present a

significant risk to the health of citizens both in the United States and abroad. In the United States, 5.1 million patients visited emergency departments for drug-related injuries in 2011, and 25% of those patient visits were attributed to illicit drugs.[3] Emergency department visits increased by nearly three hundred thousand visits per year between 2009 and 2011 for patients being treated for symptoms brought on by recreational drug use.[3] More emergency department visits can be attributed to drug abuse injuries than ever before. As such, the need for a method of detecting the specific drugs affecting a patient and an understanding of the drug's metabolic effects is necessary for the appropriate treatment of patient exposure.

The Controlled Substances Act of 1970 laid much of the groundwork for the regulation of illicit drugs. This act established guidelines for the scheduling of various drugs based on the drug's FDA-approved medical uses, potential for abuse, and potential for physical and/or psychological dependence.[9] Drugs that presented a significant threat to an individual's health without offering an appropriate therapeutic benefit—methamphetamine, cocaine, and heroine, for example—were quickly scheduled and then highly regulated by government officials. To circumvent legislation, drug manufacturers began seeking out alternative drugs that would have similar physiological effects as their scheduled counterparts, but would not be burdened by the scrutiny of government officials. These novel drugs that could avoid legislative repercussion were termed "designer drugs" and are challenging to regulate.

2.2 Designer Drugs

Many designer drugs were originally synthesized for research or medical purposes by scientists in academia or in the pharmaceutical industry. Initially these classes of drugs were unregulated and available to the public, until they were repurposed for recreational use and became drugs of abuse. A notorious example is 3,4-methylenedioxy-methamphetamine (MDMA, also known as ecstasy), a compound first synthesized in 1912 by Merck Pharmaceuticals as a parent compound to synthesize other pharmaceuticals. MDMA reappeared in the streets in the 1970s shortly after psychiatrists had begun utilizing it as a psychotherapeutic tool. Even then it was not extensively abused until the 1980s as a popular "party drug" and was federally scheduled in 1985.[10]

Designer drugs have functional similarities to other drugs of abuse but not are not structurally identical, allowing them to elude government regulation under the Controlled Substances Act. The designer drug phenomenon prompted the Controlled Substance Analogue Enforcement Act of 1986, which widened the scope of the 1970 law. The new act prohibited substances intended for human consumption that shared similar structures and physiological effects with other drugs of abuse.[11] An unfortunate side effect of the Controlled Substance Analogue Enforcement Act is that it makes many of the analogues Schedule I, which slows research on illicit drug analogues as fewer laboratories have licensing to access Schedule I drugs. This can be severely detrimental in responding to the fast-moving designer drug industry. The Oklahoma State University Forensic Toxicology and Trace Laboratory (FTTL), where this research was performed, has the necessary licensing to carry out research with Schedule I compounds and help address this current public health problem.

Even with the wider scope of the Controlled Substance Analogue Enforcement Act, two potential pathways exist for illicit drug manufacturers to circumvent the latest legislation. A manufacturer can synthesize a new compound that achieves similar stimulant, depressant, or hallucinogenic effects of its scheduled counterpart with a significantly different structure.[12] Alternatively, a manufacturer can label a drug as "not for human consumption." The latter pathway is particularly common amongst synthetic cathinones and synthetic cannabinoids, which are often labelled as "bath salts" and "spice," respectively.[4,13]

2.3. Synthetic Cathinones

Following the trend of many other designer drugs before them, synthetic cathinones are a class of psychoactive compounds derived from cathinone, a naturally occurring stimulant. Cathinone is found in the leaves of khat, which is native to parts of Africa and the Arabian Peninsula and is often chewed to achieve the desired stimulation.[14] Methcathinone, a methylated analogue of cathinone, was first synthesized in 1928[15] and represents the first of many synthetic cathinones that would later serve as "legal alternatives" to MDMA and other, previously scheduled, drugs of abuse. Synthetic cathinones are a class of compounds capable of a wide-range structural modification; the variety of structural analogues make them difficult to detect and regulate and their biological consequences even more difficult to predict.

Structurally, synthetic cathinones share a common backbone of a phenethylamine with a ketone group at the β carbon (See Figure 1). From this common backbone, the functional groups are extremely diverse, thereby giving cathinones potential for varying chemical structure and physiological effects. The trademark characteristic of synthetic cathinones is their malleable composition; as soon as a given analogue is scheduled, another is synthesized to meet consumer needs and to circumvent federal legislation. For example, methylenedioxypyrovalerone (MDPV) is capable of acting as a dopamine uptake inhibitor and was designed to be more potent than cocaine, producing intense stimulation and euphoria.[16] MDPV epitomizes the illicit drug manufacturer strategy of utilizing an existing compound that can emulate previously scheduled drugs, and repurposing it for recreational use. A different combination of functional groups could lead to a different a physiological effect and a different response to a method of detection. Synthetic cathinones' broad range of possible analogues make them ideal candidates for designer drug producers and has led to a spike in cathinone popularity in recent years.

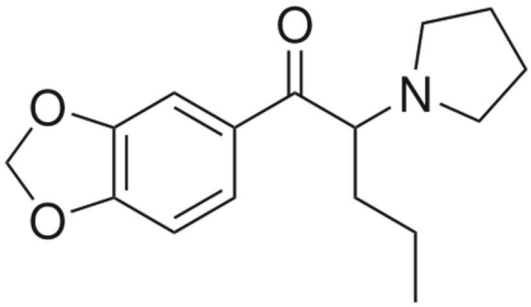

Figure 1. Structure of synthetic cathinone, MDPV.

Bath salts have begun replacing MDMA as the party drug of choice in many European countries. Mephedrone (MEPH), a synthetic cathinone analogue, is gaining popularity because of the decreasing purity of both cocaine and MDMA.[17] Consumers are able to purchase MEPH at a lower price than either MDMA or cocaine and achieve greater stimulation. Cathinones are viewed as a more reliable and safer replacement to MDMA

and cocaine, which may add to their recent popularity both in Europe and in the United States. Based on recent surveys, the main settings of use include nightclubs and parties and the Internet is the most common avenue utilized by designer drug dealers to market their product.[18]

Synthetic cathinones' popularity rise in recent years can also be attributed to information sharing, advertising, and marketing through the Internet. Online shops have increased the marketability of designer drugs because online shops are known for their adaptability to changing legislation and customer needs.[19] An online shop's flexibility, in addition to its ability to serve a wide customer base, allows illicit drug marketers to sell the most recent designer drugs to a large number of drug abusers. Another challenge presented by designer drugs is the fact that the exposure population is drastically increased when unscheduled drugs can be purchased legally in gas stations, convenience stores, tobacco shops, and head shops before legislation is able to effectively ban these structurally fluid compounds.

2.4. Synthetic Cathinone Detection

The scientific community has recognized the need to study synthetic cathinones and has developed several methods for their detection. Analytical methods have been used by various studies to detect and quantify synthetic cathinone concentrations in a variety of biological sources. Chromatographic separation, a standard technique in many analytical laboratories, consists of passing analytes of interest through a column, separating the analytes based on their affinity to the column. Due to its convenience, liquid chromatography (LC) is the most common method for separation of the various synthetic

cathinone analogues. Another chromatography method, gas chromatography (GC), is limited by the additional analytical steps required to reach the desired product compared to LC.[20] GC requires the analytes in the gas phase while LC requires that the analytes are in the aqueous phase; thus GC is only applicable if the compound is adequately volatile. Mass spectrometry (MS) is a common method for analyte quantitation and is, overwhelmingly, the most widely used method for quantifying synthetic cathinone concentrations.

Scientists have been shown that synthetic cathinones and their metabolites are deposited throughout the body after consumption. As such, a variety of biological specimens can be used in the detection of synthetic cathinones. Marinetti and Antonides were able to demonstrate that synthetic cathinones were retained in many biological samples including whole blood, plasma, urine, vitreous humor fluid, cerebrospinal fluid, bile, and tissue homogenates.[21] Each of these biological sources can be utilized in the detection of synthetic cathinones in both postmortem and human performance toxicological analyses. In addition to the above biological sources, Shah et al were able to utilize hair as a biological source for synthetic cathinone testing.[7] Hair is capable of providing long term information about the history of drug exposure, making it a potentially valuable source for forensic testing. A wide variety of samples are useful in situations where a certain sample type may become contaminated or is unavailable.

Both liquid and gas chromatography separation techniques have proven of detecting and quantifying synthetic cathinone concentrations. LC-MS/MS (liquid chromatography coupled tandem mass spectrometry) screening appears to be the most widely used method of synthetic cathinone detection.[5,6,22] Scientists have also found limited success using GC-MS (gas chromatography coupled mass spectrometry) to

dissociate various synthetic cathinone analogues.[21] Additionally, Meyer et al. found that to reliably detect some synthetic cathinones using GC-MS, enzyme digestion and derivatization were necessary.[20] The added digestion and derivatization increase both the cost and the time required to achieve successful quantitation of the synthetic cathinones. Due to its direct detection (without added derivatization), LC-MS/MS appears to be the more effective method for synthetic cathinone detection based on previous studies.

2.5. Synthetic Cathinone Physiological Effects

Permission must be granted by the Drug Enforcement Administration before researchers can acquire Schedule I drugs, contributing to the fact that few case studies have formally examined the physiological effects of these drugs. A small number of published case studies provide some insight into the symptoms that can be expected with synthetic cathinone exposure. In general, the effects of cathinone exposure are cardiovascular and neurological in nature and can include combative behavior, excited delirium, hallucinations, and abnormal rapid heart rate.[23] Prolonged symptoms can lead to permanent nervous or cardiovascular damage and eventually death.

Excited delirium is a side effect of synthetic cathinone exposure that presents a significant challenge to healthcare professionals. Drug side effects are typically only harmful to the individuals exposed to the drug, however, excited delirium makes the exposed individual a danger to themselves and others around them. Law enforcement officials and emergency health professionals are particularly vulnerable to individuals afflicted by an excited delirium as they attempt to apprehend and subsequently medically treat the affected person. This agitated state is typically characterized by hallucinations,

paranoid delusions, violent behavior, destruction of skeletal muscle, and eventually kidney failure.[24,25] The effects of these synthetic compounds have been observed from the surface, but there is little information regarding their effects within the body which would provide information about the mechanism that is bringing on the adverse drug reactions and how to safely handle these intoxicated individuals.

Many of the methods commonly used to control violent behavior, such as physical restraints, electronic control devices (TASER), and antipsychotic drugs, have the potential to lead to severe medical complications due to the exacerbation of preexisting physiological symptoms.[26] Excited delirium also makes the patients resistant to sedatives, further complicating treatment. In one case report, emergency medical professionals noted that a 30-year-old male with no prior mental disorder exhibited a "very high tolerance to sedatives and analgesics" when the patient was admitted to the emergency department exhibiting symptoms of agitation, violent behavior, and abrupt change in mental status.[27] The mechanism of this phenomenon is currently unexplained. Insight into the metabolism of synthetic cathinones may lead to a more effective way to sedate exposed individuals and safely treat them.

While the variety of biological sources available for testing demonstrates that synthetic cathinones are widely distributed throughout the body, the metabolism and elimination of the drug are not well understood. A better understanding of synthetic cathinone's mechanism of action is necessary to provide healthcare workers with the necessary information to optimize patient treatment and ensure the safety of the patient and those around them. By studying this class of drug's interaction with metabolic enzymes, a cause can be linked to the observed physiological outcome. A systematic investigation of

both the detection and metabolism of synthetic cathinones will arm healthcare providers and forensic investigators with the necessary information to counteract this dangerous designer drug.

2.6. Synthetic Cathinone Metabolism

Synthetic cathinones are relatively novel compounds, so the metabolism of many analogues is currently not well understood. Due to its popularity, MEPH has faced a more rigorous scrutiny compared to other synthetic cathinone analogues. MEPH metabolism has been hypothesized to involve an N-demethylation to the primary amine, reduction of the ketone to an alcohol, and oxidation of the tolyl to the corresponding alcohol and carboxylic acid (see Figure 2).[28] Pedersen et al. used cDNA-expressed CYP enzymes and human liver microsomal preparations to study MEPH metabolism and attributed its Phase I metabolism to cytochrome P450 2D6 (CYP2D6) with minor contributions from other NAPDH-dependent enzymes.[29] Currently, the metabolism of BUPH has only been indirectly observed by

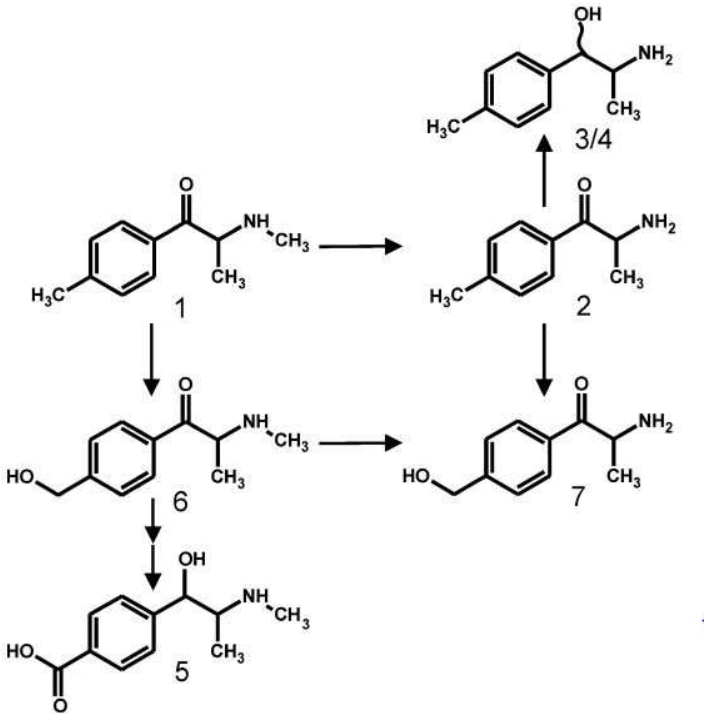

Figure 2. Proposed scheme for the Phase 1 metabolism of mephedrone in rats and humans. Metabolite 5 should only be found in human urine

comparing the excreted products to the ingested parent drug. BUPH is thought to go through a reduction of the beta-keto group and an N-dealkylation, based on metabolites found in patient urine who had been exposed to BUPH.[30] MEPH and BUPH were the selected cathinones investigated in this study.

Drug-drug interactions are a constant concern in hospital settings. Drugs that attempt to utilize the same enzyme during metabolism are going to interact based their specific active sites on the enzyme and their relative affinities for the enzyme. Cathinone ingestion commonly occurs alongside other drugs or alcohol[31,32] leaving these individuals at a heightened risk for drug-drug interactions even before physicians attempt to begin treatment of exposure. Administering drugs during treatment may worsen the symptoms associated with poly-drug toxicity. By characterizing synthetic cathinone metabolism, physicians will be able to make informed decisions and avoid harmful drug-drug interactions and adverse drug reactions which may endanger the patient. Though a public menace, synthetic cathinones are still widely understudied and further investigation of their metabolism could aid in the efforts of both detection and treatment.

2.7. Conclusion

The literature demonstrates that illicit drug exposure is a significant problem for both law enforcement officials and healthcare providers. The purpose of this research is to meet the need of forensic and clinical laboratories by developing a method for the detection and quantitation of synthetic cathinones. This detection method will then be used in a subsequent investigation of the metabolism of synthetic cathinones using human liver microsomes to address the deficit within the literature regarding the metabolic pathway of

synthetic cathinones. A greater understanding of synthetic cathinone metabolism can be used to better provide treatment to exposed individuals in both emergency and rehabilitative areas of medicine. The resulting method and metabolic investigation can be employed by law enforcement agencies and emergency medical professionals to more effectively handle individuals exposed to these dangerous compounds.

CHAPTER III

METHODOLOGY

3.1 Introduction

This method development and metabolism study was performed at the Oklahoma State University Center for Health Sciences Forensic Toxicology and Trace Laboratory, which is licensed to handle Schedule I drugs such as synthetic cathinones. Method development and metabolic inquiry was approved by the Oklahoma State University Institutional Review Board. Instrument parameters were established on a liquid chromatograph coupled mass spectrometer (LC-MS/MS) using calibrated reference standards. Metabolism studies were performed using enzyme inhibitor screening kits and human liver microsomal fractions. Following the incubation of cathinones with enzyme, the enzymatic action was observed using both fluorescence spectroscopy and mass spectrometry.

3.2 LC-MS/MS Method Development

Instrumentation utilized by this method is common in many toxicology laboratories allowing laboratories to adopt the detection method without acquiring expensive auxiliary instrumentation. The prepared calibrator samples and the microsomal fractions were analyzed with a Shimadzu (Kyoto, Japan) liquid chromatograph coupled to an AB Sciex

(Framingham, MA) triple quadrupole tandem mass spectrometer with electron spray ionization (Figure 3). Analytes were separated using a Chromegabond Wide Range C18 LC column (particle size: 5µ, pore size: 120Å, dimensions: 15cm x 2.1mm) manufactured by ES Industries (West Berlin, NJ). The separated analytes then travelled to the MS/MS where they were ionized and quantified in multiple reaction monitoring (MRM) mode with positive electron spray ionization using at least two MRM transitions for qualification.

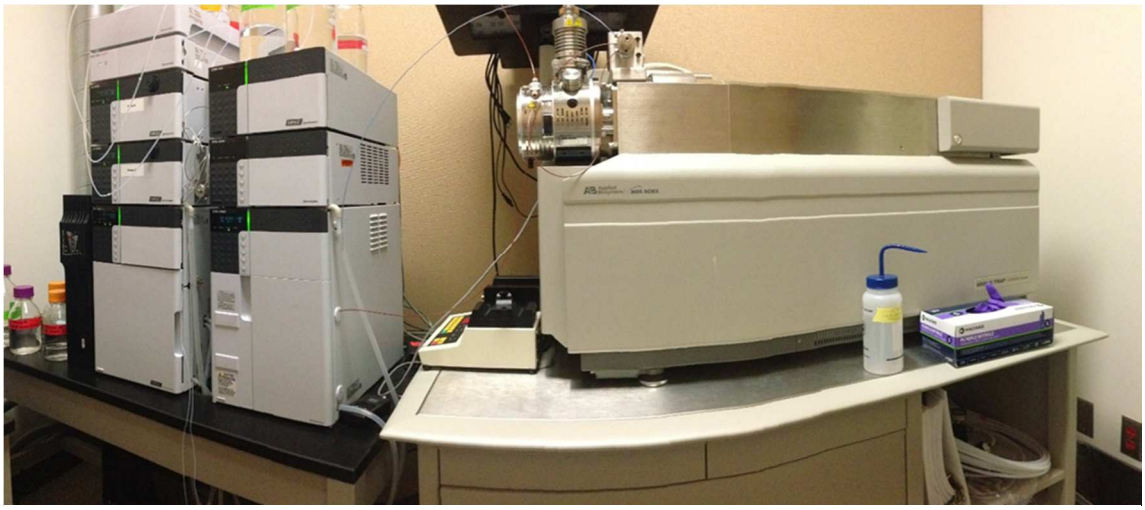

Figure 3. Photograph of LC-MS/MS used in method development.

HPLC grade acetonitrile, methanol, ammonium formate, and formic acid were purchased from VWR International (Radnor, PA). To prepare samples for injection, 20uL of a 625 ng/mL internal standard solution (except for MEPH (metabolite)-D3 which was prepared at twice the concentration of the others) and 30uL of phosphate buffer (pH 4.5) was added to 50 µL of sample. 150 µL of sample diluent made of a ratio of 95% the aqueous mobile phase (mobile phase A) and 5% of the organic mobile phase (mobile phase B) and was added to the previous 100uL for a total 250uL total prepared sample. Mobile

phase A consisted of a 2mM ammonium formate solution in water with .1% formic acid and mobile phase B consisted of a 9:1 acetonitrile:water solution with .1% formic acid.

This sample preparation was purposefully designed to mimic the conditions of the solution flowing through the instrument so that their chromatography was more consistent. Phosphate buffer was used to neutralize fluctuations in pH, which can also cause variations in chromatography. Internal standards were run in solution with samples to provide a baseline point of comparison of known concentration and allowed for the quantitation of unknown sample concentrations. This was done by comparing the instrument response (in the form of peak area ratio) of calibrators with known standard concentration to the instrument response of unknown samples. After the samples were appropriately diluted they were ready for injection onto the instrument.

3.2.1 Liquid Chromatograph Conditions

Liquid chromatography is used to separate drugs within a sample from each other by their various affinities as they travel through a functionalized column. Compounds with a high affinity for the column will "cling" to the column and move more slowly than those with a lower affinity. This process is especially important for synthetic cathinones, which can have similar fragmentation patterns in the mass spectrometer, making them impossible to dissociate from one another using a mass spectrometer alone. Chromatography was optimized using a drug standard mix and an internal standard mix. Internal standards interact similarly to separation methods as their non-deuterated counterparts and allow the instrumentation to quantify the concentration of the drug being analyzed by providing a compound for comparison that differs only in mass, not in functionality.

All drug standards and deuterated internal standards were acquired from Cerilliant (Round Rock, TX) as calibrated reference standards in methanol at 1 mg/ml and 100 µg/ml respectively (See Table 1 for complete list).

Table 1. Drug standards and internal standards and their corresponding concentrations.

Drug Standards	Concentration	Internal Standards	Concentration
3-Fluoromethcathinone	1 mg/ml	(±)-4-Methylephedrine (Mephedrone Metabolite)-D3	100 µg/ml
(±)-3-Fluoromethcathinone Ephedrine Metabolite	1 mg/ml	Alpha-PVP-D8	100 µg/ml
(±)-4-Methylephedrine (Mephedrone Metabolite)	1 mg/ml	Buphedrone Ephedrine Metabolite-D3	100 µg/ml
Alpha-PVP	1 mg/ml	Butylone-D3	100 µg/ml
Buphedrone	1 mg/ml	Ethylone-D5	100 µg/ml
Buphedrone Ephedrine Metabolite	1 mg/ml	3,4-Methylenedioxy Pyrovalerone-D8	100 µg/ml
Butylone	1 mg/ml	Mephedrone-D3	100 µg/ml
Ethylone	1 mg/ml	Methylone-D3	100 µg/ml
Mephedrone	1 mg/ml	Naphyrone-D5	100 µg/ml
Methedrone	1 mg/ml	(±)-N-Ethylcathinone Ephedrine Metabolite-D5	100 µg/ml
Methylone	1 mg/ml	Pentylone-D3	100 µg/ml
Naphyrone	1 mg/ml		
N-Ethylcathinone	1 mg/ml		
(±)-N-Ethylcathinone Ephedrine Metabolite ,	1 mg/ml		
Pentylone	1 mg/ml		
Pyrovalerone	1 mg/ml		

Drug standards and internal standards were diluted to 100 ng/ml in methanol for examination by LC-MS/MS. Chromatographic separation was a modified version of that performed by Swortwood and Boland[5] and consisted of using varying ratios of mobile phase A (consisting of a 2mM ammonium formate/.1% formic acid solution in water) and mobile phase B (consisting of a 90% acetonitrile and 10% water solution by volume with

.1% formic acid). The gradient of mobile phase A to mobile phase B proceeded as follows: 5% B up to 15% B in seven minutes, next a one minute ramp to 35% B, then an increase to 95% B over one minute where the ratio is held constant for one minute, and lastly a three minute re-equilibration at 5% B to prepare the LC for the next sample (Figure 4).

	Time	Module	Event	Parameter
1	0.01	Pumps	Pump B Conc.	5
2	7.00	Pumps	Pump B Conc.	15
3	8.00	Pumps	Pump B Conc.	35
4	9.00	Pumps	Pump B Conc.	95
5	10.00	Pumps	Pump B Conc.	95
6	10.10	Pumps	Pump B Conc.	5
7	10.10	Pumps	Total Flow	1
8	13.00	Pumps	Pump B Conc.	5
9	13.00	Controller	Stop	
10				

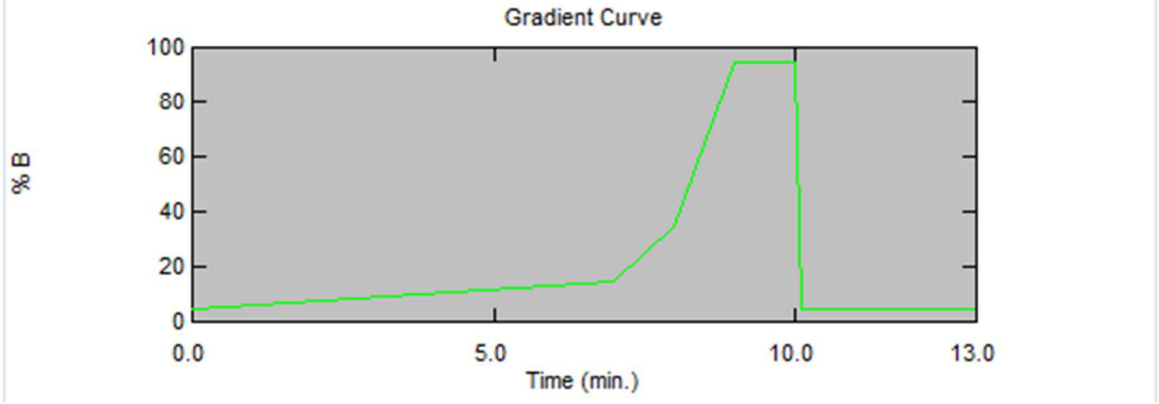

Figure 4. Diagram of the LC time program, which shows the change of mobile phase ratio as the LC separates the compounds according to their affinities to the stationary

The gradient was performed with a flow rate of .5 mL/min throughout. The column was kept at 40°C in a column oven. From the LC, the compounds flow directly into the tandem mass spectrometer so that the separated compounds can be quantified.

3.2.2 Mass Spectrometer

A mass spectrometer uses high temperatures and voltages to fragment a compound into smaller constituent components. The MS detector was optimized to detect specific fragments of each ionized compound using Lab Solutions Software which was then used to quantify the cathinones. The optimization process involves fragmenting the analytes at varying energies and recording the instrument response at each energy in terms of peak intensity. The energies and ion fragments that corresponded to the highest instrument response were included in the method. A combination of a precursor and product ion is called an MRM (multiple reaction monitoring), and at least two MRM transitions were used for each compound (See Table 2). The entrance potential for each analyte was 10V.

Table 2. Each cathinone included in the LC-MS/MS method with their corresponding optimized ion fragments and MS fragmentation energies.

Compound Name	Precursor Ion	Product Ion	Collision Energy	Collision Exit Potential	Dependent Parameters
3-FMC	182.013	163.939	19	8	56
		149.118	29	24	56
		148.405	41	24	56
3-FMC (metabolite)	184.008	151.2	31	6	51
		150.665	31	24	51
		114.8	37	18	51
Mephedrone (metabolite)	179.916	91.081	59	28	21
		116.258	39	52	21
		115.068	65	54	21
		104.962	31	6	21
		77.018	87	2	21
Alpha-PVP	233.267	91.316	33	4	61
		92	33	14	61
		77.182	69	10	61

Buphedrone	177.930	159.934	19	22	31
		130.804	33	10	31
		129.673	45	20	31
Buphedrone Ephedrine	181.091	163.039	19	10	41
		91.6	39	14	41
		92.167	39	4	41
Butylone	223.121	175.011	25	14	46
		204.989	21	10	46
		147.098	37	24	46
Ethylone	223.435	91.556	43	18	61
		118.537	43	22	61
		119.179	43	8	61
Mephedrone	178.043	144.87	29	12	51
		143.81	41	24	51
		91.092	47	12	51
		77.25	71	0	51
		119.233	31	18	51
Methedrone	194.990	162.054	29	6	51
		147.141	39	24	51
		146.8	39	6	51
Methylone	207.990	160.165	27	24	36
		159.673	27	6	36
		132.014	39	22	36
Naphyrone	282.044	141.082	35	6	81
		126.836	77	18	81
		125.872	41	4	81
N-ethylcathinone	179.090	132.77	27	6	51
		131.57	29	20	51
		130.859	37	6	51
		118.344	29	18	51
		106.037	33	16	51
N-ethylcathinone (metabolite)	180.995	139.79	11	22	51
		117.834	31	18	51
		115.839	39	18	51
Pentylone	235.975	187.887	25	8	31
		174.918	31	14	31
		131.061	51	10	31
Pyrovalerone	247.099	105.23	37	6	61
		106.247	37	16	61
		90.987	63	14	61

Mephedrone (metabolite)-D3	184.676	91	41	14	51
		98	21	14	51
		131.1	27	6	51
Alpha-PVP-D8	240.110	91.099	37	14	71
		77.387	71	0	71
		134.206	43	10	71
Buphedrone (metabolite)-D3	183.028	136.1	31	6	46
		91	37	14	46
		98	23	14	46
Butylone-D3	226.498	178.247	25	6	51
		207.859	17	12	51
		135.078	47	10	51
Ethylone-D5	227.001	179.021	27	8	51
		151.216	29	8	51
		119.139	45	18	51
MDPV-D8	284.032	134.594	41	6	71
		204.95	27	10	71
		174.9	33	8	71
Mephedrone-D3	181.023	148.208	31	10	56
		147.586	33	26	56
		144.969	27	6	56
Methylone-D3	211.003	163.029	25	24	41
		135.041	39	10	41
		91.233	55	4	41
Naphyrone-D5	287.112	216.091	27	10	81
		141.171	39	6	81
		142.16	37	10	81
N-ethylcathinone (metabolite)-D5	186.005	108.989	21	4	41
		117.955	31	18	41
		115.908	41	8	41
Pentylone-D3	239.052	191.204	27	10	61
		134.404	51	20	61
		204.914	19	20	61

3.4 Enzyme Inhibitor Screening

Enzyme screening kit and human liver microsomal fractions were purchased from Corning Inc. (Corning, NY). The CYP2D6/AMMC High Throughput kit was used to evaluate inhibition of CYP2D6 activity by quinidine, cimetidine, MEPH, and BUPH. The control drugs, quinidine and cimetidine, were chosen based on the fact that they are documented CYP2D6 inhibitors.[33] MEPH and BUPH were chosen as the cathinones to be studied because a drug standard of a metabolite was commercially available. This would allow for the observation of changes in parent concentration and any subsequent changes in metabolite concentration. The enzyme inhibition was monitored using a fluorescence plate reader manufactured by BioTek (Winooski, VT).

The enzyme screening kit utilizes the fluorescent property of 3-[2-(N,N-diethyl-N-methylamino)ethyl]-7-methoxy-4-methylcoumarin (AMMC) metabolism to investigate cathinone analogue inhibition of CYP2D6 activity. The compound of interest inhibits CYP2D6 and the AMMC is not converted to its fluorescent metabolite 3-[2-(N,N-diethyl-N-methylammonium)ethyl]-7-hydroxy-4-methylcoumarin (AMHC) producing a lower intensity of fluorescence which can be observed with the fluorescence plate reader. The assay protocol was essentially as described by the manufacturer. The analyte of interest, at concentrations varying from 1μM to .003μM, was combined with a cofactor mix in a 96-well plate and incubated for ten minutes to prepare the solution for the enzyme. The enzyme was added and the plate was covered and incubated for thirty minutes in an oven at 37^{o}C and then the reaction was stopped with a Tris Base stop solution. Each plate also contained the appropriate enzyme and assay controls (See Figure 5). The plate was then

analyzed by a plate reader (390nm excitation and 460nm emission) to determine fluorescence intensity of each well.

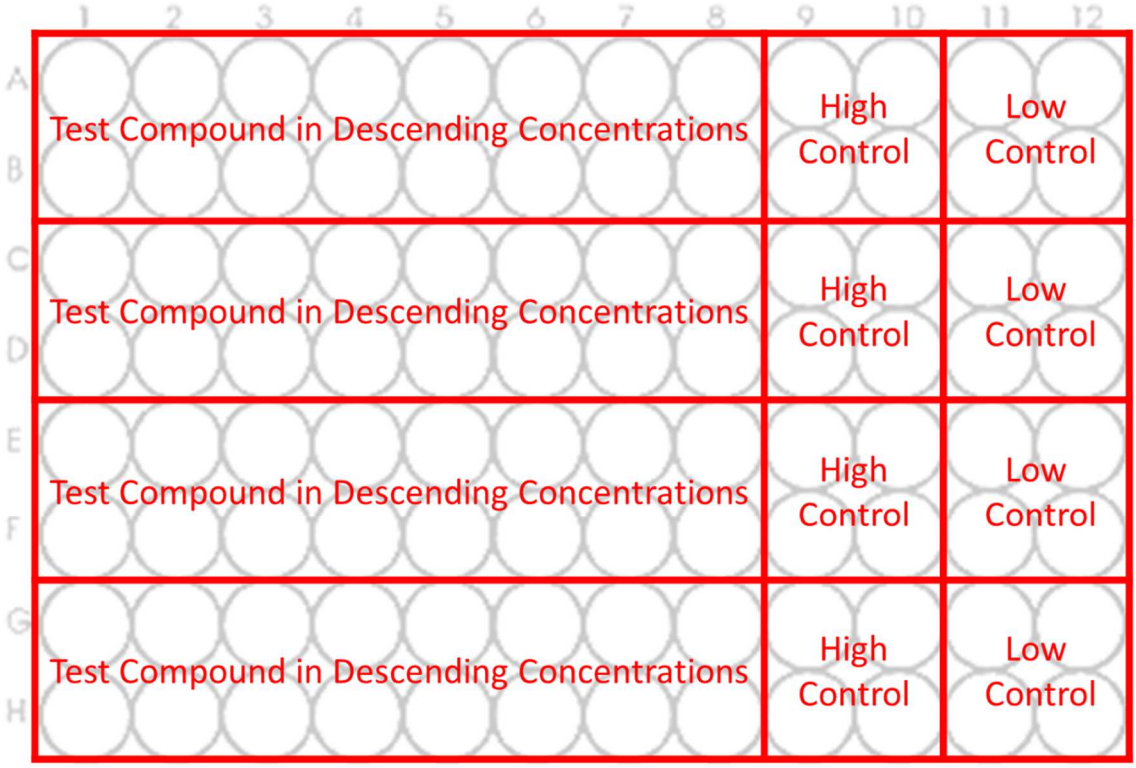

Figure 5. Diagram of CYP2D6 inhibitor screening kit plate layout.

3.5 Microsomal Preparation

A Corning Mammalian Liver Cytosol Assay was used to investigate synthetic cathinone metabolism in the presence of twenty metabolic enzymes (Table 3) over the course of an hour. The microsomal preparation was altered from manufacturer specifications to provide a higher instrument response when the samples were analyzed and for a greater enzyme action. 8.4uL of MEPH or BUPH drug stock (1 mg/ml) was diluted with HPLC water to a final concentration of 2.8µg/ml. Both cathinones were

Table 3. List of enzymes present in the microsomal preparation.

Microsomal Preparation Enzymes		
OR	CYP2C19	UGT1A4
Cytochrome b_5	CYP2D6	UGT1A6
CYP1A2	CYP2E1	UGT1A9
CYP2A6	CYP3A4	UGT2B7
CYP2B6	CYP4A11	CYP3A4
CYP2C8	FMO	CYP3A5
CYP2C9	UGT1A1	

diluted to a final concentration of 2μg/ml after the addition of all of the solutions which included: 500mM potassium phosphate buffer, acetyl-CoA, and PAPS then incubated at 37^{o}C for ten minutes to prepare the solution for the addition of the enzyme mixture. After incubation, the enzyme mixture was added to each well except the zero time point. The wells were incubated for an hour and specific wells were stopped with 100 μL of acetonitrile at 20, 40, and 60 minute time points to observe the drug-enzyme action over time (Figure 6). The wells were diluted according to the LC-MS/MS sample preparation (as discussed previously), and analyzed to determine the concentration of synthetic cathinone after incubation.

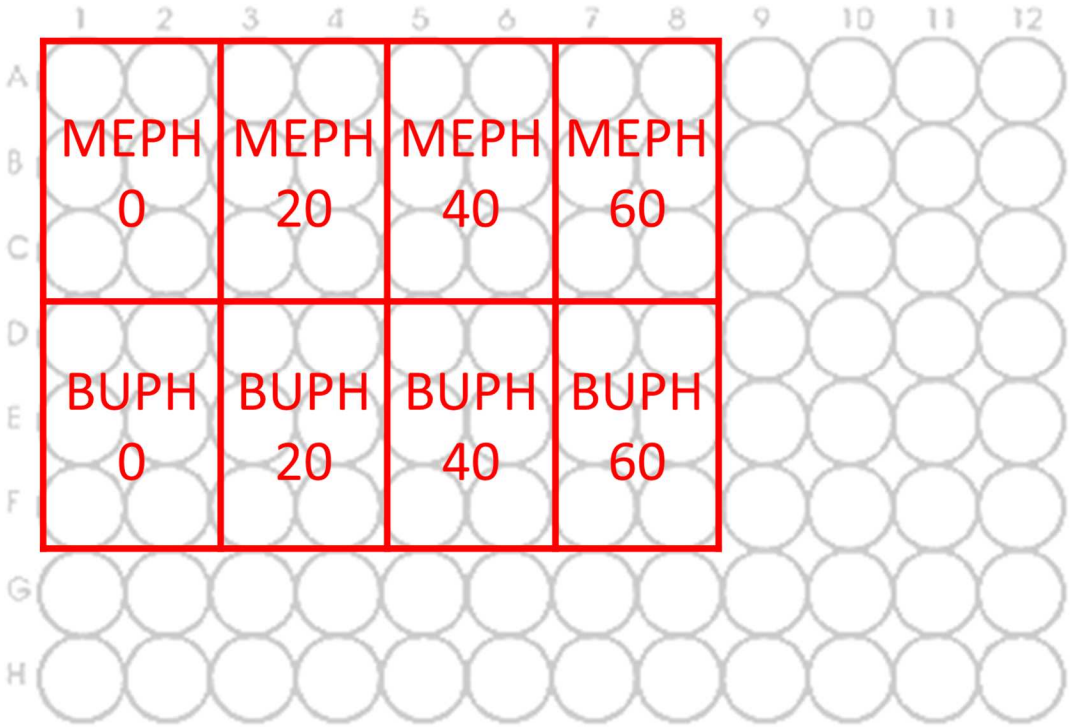

Figure 6. Diagram of microsomal preparation plate layout.

3.6 Data Analysis

The LC-MS/MS quantitation method efficiency was evaluated based on the accuracy and the upper and lower limits of detection. Limits of detection include the upper limit where the detector of the MS becomes saturated and is no longer able to accurately determine the concentration of analyte. The lower limit of detection is the smallest concentration of drug able to be accurately quantified. Accuracy refers to the method's ability measure a cathinone concentration close to that of the true value through the use of spiked samples of known concentration. A calibration curve was prepared at seven different concentrations ranging from 25ng/mL to 1ng/mL and quality control solutions were prepared separately at 15, 7.5, and 2 ng/mL to evaluate the accuracy of the concentrations being calculated. Calibrators were considered accurate if they were

calculated to be within 15% of their prepared concentration while quality controls were considered accurate if they were within 20% of their prepared concentration. Only after the LC-MS/MS was demonstrated to be accurate was it used in the microsomal preparation study.

The change in fluorescent intensity was used to determine the concentration of the inhibitor that inhibited 50% of the AMMC conversion to its fluorescent product, AMHC (IC50). The IC50s of each analyte (quinidine, cimetidine, MEPH, and BUPH) were compared statistically using nonlinear curve fitting for using GraphPad Prism Software (v6.03, San Diego, CA). The assumption of normal distribution was not made, and analyses were performed using Kruskal-Wallis analysis of variance. Potential outliers within the data were examined with the Grubb's test (α=0.05) prior to Kruskal-Wallis analysis. In the microsomal assays, one-way analysis of variance (1-way ANOVA) was performed to determine if the concentrations of the parent drug changed over time (simple time-effect).

3.7 Methods Summary

This method was systematically developed using known cathinone calibrators available commercially. Equipment, materials, and instrumentation are common and obtainable by a variety of clinical and forensic laboratories, allowing them to apply any derived methodology. Cathinones were diluted using a specific sample preparation to provide optimal peak shape and protect the instrumentation and then injected onto an LC-MS/MS for quantitation. After chromatography parameters had been established and cathinones had been successfully detected, the method was used to study solutions after

microsomal preparation to determine which enzymes are acting on MEPH and BUPH in the body.

CHAPTER IV

RESULTS

4.1 METHOD ACCURACY AND LINEARITY

Cathinone analogues are structurally similar (though not identical) from one compound to the next, making chromatographic separation of differing analogues a challenge. Chromatographic separation was the first problem to be addressed, using drug standards in methanol. Adequate chromatographic separation was demonstrated when baseline separation was achieved for each analyte peak. Afterward, prepared calibrator and quality control samples were analyzed to determine the limits of quantitation of the sample preparation being used. All prepared calibrators were diluted according to the protocol outlined in the methods section in order to simulate the conditions of unknown sample solutions. Table 3 lists the limit of quantitation of the sixteen cathinones included in the LC-MS/MS method. Limits were decided based on accuracy, precision, peak shape, and peak quality. After calibrators and quality controls were within the desired accuracy ranges, the LC-MS/MS method was deemed ready for application to the study of synthetic cathinone metabolism.

Table 4. Each synthetic cathinone included in the LC-MS/MS method and the corresponding lower limit of quantitation for each analyte.

Compound Name	Lower Limit of Quantitation
3-Fluoromethcathinone	1 ng/ml
(±)-3-Fluoromethcathinone Ephedrine Metabolite	1 ng/ml
(±)-4-Methylephedrine (Mephedrone Metabolite)	1 ng/ml
Alpha-PVP	2.5 ng/ml
Buphedrone	1 ng/ml
Buphedrone Ephedrine Metabolite	5 ng/ml
Butylone	1 ng/ml
Ethylone	10 ng/ml
Mephedrone	1 ng/ml
Methedrone	1 ng/ml
Methylone	1 ng/ml
Naphyrone	1 ng/ml
N-Ethylcathinone	1.5 ng/ml
(±)-N-Ethylcathinone Ephedrine Metabolite	1.5 ng/ml
Pentylone	1 ng/ml
Pyrovalerone	1.5 ng/ml

4.2 CYP2D6 INHIBITOR SCREENING RESULTS

The investigation of MEPH and BUPH metabolism started with an investigation into their activity on the liver enzyme typically associated with the metabolism and elimination of many xenobiotics, CYP2D6. Quinidine, cimetidine, MEPH, and BUPH inhibition of CYP2D6 was investigated using a Corning CYP2D6 inhibitor screening kit with the intent of determining their respective affinities to CYP2D6. The activity of quinidine on CYP2D6 activity was investigated since it is reportedly one of the most potent of CYP2D6 inhibitors. Cimetidine was included as a classic over-the-counter drug that has been shown to interfere with both CYP3A4 and CYP2D6 activity as an inhibitor. The representative graph below shows quinidine's inhibition curve replicates (Figure 7) as quinidine competes with AMMC for enzyme active sites. The other inhibitors examined

(cimetidine, MEPH, and BUPH) also have inhibition curves graphed in a similar manner. Predictably, the lowest concentration of test compound yields the highest fluorescence, as AMMC will occupy more CYP2D6 active sites and is converted to fluorescent AHMC.

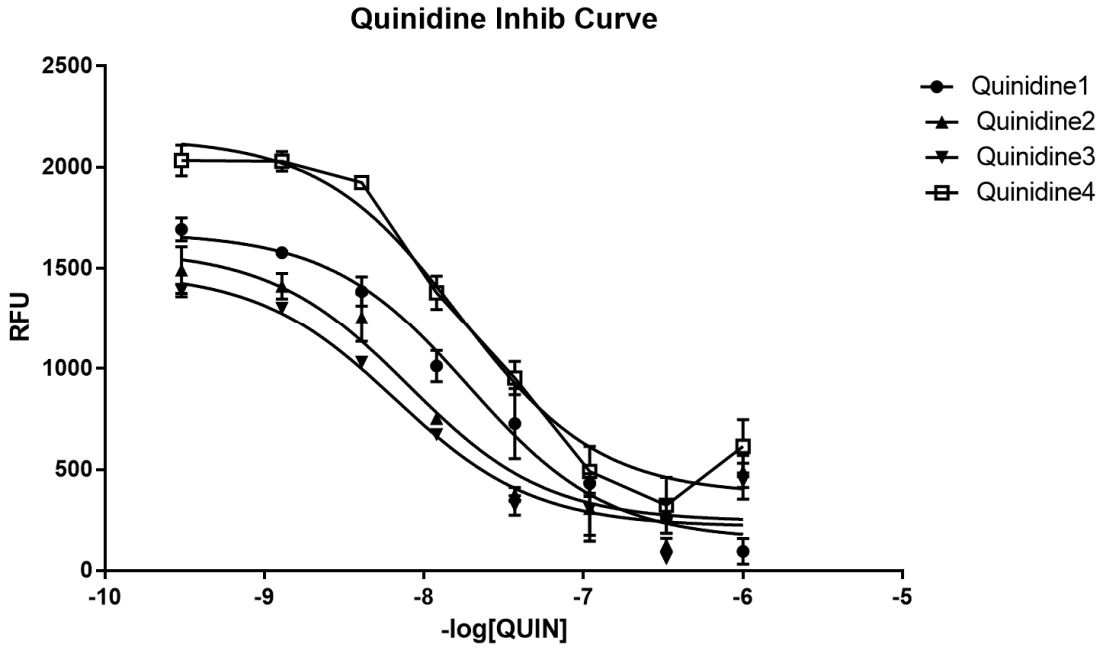

Figure 7. Nonlinear curve fitting for 4 independent assays using quinidine. Data were fit by GraphPad Prism using nonlinear regression analysis and from these plots, individual IC50 values can be determined. Each data point is the average of duplicate wells.

Collectively, analysis for each drug can then be plotted for graphical representation of CYP2D6 inhbition. Quinidine (N=4), cimetidine (N=4), MEPH (N=5), and BUPH (N=5) were all analyzed in duplicate (Figure 8).

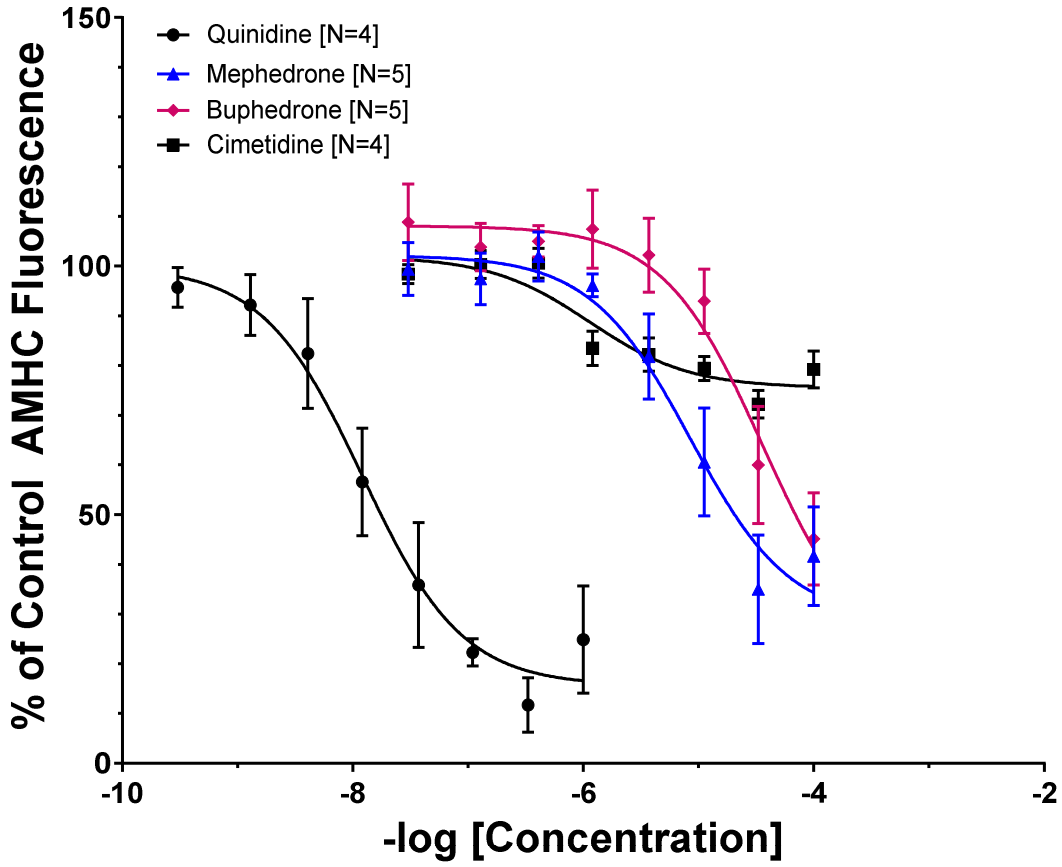

Figure 8. Composite graph of the log [analyte concentration] versus the percentage of total fluorescence observed.

Percent inhibition was calculated by subtracting the fluorescence of the blank sample (baseline fluorescence) from the fluorescence intensity at a given point (inhibited fluorescence). Dividing the resulting value by the quantity of the fluorescence achieved in the sample without inhibitor (control CYP2D6 activity) minus baseline fluorescence (see equation below).

$$\%Fluorescence = \frac{(inhibited\ fluorescence - background\ fluorescence)}{(uninhibited\ fluorescence - background\ fluorescence)}\ x\ 100\%$$

Figure 9 shows a comparison of the maximum inhibition of CYP2D6 (which would occur at the highest concentrations of inhibitor and exhibits the lowest fluorescence intensity. Maximum inhibition is a measure of drug's efficacy towards an enzyme active site.

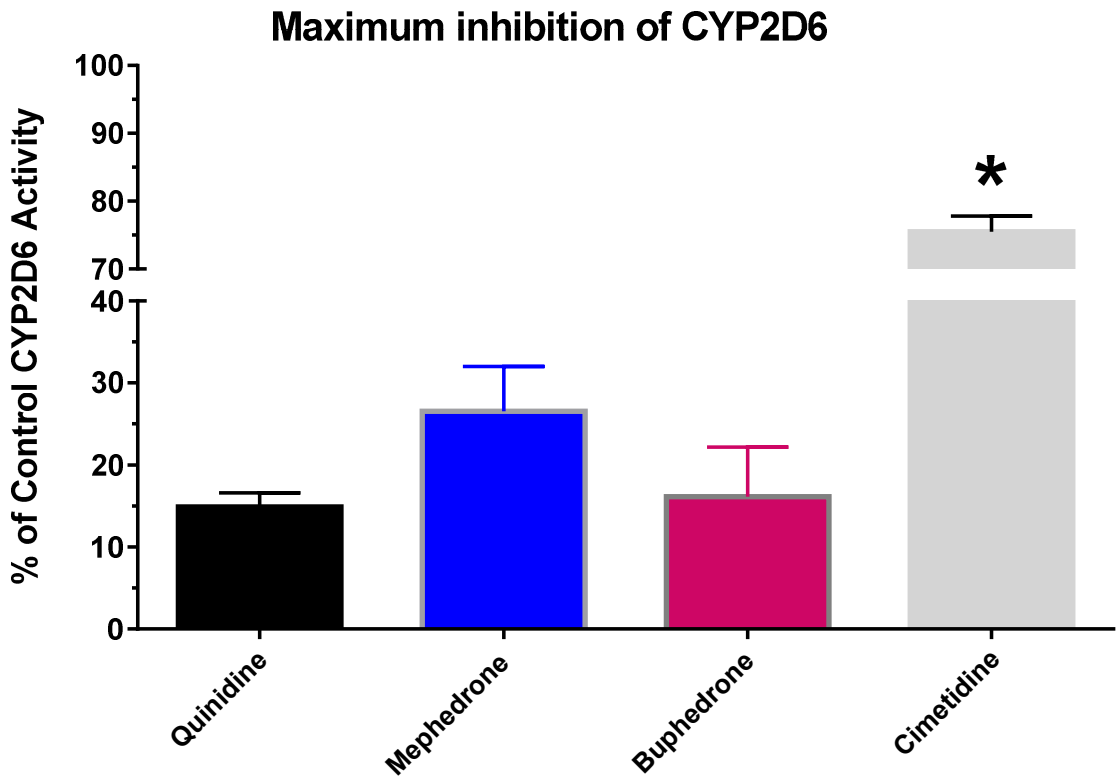

Figure 9. Graph comparing the maximum inhibition of CYP2D6 by the four drugs examined ($F_{(3, 14)}$=12.46; p=0.006). Cimetidine shows a statistically significant difference from the other three (*p<0.05).

The graph shows the cimetidine is significantly different from the other three inhibitors (p<.05). Meaning that high concentrations of cimetidine will not as readily inhibit CYP2D6 and lead to the higher fluorescence observed at high concentrations of cimetidine.

The IC50 values for each compound were calculated from the fluorescence data using an equation provided with the inhibition kit literature by Corning:

$$IC50 = \frac{(50\% - Low\%Inhibition)}{(High\%Inhibition - Low\%Inhibition)} X(High\ Conc. - Low\ Conc.) + Low\ Conc.$$

The IC50 value provides a measure of drug efficiency by representing the concentration of drug that is needed to inhibit CYP2D6 function by half. The quinidine IC50 was determined to be 12.61±3.4 nM, cimetidine was 1.1±.12 µM, MEPH was 10.1±1.0 µM, and BUPH was 61.7±16.0 µM (See Figure 10).

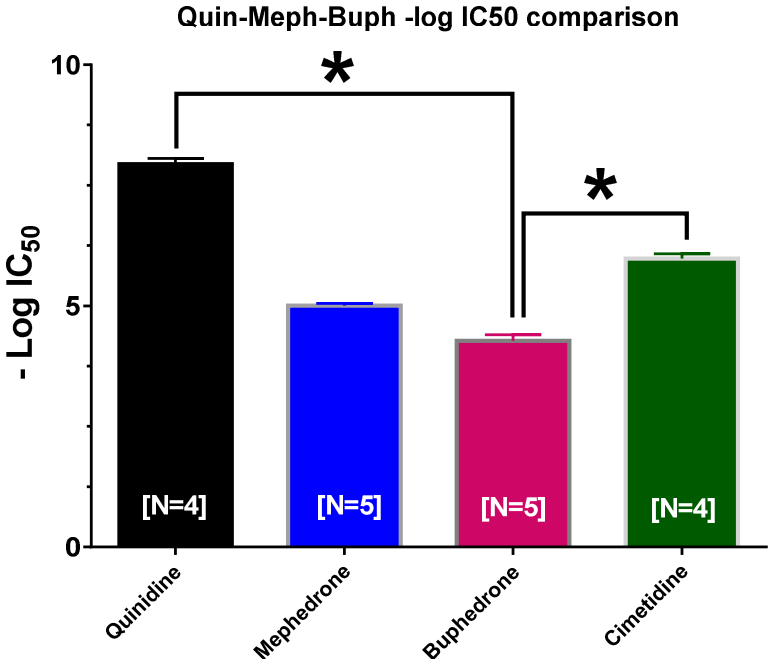

Figure 10. Quinidine IC50 was determined to be 12.61±3.4 nM, cimetidine was 1.1±.12 µM, mephedrone was 10.1±1.0 µM, and buphedrone was 61.7±16.0 µM. A comparison of the calculated IC50 values was performed using a Kruskal-Wallis nonparametric 1-way ANOVA with Dunn's Test for multiple comparisons ($F_{(3,14)}$=12.95; p=.0012). Buphedrone determined to be significantly different with from quinidine and cimetidine (*p<.05).

The IC50 value of BUPH was significantly different ($p<0.05$) from both cimetidine and quinidine using the Kruskal-Wallis nonparametric ANOVA with Dunn's Test for multiple comparisons. A higher concentration of BUPH is required to inhibit 50% of CYP2D6 activity when compared to the other three drugs. All four drugs yielded positive results for inhibition of CYP2D6 and cathinone metabolism was investigated further using a microsomal preparation that consisted of twenty hepatic enzymes for evidence of metabolism.

4.3 MICROSOMAL PREPARATION RESULTS

Human liver microsomes were incubated with MEPH or BUPH at 2µg/ml in duplicate (N=3) for 0, 20, 40, and 60. The concentration of each drug was determined using the developed LC-MS/MS method with the same standard curve Figures 11 and 12 illustrate the changes in concentration of both drugs over the course of an hour.

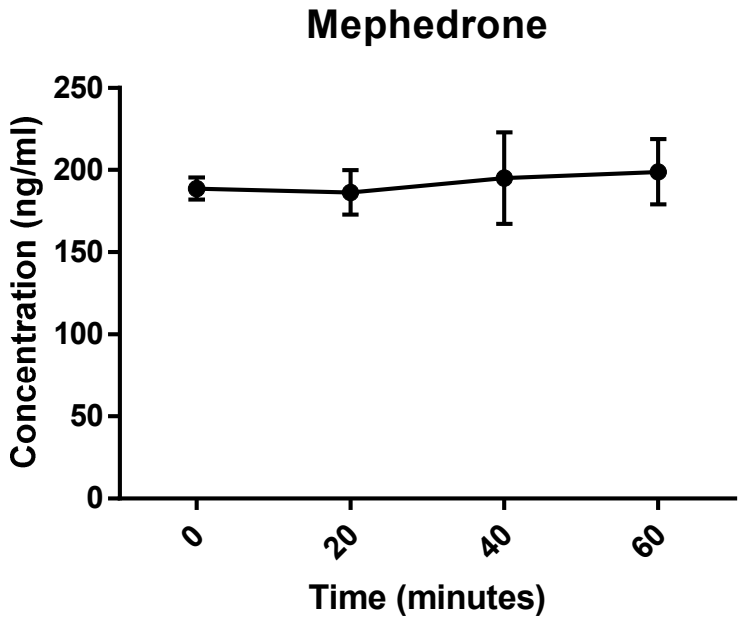

Figure 11. Plot of the change in mephedrone concentration over course of a one hour incubation with the hepatic enzyme mix.

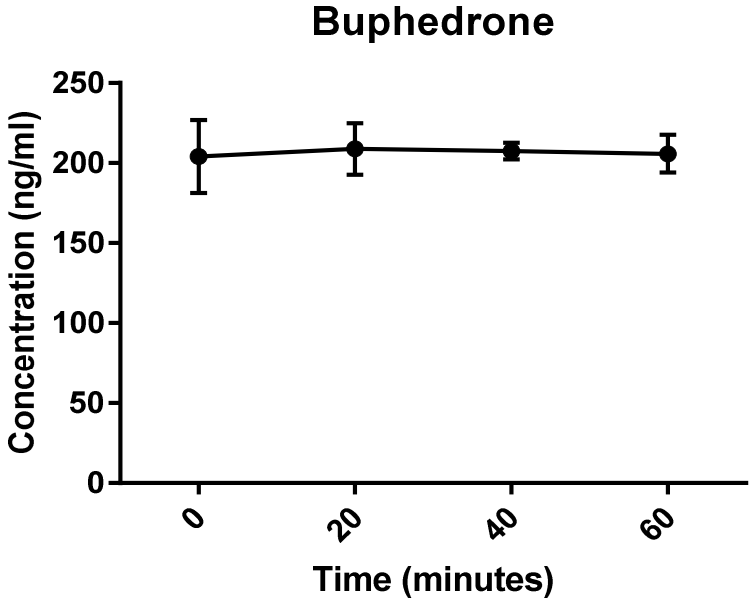

Figure 12. Plot of the change in buphedrone concentration over course of a one hour incubation with the hepatic enzyme mix.

There were no significant changes in either MEPH or BUPH contraction as a function of time (1-way ANOVA; F=.6447 and .0966 for MEPH and BUPH respectively).

4.4 CONCLUSION

The results demonstrate that the developed LC-MS/MS method is linear from a lower limit that ranges from 1 to 10 ng/ml (depending on the analyte) to an upper limit 25ng/ml for all analytes. The CYP2D6 inhibition assay demonstrated that quinidine, cimetidine, BUPH, and MEPH all inhibited CYP2D6 activity in a concentration-dependent manner. There was no apparent loss of either cathinone following incubation with the human liver microsomal preparation. One possible reason for this lack of effect would be that neither compound is directly metabolized by CYP2D6. Assays were performed essentially as described by the manufacturer. A possible confounding factor with these findings is a non-optimized reaction. It is possible that CYP2D6 protein content in the microsomes was too small to adequately metabolize either cathinone. Further study would be needed to examine the various parameters involved with optimizing the assay. The causes and implications of these findings will be discussed in the next chapter.

CHAPTER V

DISCUSSION

5.1 CATHINONE-ENZYME INTERACTION

The purpose of this research was to develop a method of detection for synthetic cathinones and to use the method to support an investigation of synthetic cathinone metabolism. The concentration of neither BUPH nor MEPH decreased when incubated with the hepatic enzyme mixture; neither cathinone was metabolized by the enzymes present. However, both compounds inhibited CYP2D6 metabolism of AMMC in the inhibition assay study with IC50 value of 10.1±1.0μM for MEPH and 61.7±16.0μM for BUPH. When considered in conjunction, these two experiments suggest that the synthetic cathinones tested inhibited CYP2D6 activity but are not metabolized. This could mean that the cathinones are affecting the catalytic site but are not being metabolized, or that they are affecting a site away from the catalytic site and are allosterically inhibiting enzyme action.

Synthetic cathinones are relatively novel compounds and their research can be a difficult task because they are scheduled controlled substances. The research available that investigates cathinone metabolism specifically is even more infrequent, attesting to this study's value as a contribution to the literature available on these dangerous compounds as they affect the body. The data in this study contradict an earlier study which have reported

CYP2D6 as the primary enzyme responsible for the metabolism of MEPH.[29] Differing enzyme concentrations, reaction times, or reaction cofactors could account for the discrepancies between the studies. The metabolic pathway of BUPH has yet to be investigated, as a result, this study provides previously unreported insight into the role of BUPH as a CYP2D6 inhibitor.

5.2 IMPLICATIONS OF CATHINONES AS CYP2D6 INHIBITORS

Drug-drug interactions are a constant concern to medical treatment officials. A medical history of all prescriptions and drugs taken recently is collected with any patient that enters the care of a physician, including recreational drug use. When the scientific community does not know how synthetic cathinones affect the body, or how the body modifies cathinones, detecting the drugs and determining the best course of treatment becomes a significant challenge. With nearly 1.3 million visits to emergency departments every year attributed to abused substances,[3] these compounds are commonplace in emergency department settings. Illicit drugs are not screened for drug-drug interactions as pharmaceuticals are, and therefore pose a significant risk to patients treated for injuries or illness while under the effects these dangerous compounds, especially when their metabolism is not well understood.

When investigating drug metabolism, phase I reactions are a likely starting point for small molecules. Some key CYPs include CYP3A4, CYP2C9 and CYP2D6. CYP2D6 is an enzyme that metabolizes a large number of xenobiotics (~25%) and adversely affecting its function can lead to toxic drug reactions resulting in patient injury.[34] The metabolic mechanism of synthetic cathinones is widely uncharacterized, leaving

individuals who take them susceptible to drug-drug interactions that can lead to adverse drug effects that can be life-threatening.

For example, tramadol and morphine, two drugs which can be used in hospital settings to treat a patient's pain should they suffer injury while under the effects of synthetic cathinones (such as during an excited delirium), are both metabolized by CYP2D6.[35-36] If a patient were exposed to synthetic cathinones and subsequently administered tramadol by a health professional (or another drug metabolized by CYP2D6) to treat any injury secondary to cathinone exposure, the substrate will remain in the plasma for a longer duration due to the inhibition of CYP2D6 by synthetic cathinones. A heightened tramadol concentration in the plasma can lead to symptoms typically associated with an overdose such as lethargy, nausea, tachycardia, or more serious symptoms if the drug-drug interaction is not anticipated.[37] By presenting emergency physicians and medical professionals with a characterization of synthetic cathinone metabolism, adverse drug reactions due to drug interaction may be prevented.

Additionally, many synthetic cathinone users are polydrug users, taking more than one drug at a time leaving them vulnerable to illicit drug-drug interactions long before they are treated for exposure.[31-32] Methamphetamine, for example, acts as a weakly binding substrate for CYP2D6 but approximately 50% of all methamphetamine metabolites excreted utilize CYP2D6.[38-39] CYP2D6 is responsible for half of the elimination of any methamphetamine consumed. When taken alongside synthetic cathinones, methamphetamine will stay in the plasma longer, increasing the likelihood that the user will experience adverse drug reactions and overdose. However, it is a possibility that other metabolic pathways would be capable of making up for the inhibition of CYP2D6 by

metabolizing more of the methamphetamine. The interaction between synthetic cathinones and methamphetamine (and other drugs such as alcohol, over the counter drugs, or drugs that may be consumed simultaneously with synthetic cathinones) needs to be investigated further.

While there was no metabolic activity observed in the hepatic enzyme mixture, there is likely activity with other enzymes. Synthetic cathinone metabolites have been observed in urine[28,30] suggesting that between ingestion and excretion, an enzymatic process (or another non-enzymatic process such as spontaneous degradation in plasma) is modifying the compound; however, currently that process is not known. The enzymes tested are those involved in Phase I metabolism, which attempts to introduce polar groups onto substrates to increase the water solubility of the compound and increase its rate of elimination.[40] It is a possibility that synthetic cathinones are acted on by enzymes involved primarily in Phase II metabolism, which typically involves conjugating xenobiotics and making them less active before excretion.[41] Although this project did not investigate the metabolism of the synthetic cathinone MDPV, Strano-Rossi et al. demonstrated that the main metabolites of MDPV were sulfated and glucoronated.[42] MEPH or BUPH could follow a similar metabolic pathway. The more information available to scientists and medical professionals about the metabolism of synthetic cathinones, the better prepared they are to handle individuals who are under the influence of these dangerous drugs.

5.3 Future Work

While there were several questions answered by this project concerning cathinone enzymatic activity, there are many other questions about synthetic cathinone metabolism that have yet to be addressed. It was discovered that synthetic cathinones act as a CYP2D6 inhibitor, yet the mechanism of inhibition is still unknown. A competitive inhibition would arise when the cathinone occupies the active site of the enzyme, preventing the substrate from accessing the site. Noncompetitive inhibition mechanism can involve an inhibitor that binds at a site separate from the substrate active site but still hampering enzyme function by allosterically altering the active site and inhibiting substrate binding. Noncompetitive inhibition could alternatively involve a cathinone covalently binding to the enzyme and inhibiting substrate binding. Determining the mechanism of inhibition could help physicians handle individuals who are exposed to synthetic cathinones more effectively. Knowing that the CYP2D6 enzyme is blockaded (and how it is blockaded) may influence treatment decisions. While tramadol and morphine are opioids which utilize CYP2D6, ketamine which provides pain relief and sedation and is metabolized primarily by CYP3A4 can be a useful alternative to avoid adverse drug effects.[43]

Further research to identify the enzyme responsible for synthetic cathinone metabolism and elimination also has many applications. An understanding of cathinone metabolism can lead to an explanation of the neurological and cardiac symptoms observed in individuals following such synthetic cathinone exposure, such as excited delirium which poses a danger to both exposed individuals and those around them.[23,27] Forensically, the detection of a drug metabolite is often preferable because it allows investigators to detect illicit drug exposure long after the parent drug has been metabolized and is no longer found

in biological samples. By identifying the enzymes involved in synthetic cathinone metabolism, it may give investigators the information they need to locate detectable metabolites and expand their window of synthetic cathinone detection. Additionally, identification of the enzymes responsible for cathinone metabolism can help predict cathinone's affect when taken simultaneously with other drugs and aid death investigations.

Another avenue of exploration includes the effects of other cathinone analogues on CYP2D6 (and other enzymes). Because they have different structures it is likely that they will react with the body differently from one analogue to the next. This could be in the form of varying affinities for CYP2D6, or action on another enzyme altogether. Different metabolic pathways would likely lead to varying physiological effects such as different symptoms observed, varying symptom intensity, and varying symptom duration. While the developed method has proven capable of distinguishing one cathinone analogue from the next during sample analysis, there is not information available on how the different analogues are going to affect someone after ingestion.

These are just a few of many avenues of research that could be explored on the topic of synthetic cathinones and their metabolism. Because such little information about their enzymatic activity currently exists, the field is wide open to future projects. With the addition of new cathinone analogues every year, this field is only going to expand.

5.4 Discussion Summary

This research illustrates that synthetic cathinones, specifically BUPH and MEPH act as CYP2D6 inhibitors by observing their enzymatic action. As an inhibitor of an

enzyme that metabolizes many xenobiotics, synthetic cathinones present a significant risk for drug-drug interactions. By studying the metabolic effects healthcare professionals can account for the inhibition of CYP2D6 and prevent adverse drug reactions. Both forensic experts and medical professionals would benefit from research further studying synthetic cathinone metabolism and identify the enzymes involved in its metabolism and elimination.

CHAPTER VI

CONCLUSION

Recent surveys demonstrate that the illicit drug problem affects millions of individuals nationwide. Synthetic cathinones are a dangerous novel drug which present a significant threat to individuals throughout the United States. Detection methods capable of quantifying drug concentration must be updated regularly to adapt to the most recent analogues produced by illicit drug manufacturers as they attempt to avoid detection and legislative repercussions. Synthetic compounds are also metabolically uncharacterized and present a problem as medical professionals attempt to treat for an illicit drug exposure the scientific community knows very little about. This may manifest in unanticipated drug-drug interactions which can potentially be life threatening. This study presents a method of detecting several of the most popular synthetic cathinones and an investigation into their metabolism that addresses the need of both the forensic and medical communities as they attempt to handle individuals who have been exposed synthetic cathinones.

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APPENDICES

A1. Observed fluorescence intensity at their corresponding concentrations of quinidine (n=4). Each repetition was a result of two wells that were averaged (four wells were averaged for the controls). From these values the percent total fluorescence was calculated.

Quinidine:	[μM]							
Concentration:	1	0.33	0.11	0.037	0.012	0.004	0.0013	0.003
Fluorescence Intensity:	7624	7780	7970	8424	8641	9009	9134	9225
	7714	7893	8038	8178	8531	8906	9168	9306
Pooled Intensity:	7669	7836.5	8004	8301	8586	8957.5	9151	9265.5
% Total Fluorescence:	5.62%	15.43%	25.23%	42.62%	59.31%	81.06%	92.39%	99.09%

Concentration:	0		Blank	
Fluorescence Intensity:	9157	9327	7521	7393
	9269	9372	7581	7800
Pooled Intensity:		9281.25		7573.75

Concentration:	1	0.33	0.11	0.037	0.012	0.004	0.0013	0.003
Fluorescence Intensity:	6668	6336	6447	6634	6998	7403	7689	7806
	6781	6384	6633	6585	6974	7572	7600	7642
Pooled Intensity:	6724.5	6360	6540	6609.5	6986	7487.5	7644.5	7724
% Total Fluorescence:	30.60%	7.91%	19.12%	23.44%	46.89%	78.11%	87.89%	92.84%

Concentration:	0		Blank	
Fluorescence Intensity:	7900	7839	6241	6204
	7768	7850	6280	6208
Pooled Intensity:		7839.25		6233.25

(A1 Continued)

Concentration:	1	0.33	0.11	0.037	0.012	0.004	0.0013	0.003
Fluorescence Intensity:	6649	6337	6461	6558	6963	7289	7586	7638
	6775	6322	6684	6626	6922	7313	7559	7682
Pooled Intensity:	6712	6329.5	6572.5	6592	6942.5	7301	7572.5	7660
% Total Fluorescence:	28.69%	3.92%	19.66%	20.92%	43.62%	66.84%	84.42%	90.09%

Concentration:	0		Blank	
Fluorescence Intensity:	7781	7774	6234	6361
	7774	7926	6208	6275
Pooled Intensity:		7813.75		6269.5

Concentration:	1	0.33	0.11	0.037	0.012	0.004	0.0013	0.003
Fluorescence Intensity:	7625	7329	7506	7998	8423	9028	9167	9082
	7811	7525	7681	8115	8540	9026	9099	9190
Pooled Intensity:	7718	7427	7593.5	8056.5	8481.5	9027	9133	9136
% Total Fluorescence:	29.74%	15.67%	23.72%	46.11%	66.66%	93.04%	98.16%	98.31%

Concentration:	0		Blank	
Fluorescence Intensity:	9173	9104	7154	7038
	9218	9191	7174	7047
Pooled Intensity:		9171.5		7103.25

A2. Observed fluorescence intensity at their corresponding concentrations of mephedrone (n=5). Each repetition was a result of two wells that were averaged (four wells were averaged for the controls). From these values the percent total fluorescence was calculated.

Mephedrone:	[μM]							
Concentration:	100	33	11	3.7	1.2	0.4	0.14	0.05
Fluorescence Intensity:	7392	8080	8629	9146	9291	9391	9417	9318
	7277	8339	8540	9163	9427	9849	9271	9600
Pooled Intensity:	7334.5	8209.5	8584.5	9154.5	9359	9620	9344	9459
% Total Fluorescence:	-30.21%	23.47%	46.47%	81.44%	93.99%	110.00%	93.07%	100.12%

Concentration:	0		Blank	
Fluorescence Intensity:	9466	9327	7845	7778
	9532	9504	7953	7732
Pooled Intensity:		9457.25		7827

Concentration:	100	33	11	3.7	1.2	0.4	0.14	0.05
Fluorescence Intensity:	6735	6527	7182	7452	7701	7922	7761	7801
	6748	6604	7044	7305	7878	7768	7803	7715
Pooled Intensity:	6741.5	6565.5	7113	7378.5	7789.5	7845	7782	7758
% Total Fluorescence:	33.61%	23.57%	54.79%	69.93%	93.36%	96.52%	92.93%	91.56%

Concentration:	0		Blank	
Fluorescence Intensity:	7854	7967	6203	6099
	7880	7925	6185	6123
Pooled Intensity:		7906.5		6152.5

(A2 Continued)

Concentration:	100	33	11	3.7	1.2	0.4	0.14	0.05
Fluorescence Intensity:	6717	6812	7064	7476	7964	7826	7752	7705
	6631	6701	7237	7617	7807	8066	7923	8317
Pooled Intensity:	6674	6756.5	7150.5	7546.5	7885.5	7946	7837.5	8011
% Total Fluorescence:	33.79%	38.14%	58.91%	79.78%	97.65%	100.84%	95.12%	104.27%

Concentration:	0		Blank	
Fluorescence Intensity:	7985	7788	6145	5898
	8004	7943	6160	5929
Pooled Intensity:		7930		6033

Concentration:	100	33	11	3.7	1.2	0.4	0.14	0.05
Fluorescence Intensity:	7822	7668	8065	8554	8619	8710	8751	8754
	7818	7713	8185	8553	8665	8693	8708	8741
Pooled Intensity:	7820	7690.5	8125	8553.5	8642	8701.5	8729.5	8747.5
% Total Fluorescence:	54.17%	47.20%	70.60%	93.67%	98.44%	101.64%	103.15%	104.12%

Concentration:	0		Blank	
Fluorescence Intensity:	8733	8615	6857	6762
	8660	8678	6963	6674
Pooled Intensity:		8671.5		6814

Concentration:	100	33	11	3.7	1.2	0.4	0.14	0.05
Fluorescence Intensity:	7876	7776	8251	8569	8676	8826	8856	8657
	7701	7708	8353	8491	8877	8860	8916	8891
Pooled Intensity:	7788.5	7742	8302	8530	8776.5	8843	8886	8774
% Total Fluorescence:	45.13%	42.68%	72.16%	84.16%	97.13%	100.63%	102.89%	97.00%

Concentration:	0		Blank	
Fluorescence Intensity:	8845	8892	7019	6969
	8875	8715	6911	6828
Pooled Intensity:		8831.75		6931.75

A3. Observed fluorescence intensity at their corresponding concentrations of buphedrone (n=5). Each repetition was a result of two wells that were averaged (four wells were averaged for the controls). From these values the percent total fluorescence was calculated.

Buphedrone:	[μM]							
Concentration:	100	33	11	3.7	1.2	0.4	0.14	0.05
Fluorescence Intensity:	7340	7693	9129	8218	8064	8126	8004	8201
	7343	7501	8283	8304	8236	8188	7898	8295
Pooled Intensity:	7341.5	7597	8706	8261	8150	8157	7951	8248
% Total Fluorescence:	56.91%	73.04%	143.06%	114.96%	107.95%	108.40%	95.39%	114.14%

Concentration:	0		Blank	
Fluorescence Intensity:	7901	8199	6454	6483
	7884	8113	6365	6459
Pooled Intensity:		8024.25		6440.25

Concentration:	100	33	11	3.7	1.2	0.4	0.14	0.05
Fluorescence Intensity:	7374	7651	8204	8033	8294	8166	8204	8296
	7165	7290	8126	8159	8265	8140	8278	8311
Pooled Intensity:	7269.5	7470.5	8165	8096	8279.5	8153	8241	8303.5
% Total Fluorescence:	46.12%	58.40%	100.86%	96.64%	107.85%	100.12%	105.50%	109.32%

Concentration:	0		Blank	
Fluorescence Intensity:	8069	8143	6387	6313
	8192	8201	6984	6376
Pooled Intensity:		8151.25		6515

(A3 Continued)

Concentration:	100	33	11	3.7	1.2	0.4	0.14	0.05
Fluorescence Intensity:	7340	7500	8118	8153	8465	8139	8282	8309
	7148	6697	7558	8066	8366	8190	8148	8484
Pooled Intensity:	7244	7098.5	7838	8109.5	8415.5	8164.5	8215	8396.5
% Total Fluorescence:	49.94%	41.34%	85.05%	101.09%	119.18%	104.34%	107.33%	118.06%

Concentration:	0		Blank	
Fluorescence Intensity:	8078	8134	6552	6319
	8080	8073	6434	6291
Pooled Intensity:		8091.25		6399

Concentration:	100	33	11	3.7	1.2	0.4	0.14	0.05
Fluorescence Intensity:	8073	8473	9126	9382	9494	9384	9393	9480
	8025	8532	9218	9342	9408	9600	9499	9352
Pooled Intensity:	8049	8502.5	9172	9362	9451	9492	9446	9416
% Total Fluorescence:	39.97%	61.07%	92.23%	101.07%	105.21%	107.12%	104.98%	103.58%

Concentration:	0		Blank	
Fluorescence Intensity:	9272	9397	7183	7188
	9199	9489	7307	7083
Pooled Intensity:		9339.25		7190.25

Concentration:	100	33	11	3.7	1.2	0.4	0.14	0.05
Fluorescence Intensity:	8019	8675	9228	9368	9354	9590	9619	9438
	7877	8713	9393	9409	9427	9537	9546	9419
Pooled Intensity:	7948	8694	9310.5	9388.5	9390.5	9563.5	9582.5	9428.5
% Total Fluorescence:	32.68%	66.07%	93.67%	97.16%	97.25%	104.99%	105.84%	98.95%

Concentration:	0		Blank	
Fluorescence Intensity:	9399	9469	7279	7237
	9385	9556	7233	7126
Pooled Intensity:		9452.25		7218.75

A4. Observed fluorescence intensity at their corresponding concentrations of cimetidine (n=4). Each repetition was a result of two wells that were averaged (four wells were averaged for the controls). From these values the percent total fluorescence was calculated.

Cimetidine:	[μM]							
Concentration:	100	33	11	3.7	1.2	0.4	0.14	0.05
Fluorescence Intensity:	8938	9140	9267	9325	9334	10042	9444	9930
	8794	9186	9242	9275	9378	10055	9198	9961
Pooled Intensity:	8866	9163	9254.5	9300	9356	10048.5	9321	9945.5
% Total Fluorescence:	63.95%	77.62%	81.84%	83.93%	86.51%	118.39%	84.90%	113.65%

Concentration:	0		Blank	
Fluorescence Intensity:	9469	9560	7447	7447
	9749	9821	7508	7508
Pooled Intensity:		9649.75		7477.5

Concentration:	100	33	11	3.7	1.2	0.4	0.14	0.05
Fluorescence Intensity:	8846	9091	9232	9392	9194	9866	9240	9879
	9079	9252	9213	9277	9280	10079	9269	9827
Pooled Intensity:	8962.5	9171.5	9222.5	9334.5	9237	9972.5	9254.5	9853
% Total Fluorescence:	71.24%	81.61%	84.14%	89.70%	84.86%	121.36%	85.73%	115.43%

Concentration:	0		Blank	
Fluorescence Intensity:	9184	9301	7538	7538
	9169	9278	7601	7601
Pooled Intensity:		9233		7569.5

(A4 Continued)

Concentration:	100	33	11	3.7	1.2	0.4	0.14	0.05
Fluorescence Intensity:	8918	9092	9221	9293	9117	9855	9217	9927
	9060	9279	9215	9282	9334	10156	9362	10035
Pooled Intensity:	8989	9185.5	9218	9287.5	9225.5	10005.5	9289.5	9981
% Total Fluorescence:	69.49%	77.90%	79.29%	82.26%	79.61%	112.99%	82.35%	111.94%

Concentration:	0		Blank	
Fluorescence Intensity:	9514	9247	7518	7518
	9230	9275	7536	7536
Pooled Intensity:		9316.5		7527

Concentration:	1	0.33	0.11	0.037	0.012	0.004	0.0013	0.003
Fluorescence Intensity:	8073	8473	9126	9382	9494	9384	9393	9480
	8025	8532	9218	9342	9408	9600	9499	9352
Pooled Intensity:	8049	8502.5	9172	9362	9451	9492	9446	9416
% Total Fluorescence:	39.97%	61.07%	92.23%	101.07%	105.21%	107.12%	104.98%	103.58%

Concentration:	0		Blank	
Fluorescence Intensity:	9293	9330	7213	7434
	9247	9370	7415	7364
Pooled Intensity:		9310		7356.5

A5. Observed concentrations from the microsomal preparation over a one hour incubation. Each repetition (n=3) was from two pooled wells.

Buphedrone

Time (min)	Observed Concentration (ng/ml)	Pooled Concentration (ng/ml)	Mean Concentration (ng/ml)
0	191.70	185.10	204.05
	178.50		
	196.80	201.40	
	206.00		
	205.90	225.65	
	245.40		
20	207.00	200.40	208.78
	193.80		
	197.90	199.40	
	200.90		
	214.90	226.55	
	238.20		
40	200.40	206.20	207.63
	212.00		
	203.40	207.60	
	211.80		
	209.10	209.10	

60	201.70	212.40	204.15
	223.10		
	196.30	204.25	
	212.20		
	195.80	195.80	

A6. Observed concentrations from the microsomal preparation over a one hour incubation. Each repetition (n=3) was from two pooled wells.

Mephedrone

Time (min)	Observed Concentration (ng/ml)	Pooled Concentration (ng/ml)	Mean Concentration (ng/ml)
0	202.10	194.30	188.73
	186.50		
	188.10	185.55	
	183.00		
	186.90	186.35	
	185.80		
20	166.00	181.40	186.42
	196.80		
	182.50	185.30	
	188.10		
	180.40	192.55	
	204.70		
40	202.10	224.15	195.15
	246.20		
	187.60	186.10	
	184.60		
	187.10	175.20	
	163.30		
60	193.30	211.55	198.92
	229.80		
	180.90	199.15	
	217.40		
	184.40	186.05	
	187.70		

VITA

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