IDENTIFYING THE CHALLENGES OF EMERGING NOVEL PSYCHOACTIVE SUBSTANCES

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by

Michael Thomas Truver

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DEDICATION

"I see now that the circumstances of one's birth are irrelevant. It is what you do with the gift of life that determines who you are." – Mewtwo

For my advisor, Madeleine Swortwood, thank you for convincing me to stay here to continue my degree and for all the opportunities and experiences you gave me. I couldn't have done it without you

For my parents, thank you for all the love and support you have given me throughout my entire college career. Thank you for believing in me and helping me in any way possible.

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ABSTRACT

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Novel psychoactive substances (NPS) are compounds similar to common drugs of abuse with slight modifications to their chemical structure. These modifications can be dangerous and sometimes fatal due the lack of knowledge and/or studies of their adverse effects. NPS are sometimes marketed as "legal highs" or "research chemicals" in order to mimic effects of illicit drugs while simultaneously bypassing legislation. Although there are many drugs that are classified as NPS, novel synthetic opioids and synthetic cannabinoids are the focus of this research. Since little is known about these novel psychoactive substances difficulties may arise when analyzing toxicological samples suspected to contain NPS. To address the emergence of novel psychoactive substances, it is necessary to properly identify issues that could hinder analyses by assessing prevalence, examining instability, determining pharmacology, and identifying potential metabolites.

The goals of this study were to 1) develop and validate a method to quantify novel synthetic opioids (NSO), buprenorphine, and heroin markers in oral fluid and apply the method to the analysis of oral fluid collected from detainees; 2) using the acquisition method from the oral fluid method, validate a blood method for the NSO and perform a long term stability study; 3) develop and validate a method to detect a NSO (U-47700) and its metabolites in plasma using a small sample volume (100 µL) and cross validate in rat plasma for a pharmacokinetic study in rats; and 4) identify metabolites for two

prominent synthetic cannabinoids *in vitro* verify metabolism with analysis of authentic urine samples.

A method was validated for the quantification of morphine, 6-acetylmorphine, buprenorphine, U-47700, U-49900, U-50488, AH-7921, MT-45, W-18, and W-15 in oral fluid and was deemed acceptable according to Scientific Working Group for Forensic Toxicology (SWGTOX) guidelines. This method was applied to analysis of oral fluid collected from detainees (n=20) in Texas detention centers participating in a drug recognition evaluatoion (DRE) certification program. Although NSO were not detected, valuable data were collected that reinforced oral fluid as viable matrix when compared to presumptive urine results and impairment observations.

A blood method was validated for seven NSO and then applied to assess the stability of these analytes over a 36-week study at four temperature conditions (-20°C,4°C, 25°C , and 35°C). The results showed minimal effect on stability at the elevated temperature during the first two weeks, indicating that these analytes would be stable in the event of improper transport/handling within this timeframe.

A method was validated for the quantification of U-47700, N-desmethyl-U-47700, and N,N-didesmethyl-U-47700 in human and rat plasma. This method was applied to a pharmacokinetic study where rats were injected with 0 (saline), 0.3, 1.0 or 3.0 mg/kg U-47700. Blood samples were collected at 15, 30, 60, 120, 240, 480 min after injection for quantification of U-47700 and its metabolites. Pharmacodynamic effects were also assessed at the same time points. It was determined that doses of U-47700 had a positive correlation with the behaviors observed which further demonstrates the analgesic effects of this novel synthetic opioid.

Finally, a metabolic study utilizing human liver microsomes was conducted to investigate 5F-MDMB-PICA and 5F-MDMB-PINACA *in vitro* metabolism. *In vitro* metabolites were verified *in vivo* by analyzing authentic case specimens. Additionally, the potency and efficacy of 5F-MDMB-PICA and 5F-MDMB-PINACA were identified by examining activity at the CB_1 receptor. The EC_{50} at the CB_1 receptor for 5F-MDMB-PICA and 5F-MDMB-PINACA were found to be comparable to each other and JWH-018. There were 22 metabolites identified for 5F-MDMB-PICA and 21 metabolites identified for 5F-MDMB-PINACA.

These studies have sought to identify toxicological issues that could arise when detecting a new NPS while providing the necessary data to the forensic toxicology community to further understand the activity and prevalence of such compounds. The challenges that arise when faced with a newly emerged NPS may be detrimental for forensic analysis and thorough characterization of new compounds is necessary for proper identification and detection.

KEY WORDS: Novel Psychoactive Substances, Novel Synthetic Opioids, U-47700, 5F-MDMB-PICA

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CHAPTER I

Introduction

The United States has been plagued by the abuse of illicit drugs for decades. According to the National Institute on Drug Abuse (NIDA), in 2013, 9.4 percent of the American population had used an illicit drug in the past month (1). Just as illicit drugs are abused, prescription drugs can also be habit forming and misused. Drug abuse can sometimes stem from addiction developed from inappropriate prescription drug use. Opioids are one of the drug classes that are commonly misused due to their pain relieving effects. Traditional opioids possess analgesic effects by binding to the μ, δ, and/or κ opioid receptor. The abuse of these addictive drugs can be partially attributed to practitioners over prescribing. According to the Food and Drug Administration (FDA), between 2014 and 2016 over 23 billion dollars were spent on analgesic opioid products (2). To combat this issue, a majority of the state governments in the United States have implemented legislation to produce prescription-drug monitoring programs (3). Although strategies have been formed to mitigate this crisis, drug-seeking individuals have found alternatives to fuel their addiction.

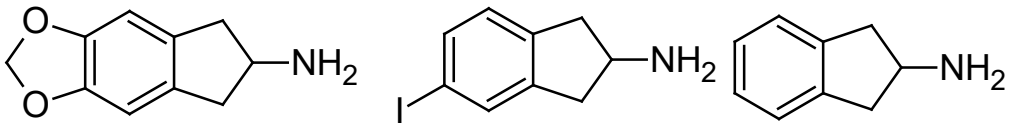
Emergence of New Psychoactive Substances

New psychoactive substances (NPS) are compounds similar to common drugs of abuse with slight modifications to their chemical structure. These modifications can be dangerous and sometimes fatal due the lack of knowledge and/or studies of the adverse effects. NPS are sometimes marketed as “legal highs” or “research chemicals” in order to mimic effects of illicit drugs while simultaneously bypassing legislation. In the United States alone, there were over 300 NPS reported to the United Nation Office on Drugs and

Crime (UNODC) by 2018 (4). NPS include drugs from the following categories: aminoindanes, designer benzodiazepines, dissociatives, psychedelic compounds, piperazines, plant-based substances, synthetic cathinones, synthetic cannabinoids, and novel synthetic opioids (5-7).

Aminoindanes

These compounds were studied in 1973 by Solomons and Sam in which they discovered the analgesic properties these compounds possessed (8). Nichols *et al*. studied the structure of MDMA (3,4-methylenedioxy-N-methylamphetamine) and synthesized MDAI (5,6-methylenedioxy-2-aminoindane) in the 1990s, which was one of the first aminoindanes (9). Other aminoindanes followed such as 2-AI (2-aminoindane), 5-IAI (5-iodo-2-aminoindane), MDMAI (5,6-methylenedioxy-N-methyl-2-aminoindane), MEAI (5-methoxy-2-aminoindane), and MMAI (5-methoxy-6-methyl-2-aminoindane) (10). Structures of some of these compounds are shown in **Figure 1.1**



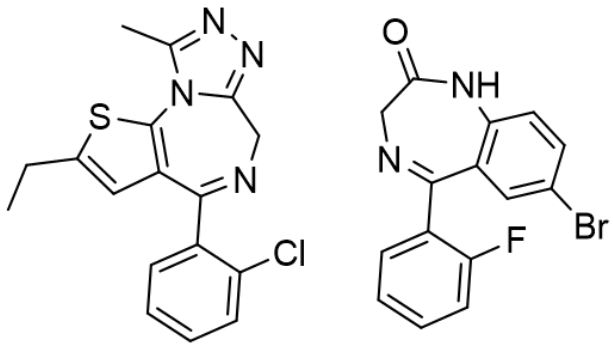
MDAI 5-IAI 2-AI

Figure 1.1 Structures of common aminoindanes

These compounds have similar adverse effects as stimulants such as hyperhidrosis, dehydration, hyperthermia, and bruxism (11). MDAI has been involved in fatal cases with concentrations in blood ranging from ~120 to 26,300 ng/mL (11,12). The case involving the highest concentration (26,300 ng/mL), the cause of death was determined to be toxicity of self-ingested MDAI (11). Other compounds such as 5-IAI and 2-AI were identified in casework in 2010 and 2011 (13).

Designer Benzodiazepines

In 2016, the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) reported that more than half a million tablets were seized that contained designer benzodiazepines such as etizolam, fonazepam, diclazepam, flubromazolam, and flunitrazolam (14). Structures of these compounds are shown in **Figure 1.2** below.



Etizolam Flubromazepam

Figure 1.2 Structures of two designer benzodiazepines

Some of these compounds have higher binding affinity to γ-aminobutyric acid $(GABA)_A$ receptors than traditional benzodiazepines, which can be dangerous if consumed with other central nervous system (CNS) depressants (15). A prominent designer benzodiazepine, etizolam, had 898 drug reports to National Forensic Laboratory Information System (NFLIS) in 2018 (16). Fatalities and acute intoxications involving designer benzodiazepines have been reported. Etizolam has been identified in fatalities with blood concentrations ranging from 25.8 ng/mL to 300 ng/mL (17-19). In a study by Høiseth *et al*, samples from drivers and individuals suspected to be impaired were analyzed for designer benzodiazepines (20). Blood concentrations for etizolam ranged

from 19.0-170 ng/mL (n=14). The study also concluded that tetrahydrocannabinol (THC) and amphetamine were often found in combination with designer benzodiazepines (20).

Dissociatives

Phencyclidine (PCP) and ketamine analogs are examples under the drug category of dissociatives. Adverse effects include confusion, delirium, and dysphoria (21). Analogs such as 3- and 4- methoxy PCP and methoxetamines were found to have higher binding affinity to the glutamate *N*-methyl-D-aspartate (NMDA) receptor. Structures for some designer dissociatives are shown below in **Figure 1.3**.

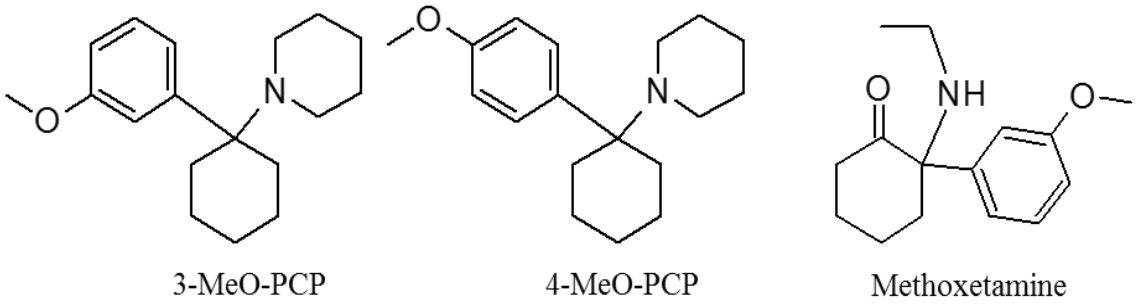
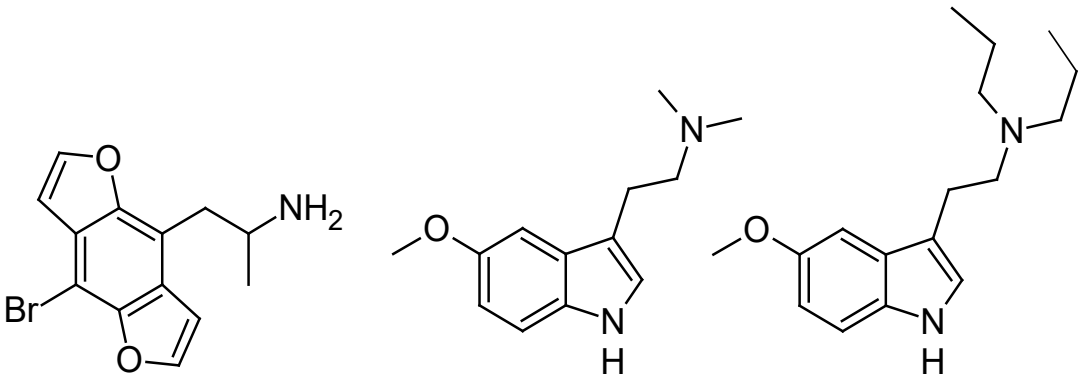


Figure 1.3 Structures of three designer dissociatives

The NMDA receptor is pharmacologically key for both PCP and ketamine (22,23). In Bäckberg *et al*, non-fatal intoxications (n=7) were reported as single substance intoxications involving 3-methoxy PCP (24). From these cases, hypertension was observed in every subject and most displayed other symptoms such as tachycardia, confusion, and hallucinations. These symptoms were also seen in other studies (25). Fatal intoxications described in Johansson *et al*, ranged from 0.05-0.38 µg/g in blood. An intoxication case from the same study detected 0.14 µg/g 3-methoxy PCP in blood (25). Methoxetamine, a ketamine analog, has been reported to have similar adverse (cardiovascular symptoms) and toxic effects to PCP analogs (26,27). Similar to PCP analogs, methoxetamine has also been found in fatalities and intoxications (26,28).

Psychedelic Compounds

NPS compounds that have emerged that possess psychedelic properties include synthetic phenethylamines and tryptamines. Users seek these compounds for hallucinogenic effects, but are unaware of the potential toxic effect that accompanies that desired experience. Structures of common designer psychedelic compounds are shown in **Figure 1.4** below.



Bromo-DragonFLY 5-MeO-DMT 5-MeO-DPT

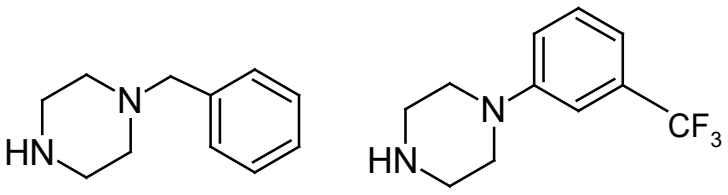
Figure 1.4 Structure of three common designer psychedelic compounds

1-(8-bromofuro[2,3-f][1]benzofuran-4-yl)-2-propanamine (Bromo-DragonFly) is a synthetic phenethylamine that was involved in a mass intoxication (n=29) at an esoteric weekend seminar (29). Some subjects displayed agitated reactions and had to be sedated in route to the hospital, while others had life-threatening reactions such as seizures. The blood concentrations ranged from 0.6-2.0 ng/mL (29). Bromo-DragonFly is a strong agonist to the serotonin receptor 5-HT_{2A}, which is the receptor that traditional psychedelic drugs such as lysergic acid diethylamide (LSD) interact with to produce psychedelic effects (30). Other intoxications and a fatality have also been associated with Bromo-DragonFly (31,32). Similar to Bromo-DragonFly, 5-methoxy-*N*, *N*-dipropyltryptamine (5-MeO-DPT) and 5-methoxy-*N*, *N*-dimethyltryptamine (5-MeO-

DMT) produce psychedelic effects due to their interactions with 5-HT receptors (33). Adverse effects for some of these tryptamine analogs include restlessness, muscle tension, and insomnia (34). In Sklerov *et al*, an autopsy case of a 25 year old male found concentrations of 5-MeO-DMT ranging from 0.15-201.6 ng/mL in postmortem matrices (blood, stomach contents, bile, urine). Concentrations in the urine and peripheral blood were 9.59 and 1.20 ng/mL, respectively. The cause of death was ruled as hallucinogenic amine intoxication (35). As of 2011, 5-MeO-DMT, is a Schedule I controlled substance (36).

Piperazines

In the early 2000s, designer piperazines arose on the internet and in night clubs, including N-benzyl-piperazine (BZP) and 1-(3-trifluoromethylphenyl)piperazines (TFMPP) (37). Both act as agonists on 5-HT_1 receptor as their mechanism of action (38,39). These drugs became popular due to their CNS stimulant-like (dilated pupils, increased heart rate and blood pressure) effects (40,41). Structures of common designer piperazines are shown in **Figure 1.5** below.



BZP TFMPP

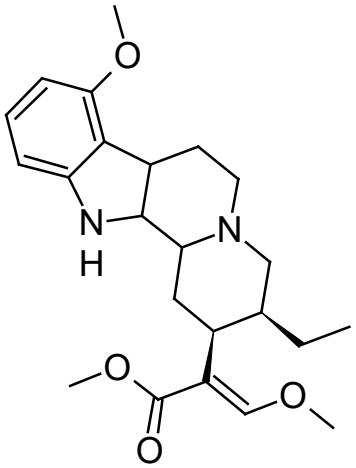
Figure 1.5 Structures of two common designer piperazines

In a study by Schechter *et al*, drug-seeking rats (trained to discriminate MDMA) chose TFMPP over MDMA when given the choice, which suggests that the drug possessed similar desired effects (42). It is not uncommon that designer piperazines are found as adulterants and/or additives in other stimulants such as caffeine or amphetamine

(43). Fatalities and nonfatal intoxications involving either of these designer piperazines are present in literature (44,45). As of 2003, both BZP and TFMPP are classified as Schedule I controlled substances in the Controlled Substance Act in the United States (46)

Plant-Based Substances

Although most NPS are synthetic derivatives of existing drugs of abuse, there are plant-based substances that fall under the category of NPS as well. Amidst the current opioid epidemic that plagues the United States, opioid users have turned to Kratom as a "legal" way to get their fix (47). Kratom is derived from *Mitragyna speciosa* Korth, which is comprised of many alkaloids with mitragynine being the most abundant alkaloid (48). The structure of mitragynine is show below in **Figure 1.6**.



Mitragynine

Figure 1.6 Structure of mitragynine

In Matsumoto *et al*, it was concluded that mitragynine might inhibit stimulation of 5-HT_{2A} receptors and/or bind to post-synaptic alpha-2 adrenergic receptors (49). Compared to morphine, mitragynine's antinociceptive effect is 13 times more potent (50,51). Trakulsrichai *et al*, performed a human study to investigate the pharmacokinetics of mitragynine (52). Participants (n=10) in this study were chronic users of Kratom and

given an exact dose of Kratom tea for 7 days and on the 8th days various amounts of Kratom tea were given and samples were collected at different times intervals. It was concluded that the pharmacokinetics of mitragynine are linear and follow a two component model (52). According to NFLIS, mitragynine was first reported to their system in 2010. From its initial report until 2014, there were 488 reports of mitragynine to their alert system (53). Since then, several fatalities involving accidental poisonings have occurred (54-57). Other substances such as O-desmethyltramadol, quetiapine, or propylhexedrine, have been found in fatalities in postmortem specimens (58-60). Intoxications involving mitragynine (including suspected driving under the influence of drugs (DUID)) have also been reported and in some cases naloxone has been used to reverse the effects of mitragynine (61-63). Buprenorphine accompanied with naloxone has been found to help Kratom users transition from their dependence of the drug (64).

Synthetic Cathinones

Synthetic cathinones emerged in the early 2000's and were sold via the internet or local smoke shops as "Bath Salts" (65). These compounds are often abused due to their euphoric effects by users looking for a drug similar to MDMA. Adverse effects include, but not limited to, hypertension, paranoia, agitation, and delusions (66,67). According to EMCDDA, there have been 130 total synthetic cathinones identified as of 2018 (14). This is the second largest substance group that the EMCDDA monitors (synthetic cannabinoids being the first) (14). Some of the first synthetic cathinones that emerged include mephedrone, methylone, and methylenedioxypyrovalerone (MDPV). Structures of these compounds are shown in **Figure 1.7.**

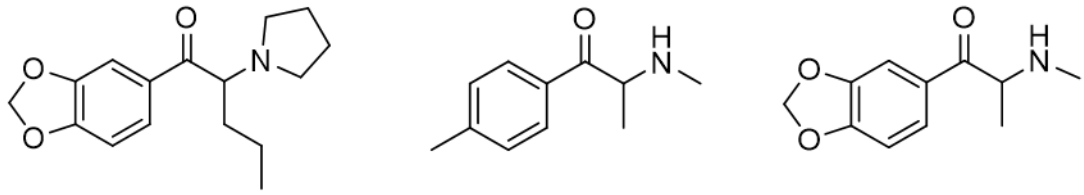


Figure 1.7 Structures of synthetic cathinones

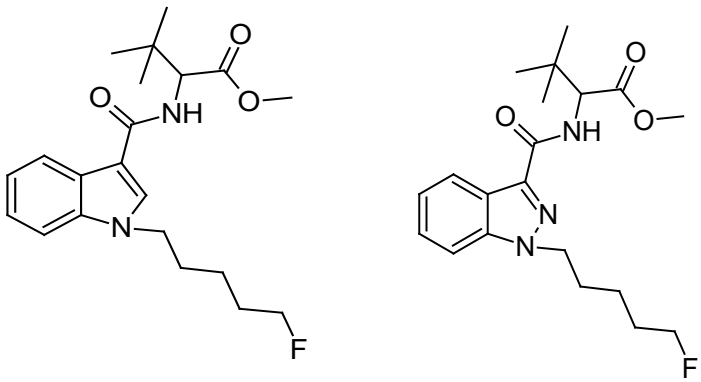
One of the first fatalities associated with MDPV involved a 40 year old man who had injected and snorted "bath salts". After consuming the "bath salts", the man ran outside and was aggressive and delusional. He was shocked with an electronic device three times by police in order to detain him. At the hospital, the decedent went into cardiac arrest and died from complications afterwards (68). MPDV toxicity and excited delirium syndrome played a role in the decedent's death. A similar case involving mephedrone intoxication was reported for a 36 year old man in the Netherlands (69). The decedent also displayed major signs of aggression (breaking windows) prior to his death. Mephedrone was detected in the femoral blood (5.1 ng/mL) of the decedent, along with cocaine and MDMA. Lusthof *et al* concluded that the fatal intake of mephedrone likely led to the excited delirium. The decedent also suffered blood loss from wounds associated with breaking the windows in his agitated state (69). Both MDPV and mephedrone were placed as Schedule I controlled substances in 2011 (70).

Synthetic Cannabinoids

Out of the other categories of NPS, synthetic cannabinoids appear to have the largest number of derivatives. As of 2017, there have been 179 synthetic cannabinoids reported to the EMCDDA. In 2017, 60% of drug-related deaths in Turkey had synthetic cannabinoids present (71). According to the 2018 NFLIS midyear report, there were

10,598 synthetic cannabinoid occurrences reported to them by June 2018. The most prominent of the synthetic cannabinoids was 5F-MDMB-PINACA or 5F-ADB accounting for 38% of the total synthetic cannabinoids identified (72).

5F-MDMB-PINACA is an indazole synthetic cannabinoid with a *tert*-leucinate group that has gained popularity in recreational use (73). In 2017, it was controlled as a Schedule I substance under the Controlled Substance Act in the United States (74). Banister *et al* examined cannabinoid receptor activity of several closely related synthetic cannabinoids and found that 5F-MDMB-PINACA was a CB_1 receptor full agonist with greater potency than the other compounds in the study (73). The fluorine substitution on the pentyl chain may increase binding affinity to the CB_1 receptor, possibly accounting for this increased potency (75). As a CB_1 receptor agonist, 5F-MDMB-PINACA should produce similar effects as THC. Adverse effects could also occur and have a greater chance of being severe due to the full agonist properties and its otherwise unknown nature (76). Structures of two synthetic cannabinoids are shown in **Figure 1.8** below.



5F-MDMB-PICA 5F-MDMB-PINACA

Figure 1.8 Structures of two synthetic cannabinoids

One of the first identifications of 5F-MDMB-PINACA was by Hasegawa *et al.* Specimens (body fluids and tissues) from a postmortem case and herbal blend packages were analyzed. The highest concentration of 5F-MDMB-PINACA was found in the stomach contents, while other fluids were below the detection limit, which might indicate a rapid death before distribution to blood. The levels of 5F-MDMB-PINACA found in the packages ranged from 0.00077-49.2 mg/g (77).

Fatalities and intoxications involving 5F-MDMB-PINACA have risen since this first identification (77-81). However, parent structures of synthetic cannabinoids are often difficult to detect in biological matrices, so metabolism studies are needed to improve detection of newly emerging synthetics. Barceló *et al* identified two phase I metabolites from five urine samples of 5F-MDMB-PINACA intoxications: products of ester hydrolysis and oxidative defluorination, respectively (78). In another study, Kusano *et al* was able to identify two additional phase I metabolites in a postmortem urine sample, ester hydrolysis in combination with oxidative defluorination and ester hydrolysis in combination with oxidative defluorination to pentanoic acid, while confirming the ester hydrolysis product (79). Both of these studies used structural elucidation of potential metabolites in authentic urine specimens. Human liver microsomes were utilized to identify possible metabolites of 5F-MDMB-PINACA by Yeter *et al* (82). From this study, 20 metabolites were identified with a recommendation of ester hydrolysis, ester hydrolysis in combination with oxidative defluorination to pentanoic acid, and hydroxylation on the aliphatic chain as suitable urine markers. Limited studies have confirmed metabolites with enzyme incubation using reference standards, but no studies have confirmed the formation of potential metabolites by human hepatocytes.

Although 5F-MDMB-PINACA has been present in fatalities and intoxications, according to the Center of Forensic Science Research and Education (CFSRE) there were no identifications of this synthetic compound in the second quarter of 2019 (83). This trend report also noted that 5F-MDMB-PICA had 64 positive samples during the second quarter. 5F-MDMB-PICA is structurally similar to 5F-MDMB-PINACA with the indazole replaced with an indole. Studies involving 5F-MDMB-PICA are limited. The first identification of 5F-MDMB-PICA was by Risseeuw *et al* from packages labelled as herbal incense (84). There has been one metabolism study performed by Mogler *et al*, using pooled human liver microsomes (HLM) (85). There were 12 potential phase I metabolites identified using the HLM and urine specimens (n=24). Of the 12 identified, ester hydrolysis was the most prominent metabolite detected (85). Receptor studies have also been reported for 5F-MDMB-PICA, indicating high potency at the CB1 receptor (73,86).

Synthetic Opioids

The current phase of the opioid epidemic in the United States is driven by synthetic opioids. Synthetic opioids can be divided into two groups, fentanyl derivatives and non-fentanyl derivatives. The fentanyl derivatives are highly potent, which makes small amounts of the substances dangerous. In the past ten years, the EMCDDA has reported 34 fentanyl derivatives (71). Cyclopropylfentanyl and methoxyacetylfentanyl were investigated in 2018 in a risk assessment and it was determined that these compounds were associated with over 90 deaths and present in over 180 seizures by law enforcement (71). In 2017, there were 1,300 seizures of synthetic opioids reported to the European Union (EU) Early Warning System. Although fentanyl derivatives made up the

majority of these seizures, non-fentanyl derivatives such as U-47700 were also present (71).

U-Series

The Upjohn Company originally developed a series of drugs in the 1970's with the intent of being used as possible analgesics. The first prominent U-series drug that was abused was U-47700. Structures of some of the prominent compounds in the U-series are shown below in **Figure 1.9**.

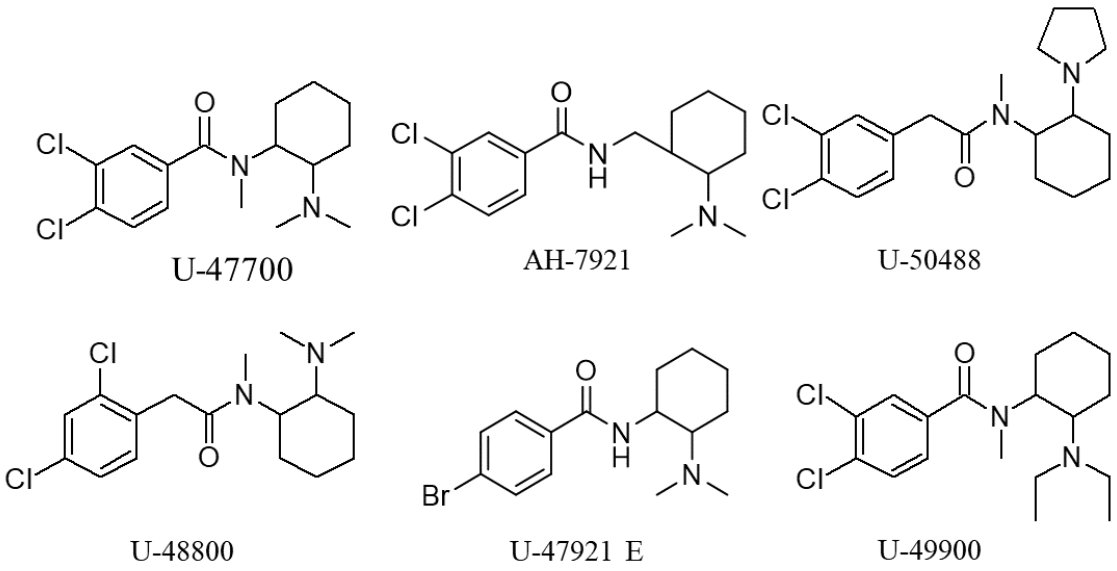


Figure 1.9 Structures of compounds from the U-series

A structural isomer of U-47700, AH-7921, was also developed in the 1970's as a potential analgesic by Allen and Hanbury (87). Other U-series drugs that have appeared on the illicit drug market are U-49900, U-50488, U-48800, and U-47921 E.

Prevalence

The first reported seizure of U-47700 was in October 2015 in the United States, while U-47700 emerged on the EMCDDA EWS in June 2016 (88,89). Once the threat of this substance was identified, it was placed into Schedule I of the Controlled Substances

Act in November 2016 (89). Between 2016 and 2017, over 80% of the states in the US had at least one seizure report of U-47700 (90). Ohio had the largest reporting (n=943) in 2017 in the United States. AH-7921 was placed under control by the EU in September 2014 (91). Similar to U-47700, AH-7921 was placed into Schedule I of the Controlled Substances Act in 2016 (92). It was first reported to NFLIS in 2013 and between 2009 and 2014, had 2 reports to NFLIS (53). To combat the challenges of legislation, other U-series drugs have appeared. According to the National Drug Early Warning System (NDEWS) synthetic opioids U-48800, U-49900, and U-50488 have appeared on the cryptomarket. These drugs are advertised to produce similar effects to U-47700 and other U-series drugs (93). Between 2018 and 2019, there were 19 cases where U-48800 was detected in a biological sample submitted to National Medical Services (NMS) (94). To date, there has only been one case were U-49900 was detected (95). The diethyl analog of U-47700 (U-49900) has not been scheduled in the United States. There has also been a report of substance found at a scene of an overdose that screened positive for U-49900, U-48800, and U-7931 E (96).

Pharmacology

The U-series drugs produce effects by binding to opioid receptors (μ, κ, or δ). U-47700 and U-48800 were found to be selective μ- opioid agonists (97). The μ receptor has been shown to be responsible for the analgesic effects morphine possesses, but also is responsible for the adverse effects of respiratory depression, dependence, and addiction associated with opioids (98). When compared to morphine, U-47700 and U-48800 are approximately 7.5 as potent (97). There have been no pharmacological studies performed involving U-47700 or other U-series drugs. Due to the receptor binding, it is

hypothesized that U-47700 and U-48800 are abused due to potential analgesic and euphoric effects. U-50488 and U-48800 are selective κ-opioid receptor ligands (99,100). AH-7921 is an agonist to both μ and κ opioid receptors, with equipotency to morphine found in rat studies (101,102). One study calculated the estimated half-life for U-47700 using blood serums at different time points of a patient on mechanical ventilation. It was approximated that the half-life of U-47700 is 6 hours (103). On international drugs forums, AH-7921 is said to produce mild sedatives effects, as well as vomiting, nausea, urine retention, reduced/ slow motor skill, and slight respiratory depression (104).

Analytical Methods

With the emergence of the U-series, various analytical methods have been developed to identify these compounds. In **Table 1.1**, methods are shown for detecting and/or quantifying compounds from the U-series. Of the 14 methods shown, most (n=10) use liquid chromatography (LC) for their separation of analytes. Although gas chromatography (GC) is commonly used in crime laboratories, compounds could be subjected to thermal degradation. The use of LC coupled with mass spectrometry (MS) offers improved sensitivity compared to GC/MS. Shoff *et al* highlights the differences between a previously used GC/MS method compared to the newly prepared LC-MS/MS method (105). There were 134 cases of carfentanil identified using the new LC method with 104 of those identifications were missed using the previous GC/MS method (105). Since little is known about NPS, eliminating the possibility of thermal degradation during analysis is key to properly identifying these compounds.

The methods described in **Table 1.1** utilize a wide variety of extraction techniques, including solid phase extraction (SPE), liquid-liquid extraction (LLE), and

protein precipitation. LLE is commonly used in forensic toxicology laboratories and long-standing analytical techniques and offers a cost-effective approach to sample preparation. Although SPE is more expensive, the benefit of eliminating some interferences from the matrix could prove advantageous, particularly for LC-MS techniques. As the pKa of NPS are generally unknown, SPE is better equipped to isolate a wide range of analytes compared to traditional LLE.

Table 1.1 Analytical methods for U-series drugs

Matrix	Analytes	Internal Standard	Extraction	Instrumentation	Linear Range (ng/mL)	LOD (ng/mL)	LOQ (ng/mL)	Reference
BL	U-47700 AH-7921	Fentanyl-d5	SPE	LC-MS/MS	0.1-10	0.019 0.042	0.1	(106)
BL	U-47700 U-50488	AH-7921-d3	SPE	LC-MS/MS	1-100 30-500	0.5	-	(107)
BL	U-47700 N-desmethyl-U-47700 U-50488 U-48800	U-47700-d3	LLE	LC-MS/MS	0.05-40	0.005 0.01 0.005 0.01	0.05	(108)
BL	U-47700 N-desmethyl-U-47700 N,N-didesmethyl-U-47700	U-47700-d6 N-desmethyl-U-47700-d3	SPE	LC-MS/MS	1-500	0.5 0.5 0.75	1.0	(109)
BL	U-47700	Fentanyl-d5	LLE	LC-MS/MS	1.6-63.5	1.6	1.6	(110)
BL	U-47700	Not Specified	LLE	HPLC-DAD	312.5-5000	50	312.5	(111)
BL	U-47700	Methadone-d9	LLE	GC-MS	50-1000	Not Specified	Not Specified	(112)

Matrix	Analytes	Internal Standard	Extraction	Instrumentation	Linear Range (ng/mL)	LOD (ng/mL)	LOQ (ng/mL)	Reference
BL BF BR U LV KD	AH-7921	MDPV-d8	LLE	GC-MS	50-2000	Not Specified	Not Specified	(113)
BL LV SC	U-47700	Hydrocodone-d3	LLE	GC-MS	20	5	20-500	(55)
BL BR LV U	U-47700	Fentanyl-d5	SPE	LC-MS/MS	Not Specified	0.5	Not Specified	(105)
BL BR LV U VF	U-47700	Not Specified	SPE	GC-NPD	25-500	25	25	(114)
BL U	U-47700 N-desmethyl-U-47700	Fentanyl-d5	SPE	LC-MS/MS	N/A	100	100	(115)
BL U	U-47700 Alpha-PVP	β-OH-Ethyl-theophyllin and methyl-clonazepam	SPE	LC-HRMS	25-3000	5	25	(116)
BL	U-47700	Fentanyl-d5	PP	LC-MS/MS	0-250	0.6	2	(117)

Matrix	Analytes	Internal Standard	Extraction	Instrumentation	Linear Range (ng/mL)	LOD (ng/mL)	LOQ (ng/mL)	Reference
U H								
OF U	U-47700	Not Specified	LLE	LC-HRMS	N/A	1	N/A	(118)
U	U-47700	Norporopoxyphene-d5	SPE	LC-MS/MS	1-1250	1	1	(119)

LOD=Limit of Detection, LOQ=Limit of quantitation, LLE= Liquid-Liquid Extraction, SPE= Solid Phase Extraction, PP=Protein Precipitation, LC=Liquid Chromatography, GC=Gas Chromatography, MS=Mass Spectrometry, HR= High Resolution, HP= High Performance, DAD=Diode Array Detector, NPD=Nitrogen-Phosphorous Detector, BL=Blood, LV=Liver, U=Urine, SC=Stomach Contents, BF=Bile Fluid, BR=Brain, KD=kidney, VF=Vitreous Fluid, H=Hair, OF=Oral Fluid, LOD and LOQ are in blood unless stated otherwise

The methods described in **Table 1.1** analyzed U-series compounds in traditional specimens for forensic toxicology, including blood and urine. Some of the methods used for postmortem casework also incorporated tissues (liver, kidney, brain). Using tissues may allow for estimations of postmortem redistribution (PMR), which is frequently unknown for NPS. There is only one method that uses oral fluid as a matrix of interest. Griswold *et al* was able to qualitatively identify synthetic opioids in both urine and oral fluid (118). When comparing the matrices, it was concluded that oral fluid was comparable to urine to the detection of synthetic opioids. Oral fluid is an appealing alternative matrix that possesses many advantages over blood and urine such as ease of use, no need for a trained professional to collect, and less likely to be subjected to adulteration. Oral fluid may also be ideal for testing individuals who are suspected for DUID as drug presence may be indicative of recent use and more likely to correlate with impairment. Alabama was the first to implement a state-wide program for incorporating oral fluid into routine DUI/D casework.

Cases

Of the compounds associated with the U-series, U-47700 is responsible for the most intoxications and fatalities reported in literature. There are minimal reports of other U-series compounds. Studies involving postmortem analysis are shown in **Table 1.2** below. Most cases also involve fentanyl derivatives such as furanylfentanyl and other illicit drugs (cocaine, methamphetamine, etc.) in addition to U-series compounds. In Garneau *et al*, one case was shown that had multiple fentanyl derivatives present. There was a total of six fentanyl derivatives (acetylfentanyl, despropionylfluorofentanyl, isobutyrylfentanyl, p-fluorofentanyl, valerylfentanyl, and furanylfentanyl) were present in

biological samples. In addition, U-47921 E was present in the urine of the decedent. Powders found at the scene were screened and came back positive for U-47700, U-48800, U-49900, U-47921 E, and other substances (96).

Table 1.2 Postmortem concentrations of U-47700 reported in literature

n	Age	Sex	U-47700 Concentration (ng/mL)	Matrix	Other Illicit Drugs Present	Notes	Reference
1	30	Male	13.8 71.0	BL U	Fentanyl (10.9 ng/mL BL)	Evidence of inhaling a white powder	(110)
1	26	Male	340 190 170 1700 360	CBL PBL VF LV U	None	Found dead in bedroom, lethal intoxication with U-47700 as only drug present	(114)
16	18-40	Male	17-490	BL	Certain cases had fentanyl derivatives also present	Five cases had both furanyl fentanyl and U-47700 present, one case had butryl fentanyl present with U-47700	(107)
2	Not Specified	Not Specified	525-1347 1393-1848 1.4-2.7 3.1-4.3 2.4-3.2 0.97-1.1	BL U KD LV LG BR	Methoxyphenidine	Both decedents were found dead in their beds	(120)
1	27	Male	146	BL	Ketamine and cannabis	Illicit drug user found dead at home	(111)
2	18,28	Male	547, 189	BL	Etizolam Oxycodone (67 ng/mL)	Both decedents were found dead at home. There was drug paraphernalia present such as plastic bags labelled as NPS compounds	(121)

n	Age	Sex	U-47700 Concentration (ng/mL)	Matrix	Other Illicit Drugs Present	Notes	Reference
1	19	Male	3040 2230	PBL U	Alpha-PVP (41.7 ng/mL BL, 62.8 ng/mL U)	Decedent was found at home with tablets of paroxetine present. Paraoxetine was present (170 ng/mL), but within a therapeutic range	(116)
1	34	Male	360	PBL	3-Fluorophenmetrazine (2400 ng/mL FBL)	Found at home, unresponsive and slumped over	(112)
1	28	Male	330	BL	Flubromazepam (10 ng/mL) Diclazepam (70 ng/mL)	Decedent had a history of illicit drug use and was found dead at home	(122)
1	Not Specified	Male	380	BL	None	History of drug addiction, found supine at apartment	(117)
1	24	Male	370	SR	Flubromazepam (830 ng/mL)	Found suffering from apnea, decedent was reanimated and hospitalized. 6 days later, mechanical ventilation was stopped	(103)

n	Age	Sex	U-47700 Concentration (ng/mL)	Matrix	Other Illicit Drugs Present	Notes	Reference
1	51	Male	290 1250 9.9 µg/g 240 570 2300 400 0.14 ng/mg	PBL CBL LV U SC BF CSF H	None	Found on hotel bathroom floor next to the toilet, spoon and syringe were present	(123)
1	31	Male	**U-49900** 1.5 2.2	 BL U	Tetrahydrofuranyl fentanyl (339 ng/mL BL, >5000 ng/mL U) Methoxy-PCP (1.0 ng/mL BL, 31.8 ng/mL U)	There was white powder found at the scene, testing confirmed it was U-49900	(95,124)
3	46	Female	54 Negative Positive	CBL PBL U	Furanyl fentanyl was present in all three cases. The case of 29 year old male also had acetyl fentanyl, despropionyl fluorofentanyl,	All three cases involved persons who had previous history of drug abuse or alcoholism. The case of 29 year old male was found with syringe in hand and empty bottles of steroids were present	(96)
	30	Male	45 26 Positive	CBL PBL U			

n	Age	Sex	U-47700 Concentration (ng/mL)	Matrix	Other Illicit Drugs Present	Notes	Reference
	29	Male	Positive Positive Positive	CBL PBL U	Isobutyrylfentanyl, p-fluorofentanyl, valerylfentanyl, and U-47931 E. Additionally substances found screened positive for U-49900 and U-48800		
2	63	Male	24	BL	Cycloproyl fentanyl (31.5 ng/mL BL) Cocaine (25 ng/mL BL) Benzoylecognine (58 ng/mL BL)	Decedent had a history of drug and was found dead at home	(125)
	57	Male	7.8	BL	Cyclopropyl fentanyl (18.5 ng/mL BL) Cocaine (130 ng/mL BL) Benzoylecognine (910 ng/mL BL) Fentanyl (6.2 ng/mL BL) Norfentanyl (5.5 ng/mL BL)	Found unresponsive in vehicle and was transported to hospital, was pronounced dead on arrival	

PBL=Peripheral Blood, CBL=Central Blood, CSF=Cerebrospinal Fluid, SR=Serum, BL=Blood, LV=Liver, LG=Lung

U=Urine, SC=Stomach Contents, BF=Bile Fluid, BR=Brain, KD=kidney, VF=Vitreous Fluid, H=Hair

The concentrations for U-47700 in postmortem casework reported ranged from 7-3040 ng/mL in blood, with a majority of the cases having concentrations <500 ng/mL. Richeval *et al* presented a case with the highest concentration of U-47700 detected in peripheral blood. The authors hypothesized reasons behind an unusually high concentration of U-47700 present. Paroxetine tablets were present at the scene and in biological specimens, therefore it was thought perhaps a paroxetine/U-47700 interaction may have occurred that would inhibit metabolism of U-47700 and increase concentrations. Another suggestion was that a high amount of U-47700 was administered prior to death.

While less commonly reported, non-fatal intoxications occurring with U-series compounds are also present in literature. Antemortem cases of U-series intoxications are shown in **Table 1.3** below. Most concentrations are below 100 ng/mL, with only three cases where the concentrations were above 200 ng/mL. These cases are within concentrations that were found in postmortem cases. Without proper knowledge of the pharmacology of these compounds, the "therapeutic" window for these compounds is unknown. One case worth noting was reported by Vogliardi *et al* (131). This study involves a man in his thirties that was found unresponsive and was comatose upon admission to the hospital. Blood was drawn from this subject shortly after admission with 94 ng/mL U-47700 present. The next day, another sample of blood was drawn which had a concentration of 5.2 ng/mL of U-47700. Pubic hair was also analyzed in this case and showed that the subject was a frequent drug user as common drugs of abuse (cocaine, MDMA, THC, etc.) were detected.

Table 1.3 Antemortem concentrations of U-47700 in literature

n	Age	Sex	U-47700 Concentration (ng/mL)	Other illicit Drugs Present	Notes	Reference
2	26, 24	Male Female	0.1 (U) Not tested (U)	None	Both were together when using substances and were taken to hospital, Male presented symptoms of opioid toxidrome, Female presented uncontrollable anxiety	(126)
1	40	Female	7.6 (SR)	Fentanyl (15.2 ng/mL in serum)	After ingesting 3 tablets thought to be "Norco", subject was hospitalized and had symptoms of opioid use such as pinpoint pupils and respiratory depression	(127)
1	22	Male	Positive (U)	None	User had a history of heroin abuse and was found unconscious by mother	(128)
1	23	Female	394 (SR) 228 (U)	None	Subject was cyanotic with respiratory depression in ambulance	(129)
1	29	Male	240 (SR)	Phenazepam (1400 ng/mL in serum)	Found unresponsive and regained consciousness before getting to the hospital	(130)
1	30s	Male	94 BL after intake 5.2 BL a day after 3.02 ng/mg (H)	Pubic hair Found cocaine, benzoylecognine, norcocaine, THC, cannabinol, mephedrone, ketamine, norketamine, MDMA	Found unresponsive and in a comatose state. Completely recovered after hospital intake	(131)

n	Age	Sex	U-47700 Concentration (ng/mL)	Other illicit Drugs Present	Notes	Reference
1	26	Male	351 (BL)	THC (3.3 ng/mL in blood) Clonazepam (6.8 ng/mL in blood) Also positive for citalopram and midazolam	Found unconscious at home with white powder residue in plastic bags, was taken to hospital and put on ventilation, subject was released 40 hrs later	(132)

Stability

There are minimal studies addressing the stability of these U-series compounds. Unknown stability of a compound can be detrimental to analysis if samples are mishandled during transport and/or improperly stored. Most stability information about the U-series compounds was acquired during assessing method validation. The stability of U-47700 and U-50488 in blood was evaluated when performing a fit-for-purpose validation by Mohr *et al* (107) . The two conditions that were evaluated were refrigerated and frozen over 10 days. Of the two concentrations tested (5 and 80 ng/mL), both were stable at the two temperature conditions over the 10 days assessed. In Qin *et al*, U-47700, N-desmethyl-U-47700, U-50488, and U-48800 were evaluated in whole blood during validation with other fentanyl derivatives (108). The temperature conditions that were evaluated were 24 hr at 4 °C and room temperature, three freeze/thaw cycles, and 1 month in the freezer at three concentrations (0.2, 5, and 30 ng/mL). All analytes were found to be stable at all temperature conditions and concentrations. At 25 ng/mL, U-47700 and its metabolites were evaluated at three temperatures (-30 °C, 3 °C and 20 °C) in blood for three weeks by Rojek *et al* (109). It was determined that U-47700 was stable at all temperatures. In Soh and Elliot *et al*, AH-7921 was found to be stable at room temperature in blood and plasma for 28 days at a very high concentration of 2000 ng/mL (133). These studies were able to successfully determine stability for some compounds of the U-series, but most were short-term stability assessments. There is no available literature on long-term stability studies involving U-series compounds.

Metabolism

Metabolism studies are key for identifying any NPS that emerges. They provide information of what potential biomarkers could be used to confirm use of a substance when the parent compound is no longer able to be detected. Metabolism for U-47700 was predicted in two studies by analyzing authentic specimens. It was suspected that demethylation occurs at the tertiary amine group and is followed hydroxylation on the cyclohexane ring (111,129). The first metabolic study of U-47700, which also included U-49900, was performed by Krotulski *et al*. Metabolites for U-47700 and U-49900 were first generated *in vitro* using human liver microsomes (HLM), then verified *in vivo* using authentic urine samples. The predicted metabolic pathway for U-47700 is shown in **Figure 1.10** below.

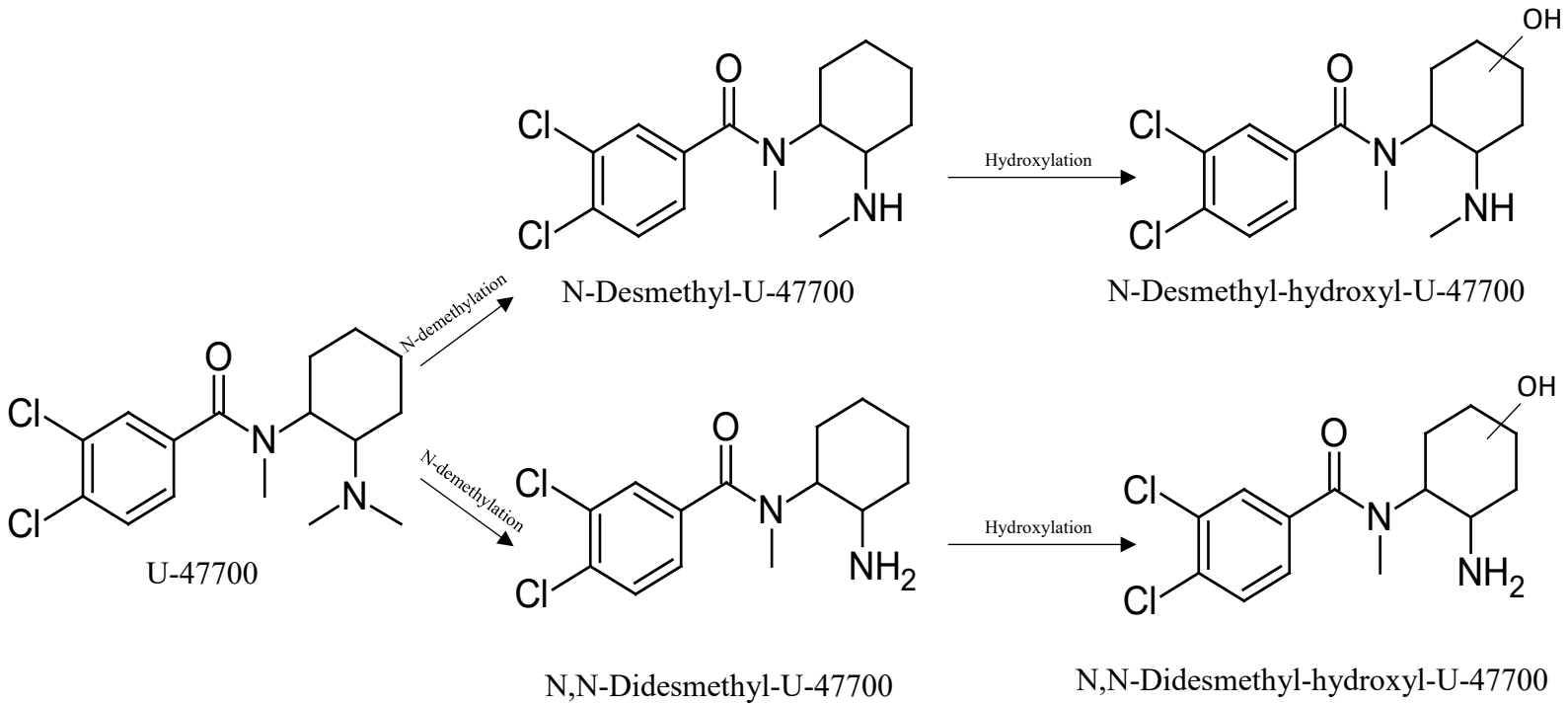


Figure1.10 Predicted metabolic pathway for U-47700 by Krotulski *et al*

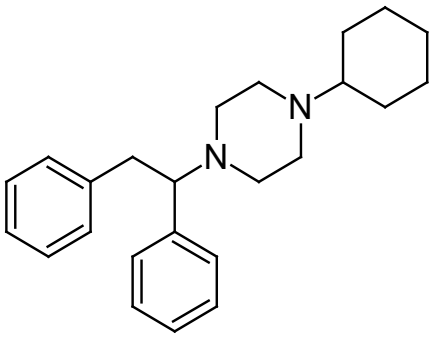
The prominent metabolite for U-47700 was identified as N-desmethyl-U-47700. U-49900 follows a similar metabolic pathway in which N-dealkylation occurs at the tertiary amine followed by hydroxylation on the cyclohexane ring or additional dealkylation or demethylation at the amide bridge (124). The prominent metabolite for U-49900 was identified was N-deethyl-U-47700. Richeval *et al*, also performed a metabolic study on U-47700 using HLMs to predict *in vitro* metabolites and verified *in vivo* using authentic samples (116). This study identified the same metabolites that were previously found, including a positional isomer of the hydroxylation on the cyclohexane ring metabolite. Rojek *et al*, developed and validated a method to quantitate U-47700 and two of its predicted metabolites (N-desmethyl-U-47700 and N,N-didesmethyl-U-47700) in blood. Only two metabolites were included as there were no commercially available reference standards for the hydroxylated metabolites. This method was then applied to 12 authentic case samples where various concentrations were identified. The use of retrospective analysis was incorporated to analyze a sample presented by Partridge *et al* (122). Using the molecular formula for the metabolites of U-47700, it was possible to go back in a previously analyzed sample and identify the presence of U-47700 metabolites. A metabolism study using hepatocyte incubations to identify metabolites for AH-7921 coupled with *in vivo* data from authentic urine was performed by Wohlfarth *et al* (134). As mentioned before, AH-7921 is a structural isomer to U-47700 and therefore it is not surprising that the two share similar abundant biotransformations. In this study, 12 metabolites were identified via hepatocyte incubations and 11 of the 12 were present in authentic urine samples. The increase in amount of metabolites produced compared to the U-47700 can be attributed to human hepatocytes used in this experiment, while the other

experiments used HLMs (116,124). Hepatocyte incubations produce metabolites closer to the abundance that occurs in the human body and are also able to produce phase II metabolites. The two most abundant metabolites identified in this study were desmethyl-AH-7921 and didesmethyl-AH-7921 (134)

Recently, a metabolic study using human liver S9 fraction on U-48800 was published by Gampfer *et al* (135). This study investigated the toxicokinetics of U-48800 by determining metabolic stability, isozyme mapping, and plasma protein binding. There were 12 phase I metabolites involving demethylation, hydroxylation, and combinations of the two identified during the *in vitro* study and 2 additional phase I metabolites identified in rat urine. Rats were used as there were no authentic human samples available to verify the predicted metabolites. It was also discovered that the isozymes CYP2C19 and CYP3A4 were responsible for the most abundant metabolites and concluded that poor metabolizers of those isozymes would be prone to higher concentrations of U-48800 and potentially toxicity (135).

MT-45

In the 1970's, the Dainippon Pharmaceuticals Company developed MT-45 as a possible alternative analgesic to morphine (136). A structure of MT-45 can be seen below in **Figure 1.11.**



MT-45

Figure 1.11. Structure of MT-45

Unlike the other synthetic opioids, MT-45 is a piperazine derivative. The Dainippon Pharmaceuticals Company also developed another piperazine derivative in the 1970's named AD-1211, which possessed analgesic and narcotic antagonist properties (137).

Prevalence

MT-45 was first reported to the EMCDDA EWS towards the end of 2013. The substance was also first report to NFLIS in 2013 (53). Shortly after, it was placed under control across the EU in 2015 (138). In 2017, the United States placed MT-45 as Schedule I controlled substance under the Controlled Substance Act (139). Between 2011 and 2016, there were three seizures of MT-45 reported to NFLIS (140). According to the EMCDDA, there were 28 fatalities involving MT-45 in Sweden between November 2013 and July 2014 (141).

Pharmacology

In 1978, Fujimura *et al*, investigated receptor activity and analgesic properties of MT-45. It was determined that MT-45 is 3.5 times more potent than morphine (142). Baumann *et al* investigated the sedative and analgesic effects of MT-45 and confirmed

that it was a MOR-1 selective agonists (143). Unusual side effects were observed by subjects presented in Helander *et al*. In these non-fatal intoxications, subject experienced loss of hearing, increased liver enzymes, and dermatitis with hair loss. Some subjects also displayed signs of thallium poisoning (Mee's lines), but no other symptoms/sign of thallium poisoning and no thallium detected (144). This is the only synthetic opioid that had these unusual side effects reported. Users on drug forums provided potential effective dosages for new users depending on the route of administration chosen. A low dose for oral administration was reported as 10 mg and a high dose of 200 mg was suggested (104). For intranasal administration, the recommended dose was 50 mg. Users also reported increased happiness and significant nodding while on the substance (104).

Methods

There is only one published validated method for MT-45. Papsun *et al* performed a fit-for-purpose validation to quantitate MT-45 using an ultra-performance liquid chromatography (UPLC)-MS/MS in blood (18). The established linear range was 1-100 ng/mL with a limit of detection of 1 ng/mL. The extraction method utilized was a traditional LLE. The precision and accuracy of their method was within ±20% CV and difference.

Cases

As previously mentioned, there were 28 fatalities reported to EMCDDA from Sweden between 2013 and 2014 (141). Of these 28 fatalities, the concentrations ranged between 8.3 and 1989 ng/mL. Using the validated method previously mentioned, Papsun *et al*, determined the concentrations of MT-45 present in single fatality case of a 35 year old man. The concentration of MT-45 was determined to be 520 ng/mL. There also the

presence of etizolam (35 ng/mL) detected in the decedents blood. Although both of these compounds were present, it was suggested that MT-45 played a more significant role in the fatality (18). Another fatality was presented by Fels *et al* that involved MT-45 (145). This case involved a 24 year old man that had been a known user of amphetamine. The decedent was found by his mother in his room where he was slumped over in a chair by a desk. There were several bags present at the scene that were labelled MT-45, methoxymetamine, and methoxphenidine. The femoral blood had a concentration of 2900 ng/mL of MT-45 present. When compared to the other fatality concentrations previously mentioned, this concentration is remarkably high. It was concluded that due to the concentration of MT-45 detected in the decedent's blood, that MT-45 had a major role in the death of the individual (145). Backberg *et al* presented a non-fatal intoxication of a 30 year old man. It was suspected that the 30 year old individual was under the influence of flubromazepam and MT-45. The urine of the 30 year old tested positive for 3-MeO-PCP, MT-45, N-ethylbuphedrone, flubromazepam, methiopropamine, α-PBP, α-PPP, and 4F-PVP. The subject was discharge within 24 hrs after fully recovering (146).

Stability

The only stability reported on MT-45 is the stability that was assessed during Papsun *et al* fit-for-purpose validation (18). The stability study did not specify the concentration of MT-45 that was used, but it concluded that MT-45 was stable in fortified blood for 30 days in the following conditions: refrigerated, room temperature, and frozen. The study also observed a possible instability of MT-45. Authentic blood samples were retested approximately 12 months after initial analysis and had degraded ~50%. Due to the unknown stability of MT-45, the authors could not conclude if this degradation was

due to the nature of the sample or the possible instability of the compound in authentic specimens (18).

Metabolism

There have been two metabolism studies involving MT-45 that have been published. Montesano *et al* conducted a metabolic study using rat hepatocytes and then verified the predicted metabolites with CD-1 mice urine of animals previously dosed with MT-45. Metabolites were first predicted *in silico* before proceeding with the *in vitro* experiments. From the hepatocyte incubations, there were 14 phase I and phase II metabolites identified. All of these metabolites were present in the *in vivo* experiments with hydroxy-MT-45 being the most abundant metabolite present. This study also assessed the mice behavior through tail flicks. It was determined that MT-45 produced comparable respiratory depression and analgesia to that of morphine. This was the first study to predict the metabolites for MT-45, but due to use of rat hepatocytes there could be differences compared to human isozymes.

McKenzie *et al* investigated the metabolism of MT-45 and an analog of MT-45 (2F-MT-45) using an array of metabolic methods (HLMs, human hepatocytes, mouse hepatocytes, and mouse and authentic human urine) (147). There was a total of 15 metabolites identified through both *in vivo* and *in vitro* experiments for MT-45. There were 12 metabolites identified for 2F-MT-45 following the *in vitro* and *in vivo* studies. The major metabolites for both compounds consisted of hydroxylations or N-dealkylation. From both of these studies, hydroxylated metabolites were suggested as potential biomarkers if the parent compound is unable to be identified (147).

W-Series

Of the previously mentioned synthetic opioids, the W-series has the least amount of previous literature available. Developed in the 1980's at the University of Alberta, the W-series consists of 32 compounds (148). There are three prominent compounds that are in the W-series. The structures of these compounds are shown below in **Figure 1.12.** W-18 gathered media attention when reports came out that this drug was "10,000 times stronger than morphine"(149) as a result of suspected involvement in 213 overdose deaths in Canada in 2015. There was no peer reviewed data to back these claims.

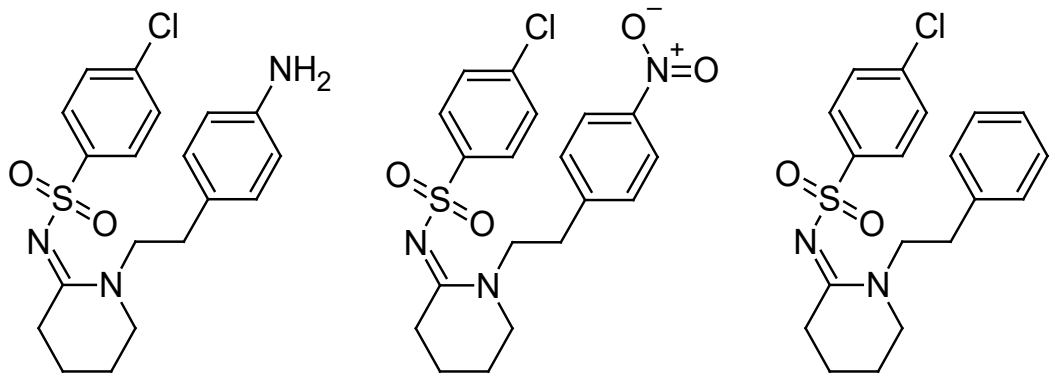


Figure 1.12 Structures of three compounds in the W-series

Prevalence

There has been a single fatality report associated with W-18 in literature. According to NDEWS, National Medical Services laboratories had a case of a 30 year old man who died from an apparent overdose in May 2016 (150). The lab was able to detect W-18 in blood through their general screen on their LC-quadrupole time of flight (QTOF). Once screened positive for W-18, it was confirmed in both urine and blood. The decedent had a history of drug abuse and also had 400 ng/mL of morphine and 13 ng/mL of 6-monoacetylmorphine present (150). After this discovery, Canada scheduled W-18 as

a Schedule I controlled substance (151). None of the W-series compounds are scheduled in the United States. In 2017, there were still forums mentioning experiences or desires of W-18 and other NPS (152). In 2019, a powder labeled “heroin #3” was analyzed by a lab in Belgium and was found to contain W-18 with a fentanyl analog (ocfentanil) (153). There have been no published methods for the detection of any of the W-series compounds.

Pharmacology

As mentioned before, original reports suggested that W-18 was a very potent opioid, but this was proven otherwise by Huang *et al*. This study concluded that there was no detectable opioid activity at the μ, δ, κ receptors from either W-18 or W-15. They also evaluated the metabolism of W-18 using both mouse liver microsomes and HLMs. They sought to see if any of the identified metabolites would perhaps have activity at any of the opioid receptors. It was concluded that W-18 is extensively metabolized. Transformation included dealkylation, hydroxylation, and nitro-reduction. Surprisingly, none of the metabolites displayed any activity to the opioid receptors.

Statement of the Problem

NPS continue to be a worldwide problem and new compounds are constantly emerging. Helander *et al* described the challenge of NPS perfectly by calling it “the hydra monster”. This Greek mythology beast would spawn three heads when one was cut off. Just like NPS, when the forensic community thinks that they are able to detect or schedule a new NPS, three new ones emerge. Although this is an extreme challenge for the forensic community, seemingly an endless fight, there are certain ways to combat these challenges in an effort to get ahead of the curve. First would be to establish the

prevalence of the compounds, in an effort to truly identify the compounds of interest that truly pose potential threats. One such mechanism for assessing prevalence is to screen oral fluid from individuals under the influence of drugs or from sensitive populations subject to routine drug testing. Next, studies evaluating the stability of compounds can help determine proper storage of samples suspected to contain NPS. Often times, a laboratory may be backlogged or lack a specific method to identify potential NPS so testing may not be complete in a timely fashion. Another way to mitigate the challenge of NPS would be to determine the pharmacology of the compounds. Understanding the pharmacokinetics and pharmacodynamics of compounds is needed in order to understand safety profiles and possible toxicity for drug users. Finally, evaluating the metabolism of a compound is useful for identifying biomarkers of recent use, particularly if the parent compound is no longer detected.

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CHAPTER II

Quantitative Analysis of Novel Synthetic Opioids, Morphine and Buprenorphine in Oral Fluid by LC–MS-MS[1]

This dissertation follows the style and format of *Journal of Analytical Toxicology*.

[1]Truver M.T., Swortwood M.J. (2018) Quantitative Analysis of Novel Synthetic Opioids, Morphine and Buprenorphine in Oral Fluid by LC–MS-MS. *Journal of Analytical Toxicology*, **42**, 554-561.

Abstract

The opioid epidemic has become a national health emergency in the United States. While heroin and prescription opioid abuse is not uncommon, synthetic opioid use has risen dramatically, creating a public safety concern. Like traditional opioids, novel synthetic opioids are abused due to their analgesic and euphoric effects. Some adverse side effects include respiratory distress, nausea, and decreased consciousness. Synthetic opioids have emerged into the illicit and online drug market, including AH-7921, MT-45, U-series, and W-series. Though originally developed by pharmaceutical companies, these substances are not well studied in humans and comprehensive analytical methods for detecting and quantifying these opioids are limited. Oral fluid is a useful biological matrix for determining recent drug use, does not require a trained medical professional, and can be collected under direct observation, deterring adulteration. The purpose of this research was to develop and validate a comprehensive analytical method for the detection and quantification of morphine, 6-acetylmorphine, buprenorphine, U-47700, U-49900, U-50488, AH-7921, MT-45, W-18, and W-15 in oral fluid collected via Quantisal™. This was achieved by solid-phase extraction followed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). The limits of detection and quantitation were 5 ng/mL and 10 ng/mL, respectively. Linearity was observed between 10 and 500 ng/mL ($R^2 \geq 0.9959$). Bias and imprecision were $<\pm 11.1\%$. Matrix effects ranged from −21.1 to 13.7%. No carryover was detected following injection of the highest calibrator. All analytes were stable (within ±15% change from baseline) under all tested conditions (24 h at room temperature, 72 h at 4 °C, and in the autosampler for 60 h at 4 °C).

Keywords: Novel Synthetic Opioids, Oral Fluid, LC-MS/MS

Introduction

The United States is experiencing an opioid epidemic, leading to record high number of overdoses. According to the Drug Enforcement Administration (DEA), there was a 576% increase in deaths related to synthetic opioids and fentanyl from 2004 to 2015 (1). Novel synthetic opioids (NSO) have the potential to be hundreds of times more potent than morphine. The increase in NSO use is due to ease of online purchasing, but also by the presence in counterfeit narcotic pills or heroin unbeknownst to the user (2).

The U-series drugs were originally developed by Upjohn ("U") Company as possible analgesics in the 1970s, but did not advance to clinical trials. Currently, the most prevalent compounds are U-47700, U-49900, and U-50488. U-47700 is a selective μ-opioid agonist approximately 7.5 times more potent than morphine (3). Street names that have been associated with this drug are "U4", "pink", and "pinky". While no human pharmacological studies have been conducted, it is believed that U-47700 is abused due to its euphoric and analgesic effects. The adverse side effects are similar to those seen with traditional opioids such as nausea, cyanosis, and respiratory depression (4, 5). U-47700 is associated with cases of intoxication (6, 7) and fatalities (8-12). In November 2016, U-47700 was classified as a Schedule I controlled substance in the US (13). U-49900 is a diethyl analog of U-47700 that is not scheduled in the US. Minimal literature is available on this compound. In Krotulski et al, the metabolism of U-47700 and U-49900 were investigated (14). There has been one reported fatality involving the ingestion of U-49900 and tetrahydrofuranylfentanyl (15). U-50488 is a highly selective κ-opioid receptor agonist studied in rat models (16). A fit for purpose validation was performed by Mohr *et al* for detecting U-50488, U-47700, and furanyl fentanyl in post

mortem cases. Of the 20 case samples, U-50488 was not detected. The abuse potential for both U-49900 and U-50488 are unknown but may rise in the future following scheduling of similar compounds.

AH-7921 is a structural isomer to U-47700 developed by Allen and Hanbury ("AH") in the 1970s as a potential analgesic, but discontinued due to its addictive properties (17). Potency and pharmacological activity is similar to that of morphine (18). It reappeared in 2012 on the Internet as a "research chemical" (19). Fatalities are not uncommon (20-23). Like U-47700, AH-7921 is a Schedule I controlled substance in the US (24).

MT-45 was developed in the 1970s by Dainippon Pharmaceuticals Company as an analgesic alternative to morphine (25). Adverse side effects include respiratory distress, unconsciousness, and temporary hearing loss in a few cases (26, 27). Online users say that the high is "opiate-like" and "a lot better than expected" (27). Severe intoxications (26) and fatalities (20, 28) continue to rise. MT-45 is also classified as a Schedule I controlled substance (29).

The W-series encompasses 32 compounds developed in the 1980s at the University of Alberta (30). In Huang *et al*, W-18 and W-15 were examined and determined to have no activity at the μ, δ, or κ opioid receptors (31). In May 2016, Canada scheduled W-18 as a Schedule I controlled substance, while it is not yet scheduled in the US (32). Very little scientific, peer-reviewed literature is available for W-18 or W-15 activity, prevalence, or intoxications (10). However, National Medical Services Laboratories has incorporated W-18 into their testing capabilities and detected it in at least one post-mortem case, in combination with morphine and 6-

monoacetylmorphine in a known heroin abuser (33). No published analytical methods are available for detection or quantification of W-18 or W-15 in biological fluids.

To date, few analytical methods are available for quantifying NSO in biological fluids. Analytical techniques published for detecting these drugs in alternative matrices such as oral fluid are minimal. The goal of this study was to develop, optimize, and validate a method to quantify multiple NSO in oral fluid. Morphine, 6-monoacetylmorphine (6-AM), and buprenorphine were also incorporated in order to identify drug mixtures or drug use trends. In the 2017 update of recommendations for toxicological investigation of drug-impaired driving, NSO were placed in the tier II category (34). Oral fluid is a useful biological matrix for determining prevalence of use or recent drug intake. Collection of oral fluid does not require a same-sex collector or a trained medical professional and can be collected under direct observation, deterring adulteration. In Griswold *et al*, NSO including U-47700 were detected in oral fluid and urine using a qualitative method on a liquid chromatography-quadrupole/time-of-flight liquid mass spectrometry (LC-QTOF-MS) in a hospital setting where heroin overdose was suspected (35). Oral fluid has been evaluated as a toxicological specimen for driving under the influence (DUID) and was deemed a feasible option for roadside or laboratory analysis (36-39).

Materials and Methods

Chemicals and Reagents

U-47700, U-49900, U-50488, MT-45, W-15, W-18, U-47700-d6, MT-45-d11, and W-18-d4 standards were obtained from Cayman Chemical (Ann Arbor, MI).

Buprenorphine, morphine, morphine-d3, 6-acetylmorphine, and AH-7921 were purchased from Cerilliant (Round Rock, TX).

Hexanes (Optima®), formic acid (Optima®), and ethyl acetate (HPLC grade) were obtained from Fisher Scientific (Fair Lawn, NJ). Methanol (LCMS grade), acetonitrile (LCMS grade), and dibasic sodium phosphate were obtained from J.T. Baker (Center Valley, MA, USA). Glacial acetic acid was obtained from Mallinckrodt Chemicals (St. Louis, MO). Quantisal™ Buffer was obtained from Immunalysis (Pomona, CA). Ammonium formate (10 M) was obtained from Sigma-Aldrich (St. Louis, MO). Concentrated ammonium hydroxide was obtained from Macron Fine Chemicals (Center Valley, MA, USA). Monobasic sodium phosphate was obtained from VWR (Radnor, PA). A Millipore Direct-Q® UV Water Purification system (Billerica, MA) was used to purify water. PolyChrom ClinII 3 cc (35 mg) solid phase extraction (SPE) columns were obtained from SPEware (Baldwin Park, CA). Oral fluid was expectorated by non-opioid users and pooled for method development and validation. Each pool of oral fluid (n=4) contained five volunteers. The pooled oral fluid was verified by running matrix extracted blanks and analyzed with acquisition method below prior to use in preparing calibrators or controls.

Instrumentation

An Agilent 1290 Infinity Liquid Chromatograph system equipped with an Agilent 6470 Triple Quadrupole Mass Spectrometer (Santa Clara, CA) was used for the analysis. Solid Phase Extraction (SPE) was performed using a CEREX-48-positive-pressure manifold (SPEware). Evaporation under nitrogen was performed using a TurboVap LV®

concentration workstation (Caliper Life Sciences, Hopkinton, MA). Data were acquired and analyzed using Agilent MassHunter software.

Preparation of Standard Working Solutions

Stock solutions were prepared at 100,000 ng/mL in methanol for each analyte. Methanolic mixed calibrator solutions containing all analytes were prepared via serial dilution creating calibrators at 10, 25, 75, 125, 250, and 500 ng/mL when fortified in oral fluid. Stock solutions were prepared at 10,000 ng/mL in methanol for each deuterated internal standard (ISTD). Mixed ISTD solution was prepared at 1000 ng/mL in methanol and contained all four deuterated compounds listed above. Mixed quality control (QC) solutions were prepared separately for low (25 ng/mL), medium (100 ng/mL), and high (400 ng/mL) controls when fortified in oral fluid. All standard solutions were stored at -20 °C in amber vials.

Extraction Procedure

ISTD solution (25 µL) was added to 1 mL of a buffer/oral fluid mixture (750 µL Quantisal buffer, 250 µL drug-free oral fluid) to achieve a final concentration of 100 ng/mL. Calibrator or QC solutions (25 µL) were added, followed by 2 mL of 0.1 M phosphate buffer (pH 6). Samples were vortexed, loaded onto SPE columns, and allowed to flow under gravity. The columns were rinsed with deionized water (1 mL) and 1 M acetic acid (1 mL). Cartridges were dried under 20 psi nitrogen for 5 min then washed with 1 mL hexane. Acidic drugs were eluted using ethyl acetate (1 mL). Following a 1 mL methanol wash, basic drugs were eluted using 1 mL of dichloromethane:isopropanol:ammonium hydroxide (80:20:5) then evaporated under

nitrogen at 50 ºC. The samples were reconstituted in 1 mL of mobile phase A: mobile phase B (95:5). A total of 5 µL was injected onto the LC-MS/MS.

Liquid chromatography

Chromatographic separation was achieved using an Agilent Poroshell 120 EC-C18 column (100 mm x 3.0 mm, 2.7 µm) equipped with a Poroshell 120 EC-C18 guard column (2.1 x 5mm, 2.7 µm). The column temperature was maintained at 40°C. Separation was achieved using gradient elution at 0.5 mL/min with 0.05% formic acid and 5 mM ammonium formate in water (mobile phase A) and 0.1% formic acid in acetonitrile (mobile phase B). Gradient elution consisted of a 2 min ramp from 95% to 80% A, then a ramp to 50% A over 7 min, followed by a 2 min high organic rinse and 2 min re-equilibration.

Mass spectrometry

Data were acquired in dynamic multiple reaction monitoring (MRM) mode with positive electrospray ionization (ESI) using a minimum of two transitions per analyte and internal standard (**Table 2.1**). Source parameters were optimized using MassHunter Source Optimizer and were as follows: gas temperature at 300 °C, gas flow at 10 L/min, nebulizer at 40 psi, sheath gas temperature at 350 °C, sheath gas flow at 12 L/min, and capillary voltage at 4500v.

Table 2.1 Optimized liquid chromatography-tandem mass spectrometry parameters for morphine, 6-acetylmorphine, U-47700, AH-7921, buprenorphine, U-49900, U-50488, MT-45, W-18, W-15 and deuterated internal standards.

Analyte	Q1 (*m/z*)	Collision energy (V)	Q3 (*m/z*)	Fragmentor (V)	Retention Time (min)	Internal Standard
Morphine	*286.3*	44	*165*	136	1.066	Morphine-d_3
	286.3	68	152	136		
6-acetylmorphine	*328.3*	44	*165*	156	2.154	Morphine-d_3
	328.3	80	152	156		
U-47700	*329.2*	37	*172.9*	117	4.559	U-47700-d_6
	329.2	61	144.9	117		
AH-7921	*329.2*	57	*144.9*	107	4.791	U-47700-d_6
	329.2	21	46.2	107		
Buprenorphine	*468.6*	44	*396.2*	200	4.9	Morphine-d_3
	468.6	150	151.9	200		
U-49900	*357.1*	37	*172.9*	120	5.102	U-47700-d_6
	357.1	65	144.9	120		
U-50488	*369.1*	49	*158.9*	115	5.49	U-47700-d_6
	369.1	37	112.1	115		
MT-45	*349.5*	20	*182*	112	6.261	MT-45-d_{11}
	349.5	93	77.1	112		
W-18	*422.1*	50	*111*	153	8.314	W-18-d_4
	422.1	50	75.1	153		
W-15	*377.1*	29	*105*	145	8.542	W-18-d_4
	377.1	97	75.1	145		
Morphine-d_3	*289.5*	44	*168.1*	136	1.066	-
	289.5	76	152	136		
U-47700-d_6	*335.2*	37	*172.9*	112	4.559	-
	335.2	57	144.9	112		

Analyte	Q1 (*m/z*)	Collision energy (V)	Q3 (*m/z*)	Fragmentor (V)	Retention Time (min)	Internal Standard
MT-45-d$_{11}$	*360.5*	20	*181.5*	132	6.261	-
	360.5	93	77.1	132		
W-18-d$_4$	*426.1*	53	*115*	143	8.314	-
	426.1	105	78.1	143		

Q1 = quadrupole 1, Q3 = quadrupole, Ions in italics indicate quantifying transitions

Validation

This method was validated according to the Scientific Working Group for Forensic Toxicology guidelines (40). Four lots of pooled oral fluid (n=5 contributors) were used as different sources for the required parameters. Linearity was determined using six non-zero calibrators over five days. Limit of quantitation (LOQ) was evaluated as the lowest non-zero calibrator (10 ng/mL) in duplicate in three different lots over three days. Limit of detection (LOD) was evaluated at half the LOQ concentration in duplicate in three different lots over three days. Bias and precision were assessed in triplicate at low, medium, and high QC concentrations over five days. Matrix effects (post-extraction addition) and recovery were evaluated at low and high QC concentrations (n=2) in three different lots. Stability was evaluated at 24 h at room temperature, 72 h at 4 °C, and in the autosampler for 60 h at 4 °C. Dilution integrity was determined by fortifying a sample at 1000 ng/mL and performing a 10x dilution (n=3). Carryover was evaluated by injecting a negative sample (ISTD only) after the highest calibrator (500 ng/mL). Interferences were evaluated at 10,000 ng/mL by fortifying into low QC samples and then extracted per above. Thirty-three common drugs were evaluated: Δ^9-tetrahydrocannabinol, alprazolam, amobarbital, amphetamine, amitriptyline, butalbital, caffeine, carbamazepine, carisoprodol, cocaine, codeine, cotinine, cyclobenzaprine, dextromethorphan, diazepam,

diphenhydramine, hydrocodone, hydromorphone, ketamine, methadone, nicotine, nordiazepam, oxazepam, oxycodone, pentobarbital, phencyclidine, phenobarbital, propoxyphene, secobarbital, tetrahydrocannabinolic acid, tramadol, and zolpidem.

Authentic Samples

Oral fluid specimens were collected via Quantisal devices from 18 anonymous detainees in a Texas adult detention center in accordance with a Sam Houston State University Institutional Review Board (IRB) approved protocol (# 2017-11-37550). All subjects gave written informed consent prior to collection. Specimens were refrigerated (4 °C) and analyzed within 72 h. Oral fluid samples (1 mL) were extracted and analyzed using the validated method as described above.

Results and Discussion

Our goal was to develop, optimize, and validate an extraction procedure and analytical method to quantify NSO in oral fluid. The assay allowed for simultaneous extraction of AH-7921, MT-45, U-series, W-series, heroin markers (6-AM, morphine), as well as buprenorphine via mixed mode SPE.

MRM transitions were optimized to minimize interferences as summarized in **Table 2.1**. Following MS optimization, chromatography was optimized by investigating several columns, mobile phase solvents, and mobile phase modifiers in order to achieve ideal separation and sensitivity. Initially, mobile phase A was only modified with 0.1% formic acid, but the W-series compounds demonstrated better peak shape and instrument response with addition of ammonium formate. Extraction was assessed in order to attain highest recoveries while minimizing matrix effects. Several elution solvents for basic (2-4% ammonium hydroxide in ethyl acetate) and acidic (n-butyl chloride, acidic methanol,

ether:toluene, and hexane:ethyl acetate:glacial acetic acid) fractions were investigated. To reduce matrix effects and improve internal standard response over the linear range, reconstitution volume was optimized to 1 mL.

To our knowledge, this is the first publication with a validated method for extraction and quantification of W-series drugs. In Shoff *et al*, W-18 was included as a target, but was not validated in their qualitative method (10). It is suspected that their extraction may not recover W-18 as only the alkaline drug fraction was collected. Our study found that both W-18 and W-15 elute with the acidic/neutral portion of the extraction. W-19 was initially included in this study, but abandoned due its alkaline nature (no suitable internal standard) and instability in the mobile phase. Unlike W-15 and W-18, W-19 eluted in the basic fraction of the extraction, possibly due to additional amine group on the phenylethyl piperidine. The analyte was suspected to have a pKa similar to the pH of the mobile phase due to shifting retention times.

Residual plots were used to determine suitable calibration models and weighting for each analyte. A linear calibration model (1/x weighting) from 10-500 ng/mL was optimal for all analytes. R2 values were ≥0.9959 (**Table 2.2**). LOD were acceptable with signal to noise ratios >3, ion ratios within ±20%, and retention times within ±0.1 min at 5 ng/mL for all validated analytes (**Table 2.2**).

Table 2.2 Linearity, limits of detection (LOD), and lower and upper limits of quantification (LLOQ, ULOQ) for morphine, 6-acetylmorphine, U-47700, AH-7921, buprenorphine, U-49900, U-50488, MT-45, W-18, and W-15 in oral fluid.

Analyte	LOD (ng/mL)	LLOQ (ng/mL)	ULOQ (ng/mL)	y-intercept (mean±SD, n = 5)	Slope (mean±SD, n = 5)	R^2 (range, n = 5)
Morphine	5	10	500	0.0118 ± 0.009	0.0192 ± 0.001	0.9975-0.9999
6-acetylmorphine	5	10	500	0.0225 ± 0.013	0.0268 ± 0.002	0.9973-0.9999
U-47700	5	10	500	0.0192 ± 0.017	0.0214 ± 0.001	0.9983-0.9998
AH-7921	5	10	500	0.0092 ± 0.009	0.0135 ± 0.001	0.9987-0.9999
Buprenorphine	5	10	500	0.0028 ± 0.002	0.0041 ± 0.000	0.9982-0.9999
U-49900	5	10	500	0.0069 ± 0.010	0.0125 ± 0.001	0.9988-0.9997
U-50488	5	10	500	0.0084 ± 0.003	0.0097 ± 0.001	0.9989-0.9995
MT-45	5	10	500	0.0160 ± 0.009	0.0084 ± 0.004	0.9962-0.9993
W-18	5	10	500	0.0090 ± 0.010	0.0139 ± 0.001	0.9983-0.9998
W-15	5	10	500	0.0189 ± 0.010	0.0128 ± 0.002	0.9959-0.9998

LOQ were acceptable with signal to noise ratios >10, ion ratios within ±20%, retention times within ±0.1 min, and bias/precision <±20% at 10 ng/mL. Chromatograms of each analyte at the LOQ are shown in **Figure 2.1**. Our morphine and 6-AM LOQ differ slightly from the proposed oral fluid cut-offs by the Substance Abuse and Mental Health Services Administration (15 and 2 ng/mL respectively), but are comparable to the limits (8 ng/mL) of a recent study evaluating field detection of drugs of abuse (37, 41). The LOQ for these heroin markers and buprenorphine are higher than previously published methods in oral fluid, but are suitable for authentic samples. In Cone *et al*, buprenorphine, morphine, and 6-AM were confirmed in authentic oral fluid samples. Buprenorphine (n = 263) had a mean concentration of 433.3 ng/mL, morphine (n = 4575) had a mean concentration of 178.9 ng/mL, and 6-AM (n = 3554) had a mean concentration of 383.2 ng/mL (42).

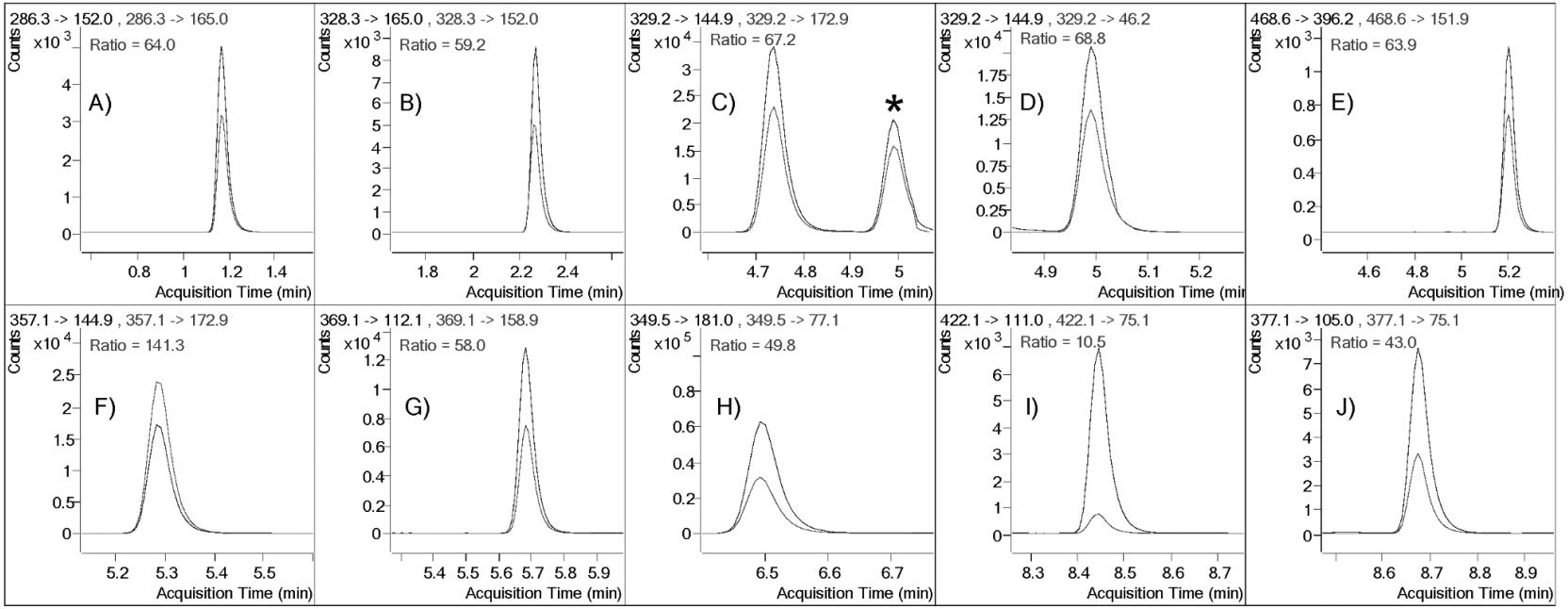

Figure 2.1 Extracted ion chromatogram of morphine (A), 6-acetylmorphine (B), U-47700 (C), AH-7921 (D), buprenorphine (E), U-49900 (F), U-50488 (G), MT-45 (H), W-18 (I), and W-15 (J) at 10 ng/mL; *Shared transitions of structural isomer AH-7921

Although there are limited studies involving detection of NSO, particularly in oral fluid, the LOQs validated in this study are anticipated to be sufficient for authentic samples. As previously mentioned, there are no validated qualitative or quantitative methods for the W-series drugs. Concentrations for MT-45, AH-7921, and the U-series drugs were 6-2900 ng/mL (20, 26, 28), 330-9100 ng/mL (20-23), and 1.5-1460 ng/mL (6-12, 15) in blood, respectively. Although these concentrations ranges are broad, it is difficult to predict oral fluid concentrations that may be encountered. Route of administration and propensity to partition into oral fluid from blood may greatly affect oral fluid concentrations. Additionally, in cases of suspected driving under the influence of drugs, oral fluid may be collected sooner than blood and may better capture recent drug use before blood concentrations are too low for detection.

Bias and precision data are summarized in **Table 2.3**. Bias ranged from -8.8 to -1.7 %, well within the acceptable limits of ±20%.

Table 2.3 Bias and precision data for morphine, 6-acetylmorphine, U-47700, AH-7921, buprenorphine, U-49900, U-50488, MT-45, W-18, and W-15 in oral fluid at three concentrations across the linear range.

Analyte	Mean bias (%, n = 15)			Between-run precision (%CV, n = 15)			Max within-run precision (%CV, n = 3)		
	Low[a]	Med[b]	High[c]	Low[a]	Med[b]	High[c]	Low[a]	Med[b]	High[c]
Morphine	-4.3	-5.3	-5.6	6.5	6.4	4.5	5.5	8.3	9.1
6-acetylmorphine	-5.8	-7.3	-6.5	6.7	6.7	5.5	8.4	11.1	9.7
U-47700	-3.9	-4.0	-3.0	5.5	5.8	4.0	5.5	8.3	6.9
AH-7921	-8.8	-7.2	-6.0	5.6	6.0	4.2	6.1	7.7	8.1
Buprenorphine	-3.3	-4.3	-2.5	6.1	6.6	4.8	6.2	10.3	9.3
U-49900	-4.1	-2.9	-1.7	5.5	5.3	4.4	4.3	7.0	7.8
U-50488	-4.2	-4.6	-2.9	5.4	5.2	4.1	4.5	7.2	7.1
MT-45	-5.1	-3.9	-4.6	5.2	5.5	3.9	5.3	6.8	6.8
W-18	-4.9	-6.1	-3.8	4.9	5.5	4.4	4.4	7.3	8.0
W-15	-5.4	-6.9	-6.1	5.4	6.1	5.7	8.7	7.8	9.3

[a] Low concentration was 25 ng/mL, [b] Medium concentration was 100 ng/mL, [c] High concentration was 400 ng/mL

Between-run precision was calculated as % coefficient of variance (CV) and ranged from 3.9 to 6.7%. The maximum within-run precision was reported and ranged from 4.3 to 11.1%CV. Matrix effects and recovery are shown in **Table 2.4**.

Table 2.4 Mean extraction recovery and matrix effect for morphine, 6-acetylmorphine, U-47700, AH-7921, buprenorphine, U-49900, U-50488, MT-45, W-18, and W-15 in oral fluid.

Analyte	Extraction efficiency (%, n = 6)		Matrix effect (%, n = 6)	
	Low[a]	High[b]	Low[a]	High[b]
Morphine	95.1	91.3	1.0	-3.7
6-acetylmorphine	87.5	87.6	13.7	2.8
U-47700	87.9	89.0	-13.0	-16.4
AH-7921	75.4	84.8	10.7	-0.9
Buprenorphine	85.2	90.4	6.5	1.1
U-49900	85.8	87.5	4.9	1.1
U-50488	77.9	86.7	7.9	1.7
MT-45	78.1	89.4	3.4	1.1
W-18	58.2	52.2	-3.6	-3.7
W-15	63.1	57.8	-21.1	-20.1
U-47700-d_6	84.6	85.6	-14.6	-16.2
Morphine-d_3	90.5	85.9	2.7	-3.2
MT-45-d_{11}	75.9	85.1	4.0	2.1
W-18-d_4	57.1	50.6	-3.5	-3.0

[a] Low concentration was 25 ng/mL, [b] High concentration was 400 ng/mL

All analytes had an extraction efficiency of >75% at low and high concentrations except the W-series. The only analyte that had matrix effects greater than ±20% was W-15 but was reproducible in different matrix lots (%CV<15%) and did not compromise validation parameters including LOD, LOQ, precision, and bias. The lower extraction efficiency and higher matrix effects could be attributed to the acidic elution of the drug. Stability assessed at 24 h at room temperature, 72 h at 4 °C, and in the autosampler for 60 h at 4 °C are presented in **Table 2.5**.

Table 2.5 Fortified and extracted samples stability (% difference) for morphine, 6-acetylmorphine, U-47700, AH-7921, buprenorphine, U-49900, U-50488, MT-45, W-18, and W-15 in oral fluid under different conditions at two concentrations.

Analyte	**24 h room temp (% difference, n = 3)**		**72 h 4 °C (% difference, n = 3)**		**60 h autosampler 4 °C (% difference, n = 3)**	
	Low[a]	High[b]	Low[a]	High[b]	Low[a]	High[b]
Morphine	-0.3	-1.6	4.9	6.2	2.5	1.2
6-acetylmorphine	1.2	-3.5	1.6	7.8	-0.7	0.2
U-47700	-0.2	-3.4	5.4	4.4	-0.5	0.8
AH-7921	7.6	4.5	9.7	8.4	0.8	2.7
Buprenorphine	5.8	-1.1	0.1	7.3	-0.6	1.8
U-49900	11.8	8.2	13.1	9.2	-5.7	0.1
U-50488	12.2	8.1	12.4	9.7	-4.1	-0.7
MT-45	0.4	-2.6	5.2	3.9	-3.3	-2.2
W-18	-4.2	-6.6	4.5	3.6	0.7	0.3
W-15	10.3	9.3	4.6	4.8	1.3	1.8

[a] Low concentration was 25 ng/mL, [b] High concentration was 400 ng/mL

Analyte concentrations within 20% of initial quantification were considered stable and no more than 13.1% difference was observed. During method development and pre-validation experiments, W-19 demonstrated significant degradation over the

course of an analytical run in the autosampler. Due to instability, this analyte was not included in validation. The 10x dilution integrity was deemed acceptable if within ±20% of the target concentration. All analytes quantified 89-95% of targeted concentration for dilution integrity.

During method development issues initially arose when assessing carryover for this method. There was significant carryover (>10% of LOQ peak abundance) for MT-45 and W-series drugs. Needle rinse parameters were optimized, including rinse time, rinse position, and rinse solution. Carryover was no longer significant after changing the needle rinse to isopropanol:methanol:water:acetonitrile (25:25:25:25). Of the 33 drugs evaluated for potential interferences, no qualitative or quantitative interferences were observed. Additionally, no endogenous interferences were observed for the various lots of pooled oral fluid from drug-free volunteers.

Fentanyl analogs are also commonly encountered in heroin and counterfeit narcotic pills. These analytes are outside the scope of this study and will be addressed in a separate publication utilizing high-resolution mass spectrometry. Further, authentic specimens are currently being collected for a separate investigation that will include impairment data. However, in the limited number of samples analyzed, only morphine and 6-AM were detected by this method. Oral fluid was collected from 18 adults: 9 males (24-42 years) and 9 females (23-47 years). From the authentic samples analyzed, morphine was detected in 4 cases and 6-AM was detected in 3 of those 4 subjects. Morphine concentrations were <LOQ, 32, 104, and 146 ng/mL and 6-AM concentrations in those same individuals were <LOD, 15, <LOQ, and 110 ng/mL.

The continued analysis of authentic samples will provide valuable data since previous literature has only qualitatively identified NSO in oral fluid.

Conclusion

A method for the simultaneous quantification of morphine, 6-AM, buprenorphine, U-47700, U-49900, U-50488, AH-7921, MT-45, W-18, and W-15 in oral fluid was optimized and met SWGTOX validation criteria. The extraction technique and LC-MS/MS parameters can also be easily translated to other biological matrices. Incorporation of heroin biomarkers and an opioid addiction treatment therapy will allow for analysis of compliance and drug use trends. This method is currently being used to determine drug trends in several groups. Analysis of oral fluid specimens from sensitive populations (i.e. DUID, prisoners, arrestees, parolees) that may be seeking alternative or substitute opioid use will allow us to examine prevalence of novel synthetic drugs in the market.

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CHAPTER III

Oral Fluid and Drug Impairment: Pairing Toxicology with Drug Recognition Expert Observations[1]

This dissertation follows the style and format of *Journal of Analytical Toxicology*.

[1]Truver M.T., Palmquist K.B., Swortwood M.J. (2019) Oral Fluid and Drug Impairment: Pairing Toxicology with Drug Recognition Expert Observations. *Journal of Analytical Toxicology*, **43**, 637-643.

Abstract

According to the Governors Highway Safety Association, drugs are detected more frequently in fatally-injured drivers than alcohol. Due to the variety of drugs (prescribed and/or illicit) and their various physiological effects on the body, it is difficult for law enforcement to detect/prosecute drug impairment. While blood and urine are typical biological specimens used to test for drugs, oral fluid is an attractive alternative matrix. Drugs are incorporated into oral fluid by oral contamination (chewing or smoking) or from the bloodstream. Oral fluid is non-invasive and easy to collect without the need for a trained professional to obtain the sample, unlike urine or blood. This study analyzes paired oral fluid and urine with drug recognition expert (DRE) observations.

Authentic oral fluid samples (n=20) were collected via Quantisal™ devices from arrestees under an IRB approved protocol. Urine samples (n=18) were collected with EZ-SCREEN® cups that presumptively screened for THC (cannabinoids), Opiates, Methamphetamine, Cocaine, Methadone, Phencyclidine, Amphetamine, Benzodiazepines, and Oxycodone. Impairment observations (n=18) were recorded from officers undergoing DRE certification.

Oral fluid samples were screened using an Agilent Technologies 1290 Infinity liquid chromatograph (LC) coupled to an Agilent Technologies 6530 Accurate Mass Time-of-Flight mass spectrometer (MS). Personal Compound and Database Libraries were produced in-house containing 64 drugs of abuse. An Agilent 1290 Infinity LC system equipped with an Agilent 6470 Triple Quadrupole MS was used for quantification of buprenorphine, heroin markers (6-acetylmorphine, morphine), and synthetic opioids.

Subjects were 23-54 years old; 11 (55%) were male and 9 (45%) were female. Evaluator opinion of drug class was confirmed in oral fluid 90% of time and in urine 85% of the time in reference to scope of testing by the LC-MS methods employed (excludes cannabis and CNS depressants). Data indicate that oral fluid may be a viable source for confirming driving under the influence of drugs (DUID).

Keywords: Oral fluid, Drug Recognition Expert, Driving Under the Influence of Drugs, Opioids

Introduction

According to the Governors Highway Safety Association (GHSA), 44% of fatally-injured drivers tested positive for drugs in 2016, which was higher than alcohol with 38% of fatally-injured drivers tested positive for alcohol. Due to the variety of drugs (prescribed and/or illicit) and their various physiological effects on the body, it is difficult for law enforcement to detect/prosecute drug impairment. According to the International Association of Chiefs of Police (IACP), there were over 30,000 Drug Recognition Expert (DRE) evaluations performed in 2017 in the US. The most abundant evaluator opinion was cannabis, then central nervous system (CNS) stimulants, followed by CNS depressants, and narcotic analgesics (1). Depending on state laws, proof of impairment could be needed in order to prosecute someone suspected of DUID. Previous studies indicate that the tests performed during DRE examinations are beneficial impairment indicators (2-4).

In 2017, there were over 8,000 certified DRE in the US (1). While training more DRE officers may help reduce DUID related fatalities, improving drug detection and identification may also aid in this public safety issue. A potential tool for improvement in drug testing is consideration of alternative matrices, such as oral fluid. Oral fluid may be collected roadside as it is non-evasive, easy to transport, and does not need a trained professional to collect. As of April 2018, states that allow oral fluid collection are Arizona, Arkansas, Colorado, Georgia, Illinois, Indiana, Kansas, Louisiana, Missouri, Nevada, New York, Oklahoma, South Dakota, Utah, and Wyoming (5). These participating states only make up 30% of the US and while it is legal for oral fluid to be collected, few states are currently collecting due to lack of capabilities. Police officers in

European countries prefer oral fluid opposed to blood, urine or sweat due to its ease of use, non-invasive nature, and lowered risk of infection transmission (6). Oral fluid collection with a roadside portable device has the benefit of screening the suspect's specimen within minutes (7-9). Krotulski *et al* demonstrated that the Alere DDS® 2 was reliable and suitably robust in the field by comparing results from the device to confirmation results and assessing true positives, true negatives, false positives, and false negatives (10). Roadside screening devices are limited in scope and sensitivity. For evidentiary testing, oral fluid can be collected with various devices, such as the Quantisal™ collection device, for comprehensive laboratory analyses such as liquid chromatography-mass spectrometry (LC/MS). With the emergence of novel psychoactive substances (NPS), analyzing oral fluid on a sensitive instrument such as a liquid chromatograph accurate mass time-of-flight mass spectrometer (LC-QTOF-MS), can allow for retrospective analysis of the accumulated data. Some NPS such as U-47700 have been detected in oral fluid samples (11).

Countries outside the US, such as Australia, Belgium, and France, have implemented either screening and/or confirmation in roadside oral fluid collection (12). Established cut-off concentrations for Δ^9-tetrahydrocannabinol (THC), CNS stimulants, and narcotic analgesics ranging from 5-25 ng/mL are implemented for confirmatory analysis in Belgium (12). This range is similar to the confirmatory analysis cut-off range (2-15 ng/mL) proposed for workplace drug testing by the Substance Abuse and Mental Health Services Administration (SAMHSA) for the same drug classes (13). Michigan recently completed a pilot study to evaluate oral fluid roadside analysis where oral fluid samples were screened with the Alere DDS® 2, collected with Quantisal collection device

(if subject consented) and compared to blood draws. Of the 92 roadside oral fluid tests, 88 were confirmed in blood or oral fluid collected by the Quantisal device. Although further evaluation will be performed, this study demonstrates the viability of oral fluid used for suspected DUID investigations in the US (14).

Our study aimed to compare presumptive urine test results and confirmatory oral fluid test results with DRE evaluations performed by officers in training in an effort to assess use of alternative matrices for toxicology during DRE examinations.

Methods

Subjects

Participants (n=20) in this study were recruited from detainee centers in Texas (Dallas and San Antonio). Demographic information can be seen in **Table 3.1**.

Table 3.1 Subject demographics

Sample	Age	Sex	Race	Location
01	37	M	Hispanic	San Antonio
02	32	F	Hispanic	San Antonio
03	27	F	Hispanic	San Antonio
04	36	F	White	San Antonio
05	23	F	Hispanic	San Antonio
06	27	M	Hispanic	San Antonio
07	34	M	Black	San Antonio
08	27	M	Black	San Antonio
09	25	F	White	San Antonio
10	36	F	Hispanic	San Antonio
11	32	F	Hispanic	San Antonio
12	47	F	Hispanic	San Antonio
13	29	M	White	San Antonio
14	24	M	Hispanic	San Antonio
15	35	M	Hispanic	San Antonio
16	42	M	White	San Antonio
17	31	F	Black	San Antonio
18	35	M	White	San Antonio

Sample	Age	Sex	Race	Location
19	54	M	Black	Dallas
20	46	M	White	Dallas

DRE Examinations

DRE examinations were performed by officers undergoing Drug Evaluation and Classification Program (DEC/DRE) certification. Part of the curriculum includes drug recognition field certification with persons under the influence of drugs. Law enforcement recruited subjects within the detention centers based on clues of impairment. Subjects were incentivized by food, water, or a phone call to participate in the training. Participation was optional and completely anonymous. Officers used the 12-step DRE protocol to perform their examinations: 1) breath alcohol test, 2) interview of the arresting officer, 3) preliminary examination and first pulse, 4) eye examination, 5) divided attention psychophysical tests, 6) vital signs and second pulse, 7) dark room examinations, 8) examination of muscle tone, 9) check for injection sites and third pulse, 10) subject's statements and other observations, 11) analysis and opinions of the evaluator, and 12) toxicological examination (15).

Urine Samples

Urine was collected during the DRE examination from subjects using a MEDTOX® EZ-SCREEN® (Burlington, NC) cup, which presumptively screened for: : amphetamines (300 ng/mL), benzodiazepines (200 ng/mL), cocaine (100 ng/mL), methadone (200 ng/mL), methamphetamine (1000 ng/mL), opiates (100 ng/mL), oxycodone (100 ng/mL), phencyclidine (PCP) (25 ng/mL), and cannabinoids (40 ng/mL). Adequate volume was verified for each specimen. Cups were read within 15 min by field

instructor while DRE examinations were continued by trainees. Urine results were blinded to trainees until DRE opinions were rendered to instructor.

Oral Fluid Specimens

Quantisal devices were used to obtain oral fluid from subjects in accordance with a Sam Houston State University Institutional Review Board (IRB) approved protocol (# 2017-11-37550). All subjects gave informed consent prior to collection. Oral fluid was collected during DRE examinations within 30 minutes of urine specimen collection. Samples were extracted and analyzed using validated methods (16,17). Oral fluid samples (1 mL) were fortified with internal standard and extracted using SPEware PolyChrom ClinII 3 cc (35 mg) (Baldwin Park, CA) solid phase extraction (SPE) cartridges. Briefly, samples were buffered (100 mM), loaded onto cartridges, then washed, and dried. For screening, basic drugs were eluted with 5% ammonium hydroxide in ethyl acetate. For confirmation, acidic and basic drugs were eluted with ethyl acetate and dichloromethane:isopropyl alcohol with 5% ammonium hydroxide, respectively. An Agilent 1290 Infinity liquid chromatograph coupled to an Agilent 6530 Accurate Mass Time-of-Flight mass spectrometer (LC-QTOF-MS) was used to screen samples for common drugs of abuse shown in **Table 3.2** below.

Table 3.2 Analytes included in PCDL categorized by DRE classification (n=64)

DRE Classification	Compounds
CNS Depressant	Alprazolam, Amytriptyline, Carisoprodol, Cyclobenzaprine, Diphenhydramine, Etizolam, Lorazepam, Zolpidem

CNS Stimulant	Amphetamine, Caffeine, Cotinine, Cocaine, Methamphetamine, Nicotine, Pseudoephedrine
Hallucinogens	25B-NBOMe, 25C-NBOMe, 25D-NBOMe, 25E-NBOMe, 25H-NBOMe, 25I-NBOMe, 25N-NBOMe, LSD, MDMA, Mesc-NBOMe
Narcotic Analgesics	4-ANPP, 6-AM, 7-hydroxymitragynine, Acetaminophen, Acetylfentanyl, AH-7921, Alfentanil, Buprenorphine, Butyrylfentanyl, Carfentanil, Cis-methyl fentanyl, Codeine, Fentanyl, Furanylfentanyl, Furanyl norfentanyl, Hydrocodone, Hydromorphone, Isobutyryl fentanyl, Meperidine, Mephedrone, Methadone, Mitragynine, Morphine, MT-45, N,N-didesmethyl-U-47700, N-desmethyl-U-47700, Norcarfentanil, Norfentanyl, Oxycodone, Oxymorphone, Remifentanil, Sufentanil, U-47700, U-49900, U-50488, Valerylfentanyl
Dissociative Anesthetics	Dextromethorphan, Ketamine, PCP

An Agilent 1290 Infinity II liquid chromatograph coupled to an Agilent 6470 triple quadruple mass spectrometer was used to confirm and quantify heroin markers and buprenorphine. Oral fluid samples were fortified with a drug mixture at 0.1, 0.25, 0.5, 1.0, 2.5, 10 and 100 ng/mL in three pooled sources assessed in duplicate over three days. The cutoff concentrations were determined to be: amphetamine (2.5 ng/mL),

methamphetamine (2.5 ng/mL), cocaine (0.25 ng/mL), morphine (1.0 ng/mL), 6-AM (1.0 ng/mL), codeine (100 ng/mL), and methadone (0.25 ng/mL)."

Limitations

Methods used in this study were optimized for the detection and quantification of synthetic opioids in order to monitor prevalence in populations (16,17). After sample collection and analysis, the authors felt that although no synthetics were detected, data aggregated from DRE examinations paired with oral fluid and presumptive urine results were important to disseminate to the forensic community. Cannabinoid use is prevalent as demonstrated by the presumptive urine cups. Although confirmatory testing would be preferred to further correlate matrices for the cannabis category, urine samples collected by the DRE training officers were promptly destroyed in line with their privacy protocol and oral fluid samples were consumed during analytical testing and therefore could not be analyzed externally. Further, THC was outside the analytical scope of the LC-MS method at the time of analysis.

Results and Discussion

The subjects were 23-54 years old and consisted of 11 males and 9 females. There were a total of 18 urine samples and 20 oral fluid samples collected and analyzed, as two subjects (08 and 16) withdrew from the DRE examinations and therefore urine specimens were not collected. **Table 3.3** summarizes the drug classes detected in urine and oral fluid, alongside the DRE opinion and self-reported drug use for each subject.

Table 3.3 Toxicological findings paired with DRE opinion

Sample	Drugs Admitted to Using	Urine	Oral Fluid	Evaluator Opinion
01	Methamphetamine, Heroin, K2	Amphetamine, Benzodiazepine, Cocaine, Methamphetamine, Opiate, THC	Amphetamine, Cocaine, Methamphetamine, Morphine (145 ng/mL), 6-AM (109 ng/mL)	Narcotic Analgesics
02	Speedballs and Ice	Amphetamine, Benzodiazepine, Cocaine, Opiate	Amphetamine, Cocaine, Codeine, Methamphetamine, Morphine (32.2 ng/mL) , 6-AM (15.2 ng/mL)	Narcotic Analgesics
03	Heroin	Opiate	Morphine	Narcotic Analgesics
04	Heroin	Amphetamine, Cocaine, Opiate, THC	Amphetamine, Codeine, Methamphetamine, Morphine (103 ng/mL) , 6-AM (6.8 ng/mL)	Narcotic Analgesics
05	Heroin and Meth	Amphetamine, Cocaine, Opiate, THC	Methamphetamine, Morphine (<LOQ), 6-AM	CNS Stimulant, CNS Depressant, Cannabis
06	Xanax, Ice, and Marijuana	Amphetamine, Methamphetamine, THC	Amphetamine, Methamphetamine	CNS Stimulant. Cannabis
07	Max (Synthetic Cannabis)	Negative	Negative	Cannabis

Sample	Drugs Admitted to Using	Urine	Oral Fluid	Evaluator Opinion
08*	-	-	Amphetamine, Methamphetamine	-
09	Methamphetamine, Marijuana, EtOH	Amphetamine, Cocaine, THC	Amphetamine, Methamphetamine	Alcohol, CNS Stimulant, Cannabis
10	LSD, Pot, and Meth	Amphetamine, THC	Amphetamine, Cocaine, Methamphetamine	Cannabis
11	Methadone, Heroin, Methamphetamine	Amphetamine, Benzodiazepine, Opiate	Amphetamine, Methamphetamine, Morphine	CNS Depressant, Narcotic Analgesics
12	Xanax	Amphetamine, Benzodiazepine, Cocaine, Methadone, Methamphetamine, THC	Amphetamine, Cocaine, Methadone, Methamphetamine	CNS Depressant, Narcotic Analgesics
13	Marijuana, Methamphetamine, Xanax	Amphetamine, Benzodiazepine, Cocaine, Methamphetamine, THC	Amphetamine, Methamphetamine	CNS Stimulant, CNS Depressant, Cannabis
14	Cocaine, Marijuana, Xanax	Benzodiazepine, Cocaine, THC	Cocaine	CNS Stimulant, Cannabis
15	Weed and K2	Amphetamine, Cocaine, THC	Amphetamine, Methamphetamine	CNS Stimulant, CNS Depressant, Cannabis
16*	-	-	Amphetamine, Methamphetamine	-
17	Klonopin, Methadone, Marijuana	Amphetamine, Benzodiazepine, Cocaine, Opiate, Methadone Methamphetamine, PCP, THC	Methadone, Methamphetamine	CNS Depressant, Hallucinogen, Narcotic Analgesics
18	Meth	Amphetamine, Benzodiazepine, THC	Amphetamine, Methamphetamine	CNS Stimulant, Narcotic Analgesics

Sample	Drugs Admitted to Using	Urine	Oral Fluid	Evaluator Opinion
19	Marijuana	Benzodiazepine, Opiate, THC	Cocaine, Morphine	Alcohol, CNS Depressant, Narcotic Analgesics
20	Heroin	Cocaine, Opiate, THC	Amphetamine, Methamphetamine, Morphine, 6-AM	Narcotic Analgesics

*participant only provided oral fluid sample

The evaluator opinion was confirmed in oral fluid 90% of the time, while confirmed in urine 85% of the time (within the scope of each test). In **Figure 3.1**, the pairing of oral fluid and urine positive identifications is depicted.

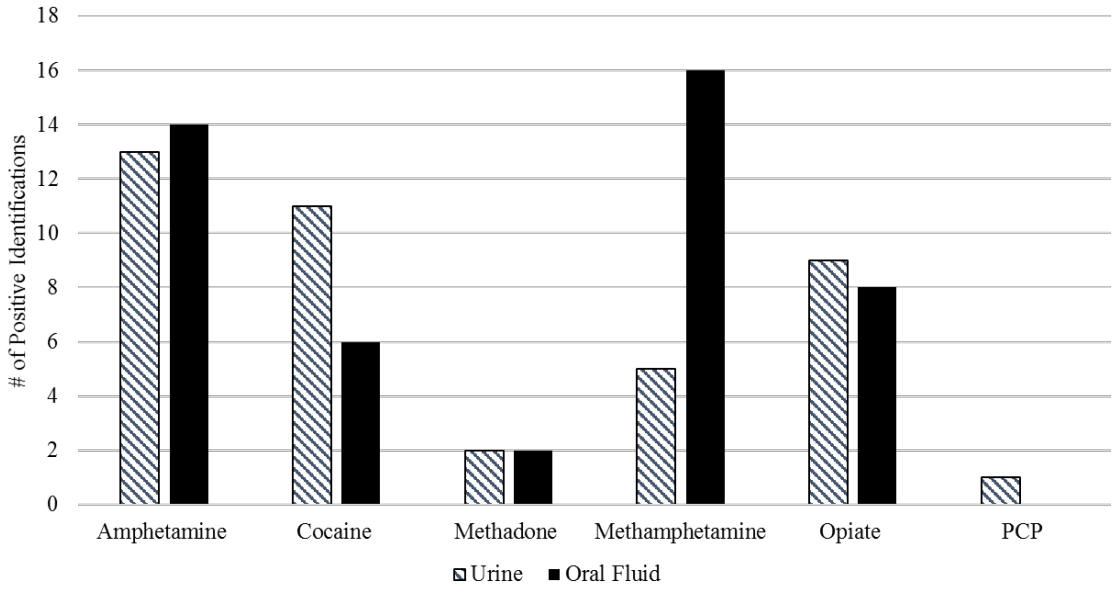

Figure 3.1 Positive identifications of analytes for urine (n=18) and oral fluid (n=20).

Low limits of detection were not achieved for all compounds due to optimization of the method to different target analytes. However, the cutoffs presented are comparable to or lower than the urine screening system. Logan *et al* reports several recommended screening method cutoff values for various drugs in blood, urine, and oral fluid (18). The recommended cutoff values range from 10- 20 ng/mL in oral fluid for compounds investigated in the present study. While not all compounds have a recommended screening cutoff value, the LODs presented here are comparable to or lower than those recommended are. These achieved lower limits of detection ensure that proper identifications can be made in oral fluid.

Cannabis

Of the 20 subjects, 9 admitted to using marijuana. With the presumptive urinalysis, 14 were positive for THC and the DRE identified cannabis intoxication for 7 subjects. Positive urine results for THC does not necessarily indicate that the subject was under the influence at the time of the evaluation, since the detection window for urine is wide. The LC-MS methods used in this study were not optimized for the detection of cannabinoids, so THC was not detected in oral fluid. Although this study was not able to detect cannabinoids in oral fluid, previous studies have detected THC in screening device and confirmatory tests (19,20).

CNS Stimulants

Methamphetamine use was self-identified by 9 of the 20 subjects, with 5 positive identifications of methamphetamine in urine and 16 positive identifications in oral fluid as seen in Figure 1. Amphetamine was detected in 13 of the urine samples and in 14 of the oral fluid samples. Differences in detection rates can be attributed to differences in detection windows between the two matrices. The two subjects that did not complete DRE examinations tested positive for methamphetamine and amphetamine, which also contributes to the differences in detection rates as there were no paired urine specimens. There were two subjects that admitted to using cocaine, while 11 were positive in urine samples and 6 positives in oral fluid. The discrepancy between the oral fluid and urine results could be attributed to the target analyte of the urine cup (benzoylecgonine) which has a longer detection window in urine.

A total of 15 subjects were positive for at least one or more CNS stimulant in urine and 16 were positive in oral fluid, but evaluators only identified CNS stimulant

intoxication for 8 subjects. Poly-drug use could cause some impairment clues to mask others (21). In **Table 3.4**, a summary of DRE examinations is shown, which includes subject's vitals, psychophysical tests, nystagmus, and physical response.

Table 3.4 Vitals, nystagmus, psychophysical tests, and physical response for subjects

	Vitals												
Sample	**Temp (°F)**	**BP (mmHg)**	**Mean HR (BPM)**	**Vertical Nystagmus**	**HGN**	**Walk and Turn**	**One Leg Stand**		**Modified Romberg Balance**		**Muscle Tone**	**Pupil Size**	**Reaction to Light**
						Clues	**Clues (Left)**	**Clues (Right)**	**Estimation of 30 seconds**	**Body Sway (in)**			
01	95.9	110/58	45	No	None	4	2	2	72	3	Flaccid	Normal	Slow
02	96.9	130/72	81	No	None	2	3	4	39	2	Flaccid	Normal	Slow
03	97.7	118/70	79	No	None	2	2	1	58	2	Normal	Normal	Normal
04	98.7	124/78	73	No	None	2	2	0	28	2	Flaccid	Normal	Normal
05	99.4	108/70	93	Yes	Present	3	2	2	18	2	-	Dilated	Slow
06	98.2	138/100	76	No	None	3	2	1	25	2	Rigid	Dilated	Normal
07	97.8	138/74	73	No	None	3	0	2	47	2	Normal	Normal	Slow
09	99.8	150/92	127	No	Present	1	1	3	25	2	Flaccid	Dilated	Normal
10	98.6	118/72	82	No	None	2	0	3	44	-	Flaccid	Normal	Normal
11	98.5	118/76	69	Yes	Present	2	2	2	22	1	Rigid	Normal	Normal
12	96.0	122/80	63	No	Present	3	3	2	25	3	Flaccid	Constricted	Little
13	97.3	144/92	103	Yes	Present	4	1	1	14	2	Flaccid	Dilated	Slow
14	97.0	142/78	65	No	None	6	2	4	30	-	Rigid	Dilated	Normal
15	97.0	152/104	94	Yes	Present	3	3	4	51	2	Flaccid	Dilated	Slow
17	97.6	112/72	60	Yes	Present	3	2	3	19	2	Flaccid	Normal	Normal
18	98.0	150/110	81	No	None	4	2	1	33	3	Flaccid	Normal	Little
19	96	98/66	76	Yes	Present	5	3	3	90	3	Flaccid	-	-
20	93.3	138/78	60	No	None	2	2	2	26	2	Flaccid	Normal	Slow

BP=Blood Pressure, HR=Heart Rate, BPM=Beats Per Minute, HGN= Horizontal Gaze Nystagmus

An example of poly-drug use with masking clues is Subject 01. He demonstrated low average heart rate (45 beats per minute, BPM), slowed estimation of time (72 s to estimate the passage of 30 s), pupils with slow reaction to light, and flaccid muscle tone. All of these clues are indicative of being under the influence of narcotic analgesics, but in both urine and oral fluid, cocaine and methamphetamine were present along with narcotic analgesics. Depending on time administered, one drug may have greater effect on subject's behavior during the time of evaluation. Subject 09 presented multiple clues of being under the influence of a CNS stimulant: elevated blood pressure (150/92) and heart rate (127 BPM), fast estimation of 30 s (25 s), and dilated pupils.

Narcotic Analgesics

The use of heroin or "speedballs" was admitted by 7 of the subjects, while there were 9 positive identifications of opiates in urine and 8 positive results of morphine or 6-acetylmorphine (6-AM) in oral fluid. Quantitative concentrations of morphine and 6-AM for Subjects 01, 02, and 04 were assessed. Morphine concentrations were 145 ng/mL, 32.2 ng/mL, and 103 ng/mL, respectively. Corresponding 6-AM concentrations were 109 ng/mL, 15.2 ng/mL, 6.8 ng/mL, respectively. There were 3 subjects that admitted to using methadone, while 2 were positive in both urine and oral fluid. A total of 10 narcotic analgesics (opiates and methadone) were identified in both urine and oral fluid. In one case, the DRE indicated narcotic analgesics but was not confirmed by either urine or oral fluid. For Subject 18, signs of stimulant use were present such as high blood pressure (150/110), but the only sign of narcotic analgesic was flaccid muscle tone and a "little" reaction to light. Due to this being a training/certification setting, a miscall would not be uncommon.

CNS Depressants

Self-reported use of Xanax was indicated by 3 subjects and 9 subjects were positive for benzodiazepines in urine. The LC-MS screening method was not optimized for benzodiazepines, so these cases were not confirmed in oral fluid. The evaluators called CNS depressant for 6 of the subjects and were confirmed in urine in 4 subjects. Subject 15 presented with clues of CNS stimulant impairment, but also had flaccid muscle tone and slow reaction to light. However, oral fluid and urine results were positive for CNS stimulants and cannabis. Subject 05 also showed clues of CNS stimulant impairment accompanied with flaccid muscle tone and slow reaction to light. Oral fluid and urine were positive for CNS stimulants, narcotic analgesics, and cannabis. It is possible that the evaluator misinterpreted the presence of a narcotic analgesic as a CNS depressant.

Hallucinogens, Inhalants, and Dissociative Anesthetics

Only one subject admitted to taking a hallucinogen (LSD) but could not be confirmed in oral fluid or urine as it was outside the scope of both methods. A hallucinogen was only identified by the evaluator for Subject 17, along with CNS depressants and narcotic analgesics. This subject demonstrated signs of poly-drug intoxication and fell asleep while providing the oral fluid sample. This subject was positive for every panel except oxycodone on the EZ-Screen urine cup. The interaction of multiple drugs may have been incorrectly interpreted as hallucinogen intoxication or perhaps a drug was missed by the toxicological testing due to limitations of both analytical tests. No inhalants were detected in either matrix and inhalant impairment was not determined in any of the examinations. As previously mentioned, the dissociative

anesthetic PCP was identified in Subject 17's urine sample but signs of impairment from that drug were not observed by the DRE.

Conclusion

Given the analytical scope of the LC-MS methods (excluding CNS depressants and cannabis), there were 20 identifications of drug class impairment in 18 subjects. The evaluator opinion was confirmed with oral fluid results in 18/20 occurrences (90%). In urine, there were 33 separate identifications of drug class impairment that were confirmed in 28 instances of DRE opinion for 18 subjects (85%). Differences in confirmation rates can be attributed to extended detection window of urine and the lack of experience by the officers due to training environment. As is the difficulty with NPS, synthetic drug use may be missed or misinterpreted by DRE examination. As such, Subject 07 admitted to using "Max" (a synthetic cannabinoid) but cannabis impairment was indicated by the officer. Synthetic cannabinoids may present as a variety of drug impairment types but were outside the scope of both the urine and oral fluid tests. Overall, toxicological results between urine and oral fluid paired with DRE evaluations were comparable. Data, while limited, add to the growing amount of literature that oral fluid may be a valuable biological specimen for identifying drugs in cases of DUID.

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CHAPTER IV

Quantification of Seven Novel Synthetic Opioids in Blood Using LC-MS/MS[1]

This dissertation follows the style and format of *Journal of Analytical Toxicology*.

[1]Lowry J., Truver M.T., Swortwood M.J. (2019) Quantification of Seven Novel Synthetic Opioids in Blood Using LC-MS/MS. *Forensic Toxicology*, **37**, 215-223.

Abstract

Purpose: The objective of this study was to develop, optimize, and validate a method for the simultaneous quantification of U-47700, AH-7921, U-49900, U-50488, MT-45, W-18, and W-15 in whole blood using liquid chromatography – tandem mass spectrometry (LC – MS/MS).

Methods: Blood samples (500 µL) were fortified with mixed calibrator or quality control (25 µL) and internal standard (10 µL) solutions. Analytes were isolated via a solid-phase extraction procedure. Analysis was performed using an Agilent 1290 Infinity II liquid chromatograph coupled to an Agilent 6470 triple quadrupole mass spectrometer. The method was validated in accordance with Scientific Working Group for Forensic Toxicology (SWGTOX) standard practices for method validation in forensic toxicology and applied to the analysis of postmortem blood specimens.

Results: Lower limits of quantification were 0.25-1 ng/mL and the upper limits of quantification were 100 ng/mL. The coefficients of determination (R^2) for the calibration curves were >0.99. Analytical bias, within-run imprecision, and between-run imprecision were within ±15%, ≤16%, and ≤17%, respectively. All analytes were found to be stable at room temperature for 24 h, refrigerated (4°C) for 72 h, and in the autosampler (4°C) for 72 h. Authentic blood samples (n=30) were analyzed using the validated method. Mean (range) U-47700 concentrations were 214 (3.2 – 1448) ng/mL in 15 cases.

Conclusions: A quantification method for seven synthetic opioids (U-47700, AH-7921, U-49900, U-50488, MT-45, W-18, and W-15) in whole blood was developed, optimized, and validated in accordance with SWGTOX standard practices for method

validation in forensic toxicology. This sensitive method was successfully applied to postmortem casework.

Keywords: Novel synthetic opioids; U-47700; W-18; W-15; method validation; LC-MS/MS

Introduction

The growing opioid epidemic has been classified by the U.S. Department of Health and Human Services as a public health emergency. Opioid abuse has been on the rise for almost two decades, with overdose deaths increasing from 8,050 to 33,091 between 1999 and 2015 according to a report by the Center for Disease Control and Prevention (CDC) (1). The increase in fatal opioid overdoses is primarily attributed to deaths involving synthetic opioids other than methadone, which according to the CDC, increased by 100% between 2015 and 2016 (2).

Numerous synthetic opioids developed in the 1970s and 1980s for their potential medical use, including U-47700, AH-7921, U-49900, U-50488, MT-45, W-18, and W-15, have recently reemerged as drugs of abuse. The Upjohn Company developed U-47700, U-49900, and U-50488 for their potential use in pain relief in the 1970s. U-47700, a μ- and κ-opioid receptor agonist, possesses a potency approximately 7.5 times greater than morphine (3, 4). U-50488 acts as an agonist on the κ-opioid receptor (5). AH-7921 was developed in 1976 by Allen and Handburys Ltd. for its analgesic properties. AH-7921, a μ- and κ-opioid receptor agonist, is equipotent with morphine (3, 6). Dainippon Pharmaceutical Co. Ltd. developed MT-45 for use as an analgesic and anti-inflammatory in the 1970s. MT-45, a μ-, κ-, and δ-opioid receptor agonist, is equipotent with morphine (6, 7). In the 1980s, a university in Canada developed a W-series of opioids, including W-18 and W-15 for their potential as analgesics (8). However, these compounds were each abandoned before advancing to clinical trials.

Overdoses have already been reported in the literature involving many of these synthetic opioids, including U-47700, AH-7921, and MT-45 (3, 7, 9-21). Overdose cases

involving U-49900, U-50488, W-18, and W-15 could be forthcoming due to their increasing popularity. The numerous deaths and potential dangers associated with these synthetic opioids, have led many countries including the U.S. to schedule these drugs as controlled substances. Currently, U-47700, AH-7921, and MT-45 are classified by the Drug Enforcement Administration as Schedule I controlled substances.

The rise in abuse of these synthetic opioids in combination with many scheduled as controlled substances has led to an increased need for analytical methods. Lack of inexpensive and simplistic analytical methods available for synthetic opioids could be contributing to delayed toxicological analysis and under-identification (22). The current analytical methods existing for these synthetic opioids are limited, but methods have been reported for U-47700, AH-7921, U-50488, MT-45, and W-18 (3, 7, 12, 17-19, 23-24). To our knowledge, methods have not yet been reported for the simultaneous quantification of these synthetic opioids or for the quantification of W-18 or W-15 in blood.

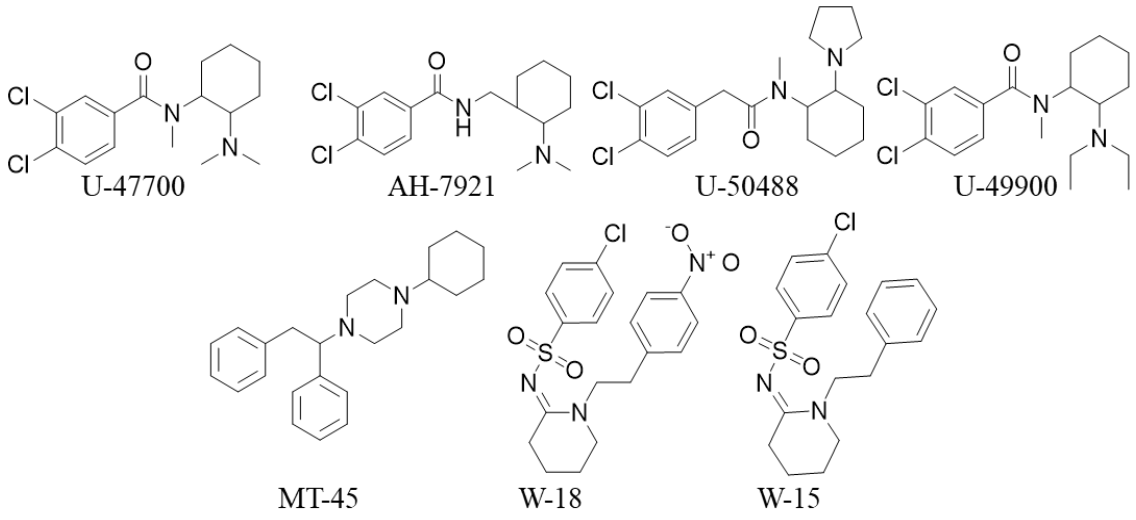

Figure 4.1 Structures of seven synthetic opioids dealt with in this study

This study focuses on the development, optimization, and validation of a quantification method for seven synthetic opioids, U-47700, AH-7921, MT-45, W-18, W-

15, U-49900, and U-50488 (**Figure 4.1**) in whole blood. This method requires minimal sample preparation prior to the isolation of these synthetic opioids via solid-phase extraction (SPE). Analysis is performed using liquid chromatography – triple quadrupole mass spectrometry (LC – MS/MS). This study provides an efficient, comprehensive method for the quantification of these seven synthetic opioids in whole blood.

Materials and Methods

Materials

U-47700, U-49900, U-50488, MT-45, W-18, W-15, U-47700-d_6, MT-45-d_{11}, and W-18-d_4 were acquired from Cayman Chemical Company (Ann Arbor, MI, USA). AH-7921 was purchased from Cerilliant Corporation (Round Rock, TX, USA). Cerex® Clin II (3 mL, 35 mg) SPE columns were acquired from Tecan (Baldwin Park, CA). Blank matrix (defibrinated bovine blood) was obtained from Quad Five (Ryegate, MT, USA). De-identified, postmortem samples from adjudicated cases were received from National Medical Services (NMS) Labs under a Sam Houston State University Institutional Review Board (IRB) approved protocol.

Ultrapure water was prepared via a Millipore Direct-Q® 3 UV Water Purification System (Billerica, MA, USA). Common chemicals used were of the highest purity commercially available.

Instrumentation

SPE utilized a CEREX-48 positive-pressure manifold (SPEware Corporation, Baldwin Park, CA). Evaporation by nitrogen gas was performed with a Caliper Life Sciences TurboVap® LV concentration workstation (Hopkinton, MA, USA). Analytes were analyzed on an Agilent 1290 Infinity II liquid chromatograph coupled to an Agilent 6470

triple quadrupole mass spectrometer (Santa Clara, CA, USA). Data were acquired using Agilent MassHunter Workstation version B.07 software.

Preparation of Standards

Individual analyte stock solutions (100,000 ng/mL) were prepared in methanol. Mixed analyte calibrator and quality control (QC) solutions were prepared from individual analyte stock solutions in methanol. The calibrators were fortified at 0.25, 1, 5, 10, 25, 50, 100 ng/mL for U-47700, AH-7921, U-49900, U-50488, MT-45, and W-15 and 1, 5, 10, 25, 50, 100 ng/mL for W-18 in blood. The low, medium, and high QC samples were fortified in blood at 0.75 (2.5 ng/mL for W-18), 20, and 80 ng/mL for U-47700, AH-7921, U-49900, U-50488, MT-45, W-18 and W-15, respectively. A mixed internal standard (ISTD) solution was prepared in methanol, resulting in 20 ng/mL for U-47700-d_6, MT-45-d_{11}, and W-18-d_4 when fortified into blood. All standard solutions were stored in amber vials at -20°C.

Sample Preparation and Extraction

Blank blood (500 µL) was fortified with mixed calibrator or QC solution (25 µL) and ISTD solution (10 µL). Blood samples were buffered with 100 mM pH 6 phosphate solution (2.5 mL), vortex mixed, and transferred onto SPE columns. SPE column washes and elutions were performed with each 2 mL volume. SPE columns were washed with deionized water, 1 M acetic acid, dried under positive pressure (5 min), and washed with hexane. Acidic and neutral analytes were eluted with ethyl acetate. A methanol wash was performed prior to the elution of basic analytes with dichloromethane/isopropanol (80/20, v/v) with 5% ammonium hydroxide. Combined elutions were evaporated under nitrogen at 50 °C, reconstituted in A/B mobile phase (80/20, v/v) (1 mL), vortex mixed, and centrifuged

(3500 rpm, 5 min). Samples were transferred to autosampler vials and injected onto the LC – MS/MS (5 µL).

Liquid Chromatography

Analyte separations were performed on an Agilent Poroshell 120 EC-C18 column (100 x 2.1 mm i.d., particle size 2.7 µm) with matching guard (5 x 2.1 mm, 2.7 µm) using a gradient elution at 0.5 mL/min. The gradient of 0.05 % formic acid with 5 mM ammonium formate in water (A) and 0.1 % formic acid in acetonitrile (B) was increased from 20 % to 50 % B over 5 min, increased to 90 % B over 2 min, held for 2 min, decreased to 20 % B over 0.10 min, and re-equilibrated for 1.90 min. The column oven was held at 40 °C.

Mass Spectrometry

Ion source parameters were optimized using Agilent MassHunter Source Optimizer software. Nitrogen drying gas temperature and flow rate were 350 ℃ and 13 L/min, respectively. Nebulizer pressure was 45 psi. Ultrapure nitrogen sheath gas temperature and flow were 350 ℃ and 12 L/min, respectively. Capillary voltage was 4500 V, and nozzle voltage was 500 V. Data were acquired in dynamic multiple reaction monitoring mode via positive electrospray ionization. A minimum of two transitions optimized by Agilent MassHunter Optimizer software was monitored per analyte and ISTD (**Table 4.1**).

Table 4.1 Optimized liquid chromatography-tandem mass spectrometry parameters for U-47700, AH-7921, U-49900, U-50488, MT-45, W-18, W-15, and internal standards, U-47700-*d*6, MT-45-*d*11, and W-18-*d*4.

Analyte	Precursor ion (*m/z*)	Product ion (*m/z*)	Collision energy (eV)	Fragmentor voltage (V)	Ret. Time (min)	Internal standard
U-47700	*329.2*	*172.9*	37	117	2.738	U-47700-d_6
	329.2	144.9	61	117		
AH-7921	*329.2*	*144.9*	57	107	2.987	U-47700-d_6
	329.2	46.2	21	107		
U-49900	*357.1*	*172.9*	37	120	3.263	U-47700-d_6
	357.1	144.9	65	120		
U-50488	*369.1*	*158.9*	49	115	3.665	U-47700-d_6
	369.1	112.1	37	115		
MT-45	*349.5*	*181.0*	20	112	4.511	MT-45-d_{11}
	349.5	77.1	93	112		
W-18	*422.1*	*111.0*	50	153	6.392	W-18-d_4
	422.1	75.1	50	153		
W-15	*377.1*	*111.0*	41	145	6.640	W-18-d_4
	377.1	75.1	97	145		
U-47700-d_6	*335.2*	*172.9*	37	112	2.733	-
	335.2	144.9	57	112		
MT-45-d_{11}	*360.5*	*181.0*	20	132	4.491	-
	360.5	77.1	93	132		
W-18-d_4	*426.1*	*115.0*	53	143	6.391	-
	426.1	78.1	105	143		

Validation

Method validation was performed in accordance with the Scientific Working Group for Forensic Toxicology (SWGTOX) standard practices for method validation in forensic toxicology (25). Calibration model was evaluated from non-zero calibrators (≥6) over 5 runs. Limit of detection (LOD) and lower limit of quantification (LLOQ) were each evaluated in 3 blood sources in duplicate over 3 runs. LOD retention time (within ±10% of calibrators), peak shape, mean signal to noise ratio (>10), and ion ratios (within ±20% of calibrators) were monitored. LOD imprecision was calculated from the mean relative response. LLOQ bias and imprecision were each evaluated from the calculated concentrations in addition to meeting or exceeding LOD criteria. Bias and imprecision were assessed at three QC concentrations in triplicate over 5 runs as target concentration (%) and coefficient of variation (CV) (%), respectively.

Carryover was evaluated by analyzing a negative sample (ISTD only) directly following a high calibrator over 5 runs. Low QC samples were fortified with common drugs of abuse (n = 32) to assess interferences (**Table 4.2**).

Table 4.2 Common drugs of abuse for interference studies

Alprazolam	Amobarbital	Amphetamine	Amitriptyline
Butalbital	Caffeine	Carbamazepine	Carisoprodol
Cocaine	Codeine	Cotinine	Cyclobenzaprine
Dextromethorphan	Diazepam	Diphenhydramine	Hydrocodone
Hydromorphone	Ketamine	Methadone	Nicotine
Nordiazepam	Oxazepam	Oxycodone	Pentobarbital
Phencyclidine	Phenobarbital	Propoxyphene	Secobarbital
THC	Tetrahydro-cannabinolic acid	Tramadol	Zolpidem

Ionization suppression and enhancement and extraction recovery were evaluated from pre-extraction and post-extraction addition of six blood sources fortified at low and high QC concentrations (26). Ionization suppression and enhancement were calculated as the ratio of the mean area of post-extraction fortified samples to neat samples, minus one, as a percentage. Extraction recovery was determined as the percentage of the ratio of the mean area of pre-extraction to post-extraction fortified samples.

Dilution integrity (1:10) was evaluated at 2.5 times the high QC concentration in duplicate. Analyte stability was assessed in fortified blood at low and high QC concentrations in triplicate at room temperature (20 °C) for 24 h and 4°C for 72 h, as well as extracted samples in the refrigerated autosampler (4 °C) for 72 h. Bias and imprecision were assessed by comparing mean concentrations of fresh QC samples to mean concentrations at the various conditions for fortified stability and to mean initial (t_0) concentration for the processed sample stability.

Postmortem Samples

A total of 30 postmortem blood samples were received in the absence of demographic or case information. Due to the viscosity of postmortem samples, an additional centrifugation step was added to sample preparation before loading onto the SPE columns to prevent clogging. Samples (500 µL) were fortified with ISTD and extracted as described with calibrators and QC samples. Dilutions, when needed, were prepared using blank whole blood.

Results and Discussion

Optimization of the extraction method involved the assessment of various acidic/neutral (ethyl acetate, ether/toluene (50:50, v/v), acidic methanol, hexane/ethyl

acetate/acetic acid (49:49:2, v/v/v), and n-butyl chloride) and basic (2% ammonium hydroxide in ethyl acetate, and 5% ammonium hydroxide in dichloromethane/isopropanol (80/20, v/v)) elution solvents, the addition of a dichloromethane wash, and the evaluation of various wash and elution volumes. The final extraction method achieved improved matrix effects and extraction efficiency, particularly for the analytes eluting in the acidic/neutral fraction (W-18 and W-15) (27). Chromatography optimization included the addition of ammonium formate to the aqueous mobile phase, which resulted in improved peak shape and response for W-18 and W-15.

LODs, LLOQs, upper limits of quantification (ULOQ), and coefficients of determination (R^2) from 5 runs are summarized in **Table 4.3**.

Table 4.3 Limit of detection (LOD), lower limit of quantification (LLOQ), upper limit of quantification (ULOQ), and mean *y*-intercept, slope, and coefficient of determination (R^2) for U-47700, AH-7921, U-49900, U-50488, MT-45, W-18, and W-15

Analyte	LOD (ng/mL)	LLOQ (ng/mL)	ULOQ (ng/mL)	y-intercept (mean ± SD, *n* = 5)	Slope (mean ± SD, *n* = 5)	R^2 (range, *n* = 5)
U-47700	0.125	0.25	100	0.0009 ± 0.0007	0.0510 ± 0.0045	0.9967-0.9996
AH-7921	0.125	0.25	100	-0.0010 ± 0.0015	0.0344 ± 0.0029	0.9957-0.9999
U-49900	0.125	0.25	100	-0.0004 ± 0.0017	0.0434 ± 0.0037	0.9971-0.9999
U-50488	0.125	0.25	100	-0.0004 ± 0.0007	0.0138 ± 0.0011	0.9968-0.9996
MT-45	0.125	0.25	100	0.0012 ± 0.0017	0.0648 ± 0.0046	0.9942-0.9996
W-18	0.25	1	100	0.0005 ± 0.0122	0.0767 ± 0.0073	0.9982-0.9996
W-15	0.125	0.25	100	0.0043 ± 0.0055	0.0860 ± 0.0087	0.9939-0.9996

SD standard deviation

LODs were 0.125 ng/mL (U-47700, AH-7921, U-49900, U-50488, MT-45, and W-15) and 0.25 ng/mL (W-18). LLOQs were 0.25 ng/mL (U-47700, AH-7921, U-49900, U-50488, MT-45, and W-15) and 1.0 ng/mL (W-18). Calibration curves were comprised of seven non-zero calibrators (U-47700, AH-7921, U-49900, U-50488, MT-45, and W-15) and six non-zero calibrators (W-18). SWGTOX requires a minimum of six non-zero calibrators be used, which was met for each analyte in this method. AH-7921, U-49900, U-50488, W-18, and W-15 utilized linear calibration models with $1/x$ weighting, while U-47700 and MT-45 necessitated $1/x^2$ weighting. The calibration curves were considered acceptable with all R^2 >0.99.

Recent methods for U-47700 have LLOQs of 1, 1.6, and 312.5 ng/mL (3, 11, 17). A method reported for AH-7921 obtained an LLOQ of 10 ng/mL (21). Methods for U-50488 and MT-45 each reported an LLOQ of 1 ng/mL (7, 17). This method achieved LLOQs of 0.25 ng/mL for each analyte, except W-18 (1.0 ng/mL) (**Table 4.3**). The increased sensitivity of this method could potentially be used to quantify low levels of these analytes, which were previously undetected.

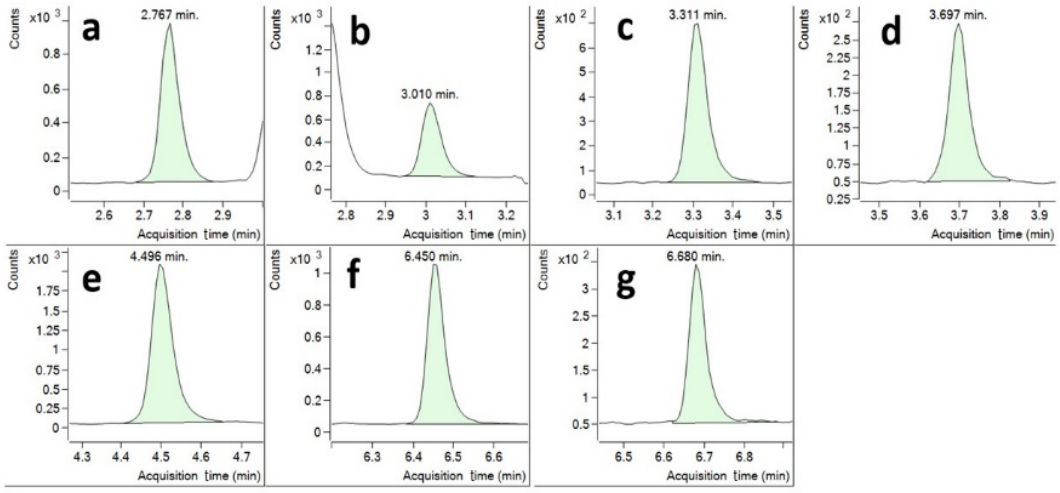

Figure 4.2 Multiple reaction monitoring chromatograms for quantifying transitions for **a** U-47700, **b** AH-7921, **c** U-49900, **d** U-50488, **e** MT-45, **f** W-18, and **g** W-15 at their LLOQs (0.25 ng/mL for U-47700, AH-7921, U-49900, U-50488, MT-45, and W-15 and 1 ng/mL for W-18)

The LOD relative peak area imprecision (≤18.6%) and mean signal to noise (≥10.6) demonstrated acceptable reproducibility and analyte response. LLOQ demonstrated acceptable bias and imprecision which were within ±16.9% and ≤13.7% CV, respectively. Representative chromatograms for each analyte at their LLOQs are represented in **Figure 4.2** above. This method met acceptable bias for three QC concentrations, which were each within ±14.5% (**Table 4.4**). Within-run and between-run imprecisions met acceptable criteria at ≤16.0 and ≤16.1%, respectively (**Table 4.5**).

Table 4.4 Bias results at three quality control (QC) concentrations for U-47700, AH-7921, U-49900, U-50488, MT-45, W-18, and W-15

Analyte	Bias (%, *n* = 15)		
	Low[a]	Med[b]	High[c]
U-47700	-12.6	-0.8	2.1
AH-7921	-8.9	-1.5	0.6
U-49900	-11.6	-0.5	2.4
U-50488	-7.1	-0.8	2.8
MT-45	-14.5	2.0	3.2
W-18	-13.0	3.1	2.6
W-15	-12.1	5.8	3.8

[a]Low concentration: 0.75 ng/mL for U-47700, AH-7921, U-49900, U-50488, MT-45, and W-15, 2.5 ng/mL for W-18
[b]Med concentration: 20 ng/mL for U-47700, AH-7921, U-49900, U-50488, MT-45, W-18, and W-15
[c]High concentration: 80 ng/mL for U-47700, AH-7921, U-49900, U-50488, MT-45, W-18, and W-15

Table 4.5 Imprecision at three QC concentrations for U-47700, AH-7921, U-49900, U-50488, MT-45, W-18, and W-15

Analyte	Imprecision (%CV, *n* = 15)								
	Mean within-run			Maximum within-run			Between-run		
	Low[a]	Med[b]	High[c]	Low[a]	Med[b]	High[c]	Low[a]	Med[b]	High[c]
U-47700	3.8	5.8	7.7	7.7	9.1	13.7	5.5	11.4	8.2
AH-7921	4.5	4.3	7.8	6.4	9.1	15.8	5.5	10.7	8.4
U-49900	5.2	5.9	7.5	9.0	9.4	15.5	6.2	11.9	7.7
U-50488	3.0	5.4	7.3	7.0	9.5	16.0	7.8	10.9	7.8
MT-45	4.0	7.3	7.2	7.7	11.2	11.7	5.3	15.4	9.2

Analyte	Imprecision (%CV, n = 15)								
	Mean within-run			Maximum within-run			Between-run		
	Low[a]	Med[b]	High[c]	Low[a]	Med[b]	High[c]	Low[a]	Med[b]	High[c]
W-18	4.8	6.0	6.7	9.3	9.9	11.9	5.9	16.1	8.9
W-15	7.6	5.5	7.7	13.0	9.1	12.3	8.5	15.2	10.1

Carryover was negligible with analyte responses from carryover experiments <20% of the LOD. The extracted low QC samples fortified with various interferences were accurately quantified within ±19.9%. Therefore, interference from 32 common drugs of abuse (**Table 4.2**) was insignificant. Endogenous interferences were undetected in three different blank blood sources and no significant peaks for the analytes of interest were observed in negative (ISTD only) samples.

Ionization suppression and enhancement for each analyte (±20.8%) met acceptable criteria, except for U-50488 low QC (28.4%) (**Table 4.6**). Although ionization suppression and enhancement exceeded requirements, reproducibility was demonstrated between 6 blood sources (≤12.4% CV). Extraction recoveries (62.6-96.6%) were comparable between analytes and their matched deuterated ISTDs (**Table 4.6**). For analytes with no commercially available deuterated ISTD, the ISTD with the closest matrix effects, extraction elution fraction, and extraction recovery was chosen and met acceptable criteria for accurate quantification.

Table 4.6 Ionization suppression and enhancement and extraction efficiency results for U-47700, AH-7921, U-49900, U-50488, MT-45, W-18, W-15, U47700-d_6, MT45-d_{11}, and W18-d_4

Analyte	Ionization Suppression/Enhancement (%, n = 6)		Extraction Efficiency (%, n = 6)	
	Low[a]	High[b]	Low[a]	High[b]
U-47700	12.7	1.5	88.1	92.0
AH-7921	19.7	-2.7	80.3	88.2
U-49900	6.6	-1.3	88.0	89.3
U-50488	28.4	-2.9	85.2	91.2
MT-45	7.9	-3.4	78.5	85.9
W-18	11.8	4.8	63.1	66.5
W-15	15.4	12.7	72.3	72.5
U47700-d_6	8.8	10.8	86.0	96.6
MT45-d_{11}	10.8	11.0	76.4	88.3
W18-d_4	15.9	20.8	62.6	67.6

[a]Low concentrations: 0.75 ng/mL for U-47700, AH-7921, U-49900, U-50488, MT-45, and W-15; 2.5 ng/mL for W-18
[b]High concentrations: 80 ng/mL for U-47700, AH-7921, U-49900, U-50488, MT-45, W-18, and W-15

Dilution integrity samples fortified at 2.5 times the high QC were accurately quantified (±17.1%), with the exception of W-18 (-23.8%). All analytes were found to be stable under each condition assessed: 24 h room temperature (-7.9 to 7.3%), 72 h at 4°C (-16.8 to 0.2%), and 72 h autosampler (-3.8 to 1.5%) (**Table 4.7**).

Table 4.7 Bias results for the stability of U-47700, AH-7921, U-49900, U-50488, MT-45, W-18, and W-15 at 24 h room temperature, 72 h 4°C, and 72 h in a refrigerated autosampler

Analyte	Fortified 24 h room temp. (%bias, *n* = 3)		72 h 4°C (%bias, *n* = 3)		Extracted 72 h autosampler (%bias, *n* = 3)	
	Low[a]	High[b]	Low[a]	High[b]	Low[a]	High[b]
U-47700	7.3	-2.0	-7.9	-11.5	-1.6	-3.3
AH-7921	-7.8	-0.3	-10.7	-10.9	-3.8	0.0
U-49900	6.9	0.8	-7.9	-10.5	1.5	-1.7
U-50488	7.0	3.0	-11.2	-9.5	-1.6	-1.1
MT-45	-7.9	-6.8	-12.1	-16.8	-0.4	-0.9
W-18	7.1	-2.8	0.2	-15.2	0.3	1.3
W-15	3.7	0.9	-2.1	-10.8	-1.1	1.2

[a]Low concentrations: 0.75 ng/mL for U-47700, AH-7921, U-49900, U-50488, MT-45, and W-15, 2.5 ng/mL for W-18
[b]High concentrations: 80 ng/mL for U-47700, AH-7921, U-49900, U-50488, MT-45, W-18, and W-15

Of the 30 postmortem samples, 15 were positive for U-47700 (**Table 4.8**). These samples were received without any temperature preservation and were exposed to extreme Texas temperatures for >8 h. Due to this, drug concentrations may have been affected. U-47700 concentrations ranged from 3.2-1450 ng/mL with median and mean of 109 and 214 ng/mL, respectively.

Table 4.8 Concentrations of U-47700 in postmortem blood samples

Sample	Source (blood)	U-47700 concentration (ng/mL)
1	Cardiac	1450[a]
2	Peripheral	ND
3	Subclavian	ND
4	Femoral	135[b]

Sample	Source (blood)	U-47700 concentration (ng/mL)
5	Femoral	ND
6	Peripheral	ND
7	Peripheral	133[b]
8	Cardiac	ND
9	Not Specified	190[b]
10	Peripheral	ND
11	Iliac	425[b]
12	Femoral	ND
13	Iliac	36.0
14	Femoral	ND
15	Cardiac	ND
16	Peripheral	ND
17	Peripheral	3.2
18	Femoral	4.2
19	Peripheral	ND
20	Cardiac	354[b]
21	Femoral	ND
22	Iliac	60.6
23	Femoral	101
24	Femoral	ND
25	Femoral	43.1
26	Femoral	ND
27	Femoral	53.7
28	Femoral	118[b]
29	Femoral	ND
30	Iliac	109[b]

ND not detected
[a] One hundred-fold dilution
[b] Ten-fold dilution

The concentration from Sample 1 (1450 ng/mL) is comparable to the concentration found in Elliot *et al* (11), although the blood source for that sample was peripheral blood while this sample was from cardiac blood (8). It can be anticipated that if a peripheral blood sample was paired with the cardiac sample of Sample 1, the concentration could be lower due to the "modest potential" of postmortem redistribution determined by McIntyre *et al* (15). All of our U-47700 concentrations were comparable to those reported in previous studies (1, 9, 13-15). Fatalities involving U-47700 are associated with blood concentrations

ranging from 0.090-1460 ng/mL (3, 11, 14-17, 28), while driving intoxication cases were 3.4-16 ng/mL (29). Cases in which fentanyl analogs were also detected, were reported to have U-47700 concentrations from 0.090-490 ng/mL. While not detected in these samples, AH-7921 and MT-45 blood concentrations in fatalities were 330-9100 ng/mL (18, 20, 21) and 520-2900 ng/mL (7, 21), respectively. The linear range of this method is sufficiently low for the detection of these analytes in the majority of the reported cases. For cases with unexpectedly high concentrations, dilution was assessed and met acceptable criteria and would be useful for quantification in those scenarios as well. Therefore, this method is applicable for the analysis of novel synthetic opioids (NSO) in acute and fatal intoxication cases.

Conclusions

A comprehensive method for the quantification of U-47700, AH-7921, U-49900, U-50488, MT-45, W-18, and W-15 in blood using LC-MS/MS was successfully developed, optimized, and validated. To our knowledge, this is the first quantification method reported for W-18 and W-15 in blood. This method required minimal sample preparation and achieved increased sensitivity as compared to previously reported studies. Applications of this method include analyses of NSO driving intoxication and fatal intoxication cases.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflicts of interest.

Ethical Approval This article does not contain any studies with animals performed by any of the authors. The blank blood samples were acquired by healthy volunteers after obtaining informed consent.

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CHAPTER V

Long-Term Stability of Novel Synthetic Opioids in Blood[1]

This dissertation follows the style and format of *Journal of Analytical Toxicology*.

[1]Truver M.T., Swortwood M.J. (2019) Long-Term Stability of Novel Synthetic Opioids in Blood.

Submitted to *Forensic Science International*

Abstract

Recently, there has been an increase in overdose deaths due to novel synthetic opioids (NSO). Due to backlogs experienced by many forensic laboratories, it is important to understand drug stability in a variety of storage conditions. The objective of this study was to investigate the stability of AH-7921, U-47700, U-49900, U-50488, MT-45, W-15, and W-18 in blood at various temperatures over a 36-week period. NSO were generally stable over the 36-week period (66%-118%) at low and high concentrations when blood samples were stored in the refrigerator or freezer. Most analytes were stable for at least 2 weeks at room temperature (77%-120%). At the elevated temperature (35°C), analytes were generally stable for at least 14 days (75%-109%). This study has determined the stability of several NSO at various temperatures over a 36-week period. These results reflect the forensic significance of keeping samples stored at proper temperatures. Blood samples suspected to contain synthetic opioids should be stored refrigerated or frozen, when possible, in order to preserve analyte stability, especially at low concentrations.

Keywords: Novel Synthetic Opioids, Stability, MT-45, U-47700, U-series, W-18, W-series

Introduction

Novel psychoactive substances (NPS) have had detrimental impact on drug abuse worldwide. Under the umbrella of NPS, novel synthetic opioids (NSO) have emerged as fentanyl and non-fentanyl derivatives. According to United Nations Office on Drugs and Crime (UNODC), there were 62 different synthetic opioids reported to their Early Warning Advisory (EWA) by 2019, resulting in a 110% increase from the previous three years (1). Between 2016 and 2017, there were over 28,000 synthetic opioid related overdose fatalities in the United States (2). During the same period of time, there were over 15,000 overdose deaths involving heroin in the United States as well. These synthetic opioids have the potential to be dangerously more potent than natural opioids.

NSO such as U-47700, MT-45, and AH-7921 are just a few compounds that have emerged in casework across the nation. U-47700 is part of the U-series of drugs developed by the Upjohn Company in the 1970's. Other compounds in the U-series include U-49900 and U-50488. While there are very few reports involving U-49900 and U-50488 (3-5), U-47700 has been involved in numerous fatalities and intoxications (3,6-15). The stereoisomer of U-477700, AH-7921, and MT-45 have also been associated with fatalities and intoxications (16-21). The W-series was developed in the 1980s by the University of Alberta and consisted of 32 compounds, including W-18 and W-15. There is minimal information available about these compounds. Recently, a report in Belgium had identified a heroin-like powder to contain W-18 (22). Structures of the seven NSO mentioned above are shown in **Figure 5.1** below.

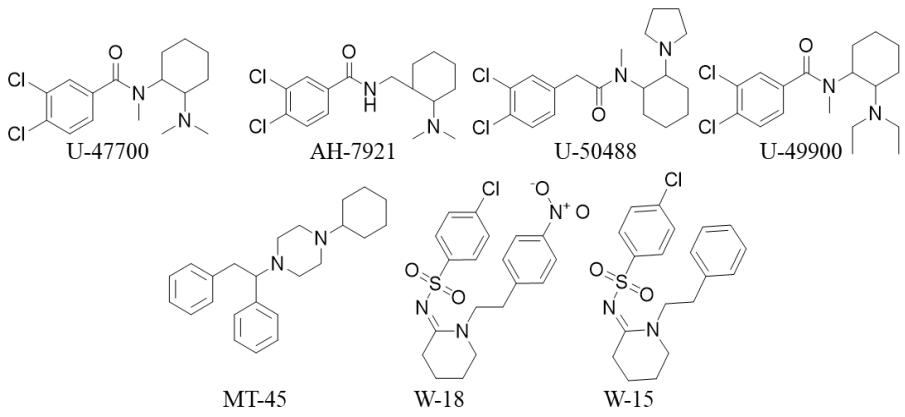

Figure 5.1 Structures of seven novel synthetic opioids

While long-term stability studies are limited for these NSO, some analytes have been evaluated in various conditions. Mohr *et al* evaluated the stability of two of the U-series drugs, U-47700 and U-50488, when performing a fit for purpose validation (3). Two concentrations (5 ng/mL and 80 ng/mL) in blood were evaluated at refrigerated and frozen temperatures over 10 days. It was concluded that both analytes were stable over the 10 days at both temperatures. Stability of both of these analytes along with other fentanyl derivatives was also evaluated by Qin *et al* (23). Stability was assessed at three concentrations (0.2 ng/mL, 5 ng/mL, and 30 ng/mL) in whole blood at four different conditions: 1 month in the freezer, 3 freeze/thaw cycles, 24 hr at 4°C, and 24 hr at room temperature. At all four conditions and at the three concentrations, both analytes remained stable. U-47700 was also evaluated by Rojek *et al* at 25 ng/mL in three different temperatures (-30°, 3°C and 20°C) for 21 days in blood (13). It was observed that U-47700 was stable over the 21 days, even at the ambient temperature of 20°C. Similarly, the stereoisomer of U-47700 (AH-7921) was found to be stable at a higher concentration (2,000 ng/mL) at room temperature (20-23°C) for 28 days in blood and plasma (24). Papsun *et al* evaluated MT-45 stability in fortified blood and determined that the compound was stable up to 30 days at frozen, refrigerated, and room temperature

(20). Case samples from this experiment (blood, urine, bile, and vitreous humor) were also retested approximately 12 months after original testing and MT-45 concentrations had degraded by approximately 50%. However, this degradation could not be determined and may be due to the nature of the authentic sample.

Due to backlogs experienced by many forensic laboratories, it is important to understand drug stability in a variety of storage conditions. In this study, the goal was to investigate the stability of AH-7921, U-47700, U-49900, U-50488, MT-45, W-15, and W-18 in blood at various temperatures over 36 weeks.

Materials and Methods

Chemicals and Reagents

All standards and deuterated internal standards: MT-45, U-47700, U-49900, U-50488, W-15, W-18, U-47700-*d*6, MT-45-*d*11 and W-18-*d*4 were purchased from Cayman Chemical (Ann Arbor, MI, USA). Reagents and solvents for extraction were analytical grade or higher. LCMS grade methanol (J.T. Baker, Center Valley, MA, USA) and Optima® formic acid (Fisher Scientific, Fair Lawn, NJ, USA) were used for mobile phase preparation. Bovine blood preserved with sodium oxalate was purchased from Quad Five (Ryegate, Montana, USA). Cerex® Clin II (3 mL, 35 mg) solid-phase extraction (SPE) columns were purchased from SPEware (Baldwin, Park, CA, USA). A Millipore Direct-Q UV Water Purification system (Billerica, MA, USA) was used to purify water. BD Vacutainer™ tubes (10 mL, 16 x 100mm) without preservative were acquired from VWR (Radnor, PA, USA).

Instrumentation

Extraction was performed using a CEREX-48-positive-pressure manifold (SPEware). An Agilent 1290 Infinity II Liquid Chromatograph system equipped with an Agilent 6470 Triple Quadrupole Mass Spectrometer (Santa Clara, CA, USA) was used for analysis. Data were analyzed and acquired using Agilent MassHunter software. A Turbovap LV® concentration workstation (Caliper Life Sciences, Hopkinton, MA, USA) was used for evaporation by nitrogen.

Liquid chromatography

Separation was achieved using our previously published method with limit of detection and quantification at 0.125 and 0.25-1 ng/mL, respectively (25). Mobile phase A consisted of 5 mM ammonium formate with 0.05% formic acid in water and mobile phase B consisted of 0.1% formic acid in methanol. An injection volume of 5 µL was used.

Preparation of Blood

Blood (100 mL) was fortified with aqueous standards at 0.75 ng/mL (2.5 ng/mL for W-18) and 80 ng/mL to represent low (LQC) and high quality control (HQC) concentrations, respectively. The fortified blood was equally disbursed into empty vacutainer tubes and placed into appropriate temperature settings: frozen (-20°C), refrigerated (4°C), room temperature (~25°C), and elevated temperature (35°C). Aliquots were taken from each tube immediately after preparation to represent T_0. A total of 9 time points were analyzed in duplicate in this study (T_0, 3, 14, 21, 28, 56, 84, 112, and 252 days).

Extraction

Samples were extracted using a previously validated method (25). An additional protein precipitation step was added to reduce solvent usage and time. Blood (0.5 mL) was fortified with internal standard then ice cold acetonitrile (1 mL) was added. Samples were then centrifuged (2360 x *g*) for 5 mins and decanted in a new tube. Briefly, samples were buffered (100 mM phosphate buffer, pH 6), loaded onto Cerex® Clin ll cartridges, then washed, and dried. Acidic and basic drugs were eluted with ethyl acetate and dichloromethane: isopropyl alcohol with 5% ammonium hydroxide, respectively. Combined elution fractions were dried under nitrogen at 50°C and reconstituted in 0.25 mL of A/B mobile phase (60:40, v/v).

Results

The range of concentrations from the two aliquots are represented as error bars with the mean of each set represented by the data point in the following figures. The mean of the concentrations from time zero (T_0) was used as a baseline for the stability experiment. Stability was accessed as %difference from the established baseline concentration and deemed acceptable between ±20%.

Stability at Frozen Temperature (-20°C)

The seven analytes were evaluated at -20°C at two concentrations (LQC and HQC) as shown in **Figure 5.2** below.

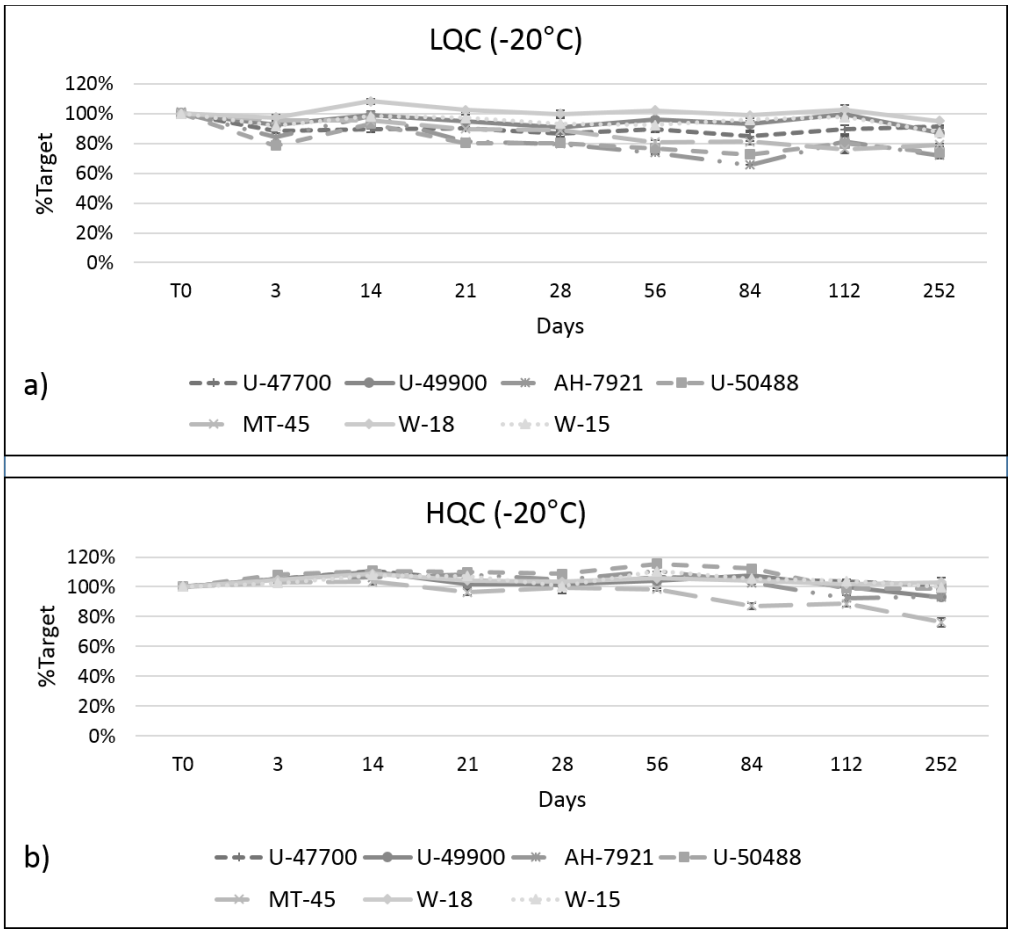

Figure 5.2 Frozen blood stability of U-47700, U-49900, AH-7921, U-50488, MT-45, W-18, and W-15 at a) 0.75 ng/mL (2.5 ng/mL for W-18) (LQC) and b) 80 ng/mL (HQC)

U-47700, U-49900, W-18, and W-15 were stable through the 36-week evaluation at both concentrations. At the low concentration, AH-7921, U-50488, and MT-45 were stable for at least 28 days at the frozen temperature. There was slight variability seen after the 28-day time point, although never exceeding more than 30% loss in the low concentration. In the HQC, all analytes were stable for the entire evaluation except for MT-45, which demonstrated 24% loss by the last time point compared to T_0.

Stability at Refrigerated Temperature (4°C)

Analytes were evaluated at 4°C at two concentrations (LQC and HQC) as shown in **Figure 5.3** below.

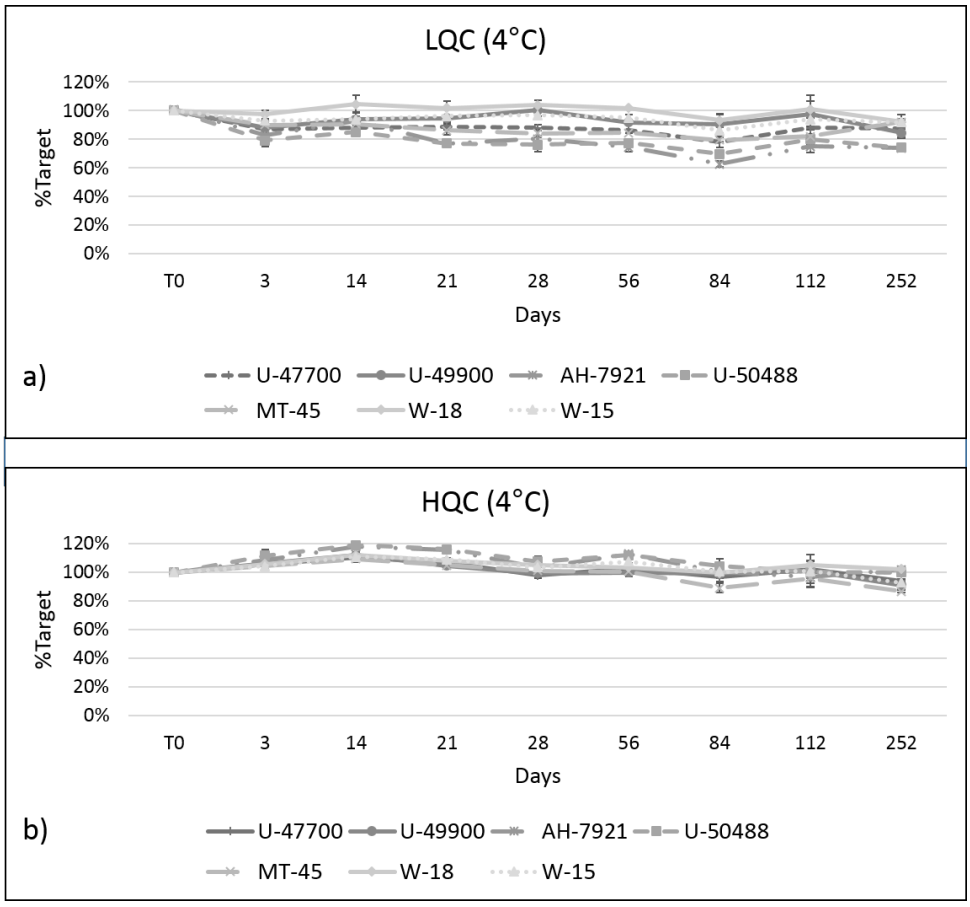

Figure 5.3 Refrigerated blood stability of U-47700, U-49900, AH-7921, U-50488, MT-45, W-18, and W-15 at a) 0.75 ng/mL (2.5 ng/mL for W-18) (LQC) and b) 80 ng/mL (HQC)

All analytes except for AH-7921 and U-50488 were stable at the low concentration throughout the 36-week experiment. AH-7921 and U-50488 were stable for 28 days then began to decline, although the loss never exceeded 37%. At the high concentration, all analytes were stable at every time point.

Stability at Room Temperature (25°C)

Analytes were evaluated at room temperature and the results are shown in **Figure 5.4** below.

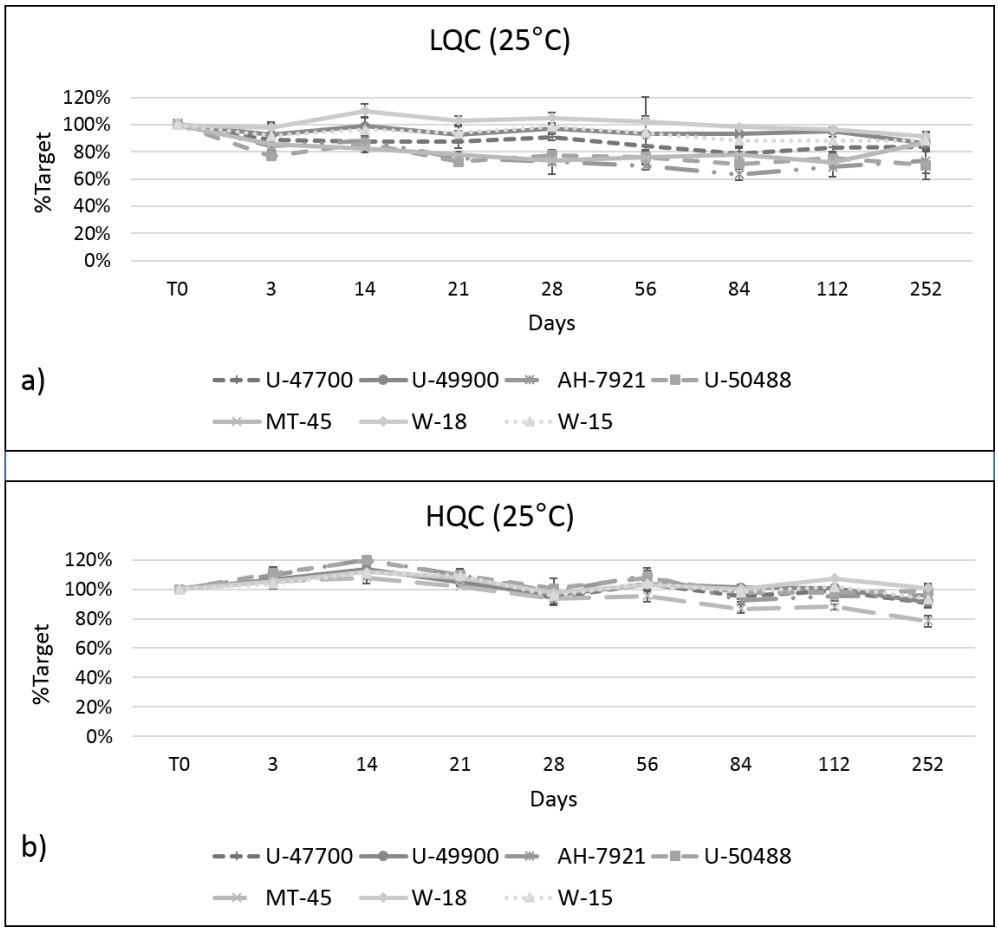

Figure 5.4 Room temperature blood stability of U-47700, U-49900, AH-7921, U-50488, MT-45, W-18, and W-15 at a) 0.75 ng/mL (2.5 ng/mL for W-18) (LQC) and b) 80 ng/mL (HQC)

At room temperature, all analytes were stable during the first 14 days at the low concentration. After 21 days, AH-7921 and U-50488 fell below acceptance criteria. After 28 days, MT-45 also fell out of acceptance criteria. At the high concentration, all analytes were stable at all time points except MT-45 at the last sampling (78%).

Stability at Elevated Temperature (35°C)

Analytes were evaluated at an elevated temperature to simulate if a sample was subject to high heat during transportation. Stability of all seven analytes at the elevated temperature is shown in **Figure 5.5**.

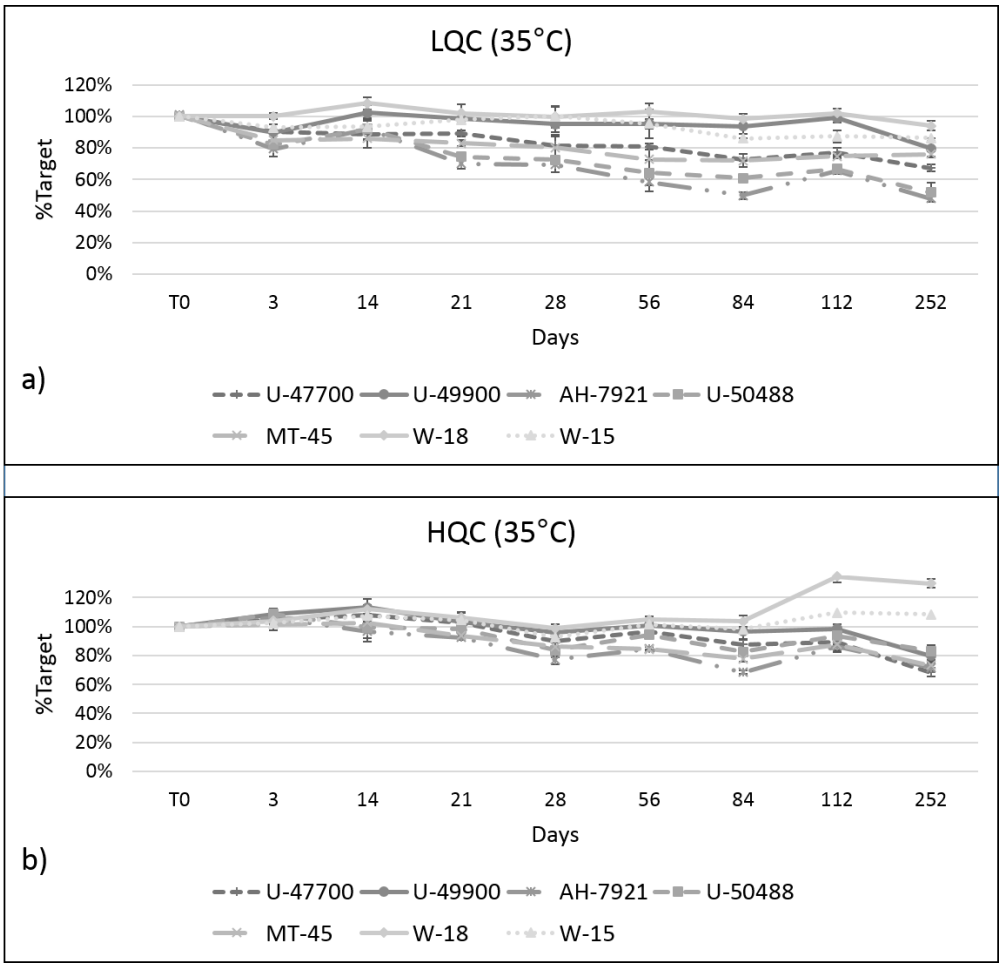

Figure 5.5 Room temperature stability of U-47700, U-49900, AH-7921, U-50488, MT-45, W-18, and W-15 at a) 0.75 ng/mL (2.5 ng/mL for W-18) (LQC) and b) 80 ng/mL (HQC)

Similar to the room temperature condition, all analytes were stable for two weeks at the low concentration. AH-7921 and U-50488 were deemed unstable (70 and 74%) after 21 days and MT-45 at 28 days (79%). At the high concentration, all analytes except AH-7921 and MT-45 were stable for 112 days. AH-7921 and MT-45 were stable until 84

days. U-47700 and U-49900 were deemed unstable at 252 days. U-50488 was the only analyte that was stable throughout the 36 weeks. There was enhancement observed in the W-series beginning at 112 days which resulted in concentrations >100% compared to T_0.

Discussion

All analytes were stable at the 4 evaluated conditions in blood for two weeks at the low concentration. There was variability observed in the low concentration for AH-7921, U-50488, and MT-45. The only study that has evaluated any of these analytes at a low concentration similar to the current study was Qin *et al*, with 0.2 ng/mL (23). The findings from the current study are aligned with their U-47700 and U-50488 short term stability studies. The stability of U-47700 is comparable to previous studies where it was determined to be stable for at least for 21 days frozen, refrigerated, and at room temperature (3,13). The only previous study involving stability of MT-45 was Papsun *et al*. While our findings for MT-45 stability are similar to those assessed in fortified samples, the instability in the authentic samples has yet to be replicated *in vitro*. In general, most analytes were stable at the high concentration throughout the study with the exception of the elevated temperature. Additional experiments were done to assess increasing concentration observed with the W-series analytes. It was determined that there was suppression of the internal standard from the matrix by plotting the response of the internal standard over time for each temperature condition. An additional discovery during this evaluation was that there was a difference in concentrations between vacutainers that had been opened compared to those that were sampled for the first time. Multiple vacutainers were placed in each temperature setting and were used until depleted with duplicates taken from the same tube. Therefore, when switching to a new

tube, there would be variability in the concentration. Although the difference was not large, this could contribute to the variability of analytes at the low concentration and may not necessarily indicate true degradation.

Conclusions

This study determined the stability of several NSO at various temperatures over a 36-week period. To the authors knowledge, this is the first long-term stability study involving these seven NSO and the data support the forensic significance of proper sample storage. However, blood samples suspected to contain synthetic opioids, particularly at low concentrations, should be stored refrigerated or frozen, when possible, in order to preserve analyte stability. Samples should be analyzed within 4 weeks in order to capture true concentrations, although analyte loss did not exceed 40% beyond this timeframe.

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CHAPTER VI

Quantification of U-47700 and its Metabolites in Plasma by LC-MS/MS[1]

This dissertation follows the style and format of *Journal of Analytical Toxicology*.

[1]Smith C.R., Truver M.T., Swortwood M.J. (2019) Quantification of U-47700 and its Metabolites in Plasma by LC-MS/MS. *Journal of Chromatography B*, **1112**, 41-47.

Abstract

Novel Synthetic Opioids (NSO) have caused a recent epidemic both nationally and globally. NSO have gained popularity in the illicit drug market and have brought about an increase in fentanyl and its derivatives, as well as other chemically unrelated opioid agonists. U-47700, a non-fentanyl analog analgesic opioid, was first developed by The Upjohn Company and has a reported potency of 7.5 times that of morphine. Like many NSO, U-47700 is usually sold as a research chemical that can be purchased online but can also be found in "Gray Death" which is a mixture of fentanyl(s), heroin, and U-47700. With the emergence of these NSO, there is a need for laboratories to be able to detect these drugs in various matrices. In this study, a liquid chromatography tandem mass spectrometry (LC-MS/MS) method was optimized and validated to detect and quantify U-47700 and its metabolites, N-desmethyl-U-47700 and N,N-didesmethyl-U-47700, in 100 µL human plasma using an optimized solid phase extraction procedure. A small sample size (100 µL) was utilized for a future pharmacokinetic study in rats. The method was validated according to SWGTOX guidelines, including: precision and bias, linearity, carryover, interferences, matrix effects, limit of detection (LOD), limit of quantification (LOQ), dilution integrity, and stability. The LOD were 0.05 ng/mL for U-47700 and N-desmethyl-U-47700 and 0.1 ng/mL for N,N-didesmethyl-U-47700. Linear ranges for U-47700 and N-desmethyl-U-47700 were 0.1-100 ng/mL and 0.5-100 ng/mL for N,N-didesmethyl-U-47700. Matrix effects were analyzed following the post-extraction addition approach and were <5%, indicating little ion suppression or enhancement. Extraction recovery was >79%. Analytes were stable in all conditions and no endogenous or exogenous interferences were detected. This method was cross-

validated in rat plasm with bias (2.1-6.2%) and precision (-14.7-15.7%) within acceptable limits. Matrix effects and extraction efficiency was comparable to human plasma validation. Postmortem whole samples (n=15) were analyzed with the validated method. U-47700, N-desmethyl-U-47700 and N,N-didesmethyl-U-47700 concentration ranges were 1.1-1367 ng/mL, 4.0-1400 ng/mL and 0.3-658 ng/mL, respectively.

Keywords: U-47700, LC-MS/MS, Novel synthetic opioids

Introduction

Novel psychoactive substances (NPS), the synthetic substitutes for classic drugs of abuse, are a worldwide problem. With the ease of internet purchases and increased manufacturing capacity of foreign countries, NPS are emerging at a rapid rate (1). It is believed that NPS are a trending problem to bypass current drug control laws by being marketed as legal alternatives to controlled drugs (2,3). NPS are often considered a "safer" alternative to illicit drugs (4). Though the most common NPS are stimulants and cannabinoids, there has been an increase in the number of novel synthetic opioids (NSO) in the drug market across the nation (5). These NSO are commonly encountered in heroin or counterfeit pain pills (6) and are fairly easy to obtain (7,8). Opioids are highly addictive drugs and remain a major problem in the United States (7). In 2016, over 40,000 fatal drug overdoses in the US were related to opioids (9). However, identification of NSO is challenging for toxicologists due to unknown compounds, lack of reference materials, and low concentrations in biological samples due to high potency. Additionally, pharmacology is unknown or data are limited, therefore metabolites and windows of detection are not well known. NSO are a serious issue for the public who are unaware of the dangers associated with these drugs (10,11) and users often administer the synthetic drugs unknowingly (6).

An emerging NSO that is considered a non-fentanyl benzamide and a heroin adulterant is 3,4-dichloro-N-[-2-(dimethylamino) cyclohexyl]-N-methyl benzamide, or U-47700. In the 1970s, this drug was originally investigated by The Upjohn Company as a chemical with analgesic and therapeutic properties (6,12) but was never made available commercially for medical use or studied in humans (7,12). U-47700 is a selective μ-

agonist with a potency of the (1R, 2R) isomer of 7.5 times greater than morphine (12-14). Agonists which preferentially bind to the μ-receptors over delta (ϱ) or kappa (ƙ) receptors are more likely to be associated with severe respiratory depression and increased addiction potential (15). U-47700 exhibits opioid-like properties with analgesic and euphoric effects (12). On the streets, this drug is commonly known as "pink" (16). U-47700 is commonly detected with fentanyl or fentanyl analogs (17-19). When these two drugs are cut into heroin, the combination is known as "Gray Death." U-47700 can be administered via oral consumption, inhalation, insufflation, intravenous injection, or intrarectal administration (12). Common effects of this drug include those that are similar to opioids including analgesia, sedation, euphoria, itching, hypothermia, respiratory depression, miosis and epistaxis (12). U-47700 overdose, like fentanyl, can be reversed by naloxone (19-21). In 2016, U-47700 was emergency classified as a Schedule I compound by the Drug Enforcement Administration (DEA) (7,22). There is very little pharmacological data published for U-47700, which makes detection and data interpretation difficult (12). However, it is known that this newly emerging drug has led to at least 46 fatalities and has been associated with 88 seized drug samples submitted to state and national crime laboratories in the United States (3,7,12).

In order to extend detection windows for NPS, toxicologists often seek to identify metabolites in biological samples. However, without controlled administration studies in humans, this is a difficult challenge and requires extensive use of *in vitro* metabolic assays or analysis of urine specimens after suspected U-47700 intake. Elliott *et al* were the first to report U-47700 related fatalities as well as identify potential metabolites in blood and urine (23). Additionally, one study also identified the metabolites in urine and

serum using high resolution LC-MS (20). They also concluded that 4N-desmethyl-U-47700 (dm-U-47700) and N-didesmethyl-U-47700 (ddm-U-47700) were the major phase I metabolites. Confirmation of the major metabolites is key to understanding pharmacology of the drug and interpreting toxicological data. In a separate study, those same metabolites were also identified in casework, though the true identity could not be confirmed without examining metabolism directly (21). Krotulski *et al* performed *in vitro* metabolism with human liver microsomes and confirmed in authentic urine: N-desmethyl-U-47700 (dm-U-47700), N,N-didesmethyl-U-47700 (ddm-U-47700), N-desmethyl-hydroxyl-U-47700, and N,N-didesmethyl-hydroxyl-U-47700, with the major metabolite being dm-U-47700 (13).

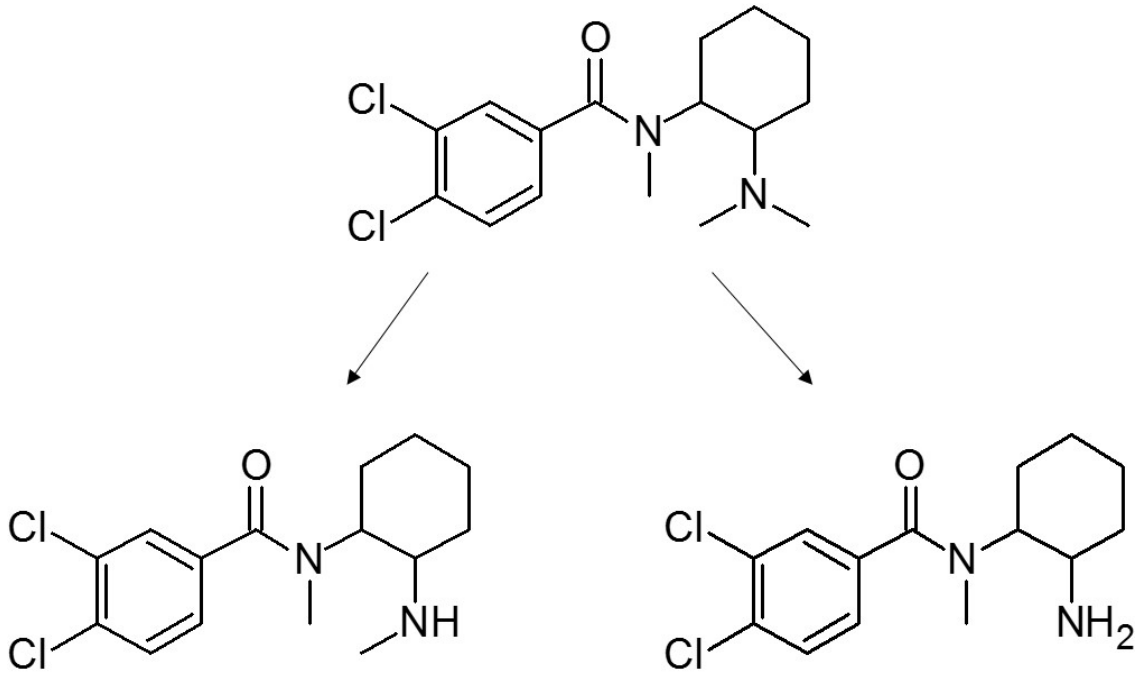

Figure 6.1 Structure of U-47700 (top), N-desmethyl-U-47700 (bottom, left) and N,N-didesmethyl-U-47700 (bottom, right). Metabolic pathways follow N-demethylation

The presence of dm-U-47700 and ddm-U-47700 (structures in **Figure 6.1**) were confirmed by liquid chromatography coupled to a time of flight quadrupole (LC-QTOF).

Lastly, Richeval *et al* found dm-U-47700, ddm-U-47700, and N-desmethyl-hydroxy-U-47700 using human liver microsomes *in vitro* experiments. They also were able to detect two N,N-didesmethyl-hydroxy-U-47700 metabolites in a urine sample from an authentic case (24). There are currently no reference standards available for the hydroxylated metabolites. However, there are no fully validated, routine analytical methods available to detect and quantify U-47700 and its metabolites.

Previous studies have detected potential metabolites of U-47700 using gas-chromatography mass-spectrometry (GC-MS), liquid chromatography coupled to a quadrupole time-of-flight mass spectrometer (LC-QTOF), or LC-tandem mass spectrometry (LC-MS/MS). U-47700 has been detected in blood (14,17,23,28,30,31), serum (20,27), and urine (20, 21, 23-24,27, 29, 30). To date, no methods exist for the quantification of U-47700 and its metabolites.

The goal of this study is to develop and validate an analytical method to detect and quantify U-47700 and its metabolites in plasma at low limits of quantification in a small sample size (100 µL). The ultimate goal of the research is to examine U-47700 and its metabolites in rat plasma following controlled intravenous drug administration. Animal models are essential to understanding pharmacokinetics (PK) and pharmacodynamics (PD) of NPS in an effort to understand pharmacology of these rapidly emerging compounds. The data from the rat study will be paramount to understanding effects and metabolism of this drug as very little information is currently available.

One of the major goals of this study was to develop, optimize, and validate a quantitative method for analysis of U-47700 and its metabolites in human plasma using a small sample size (100 µL). The practical application of this study is application to a rat

study with controlled intravenous U-47700 drug administration. Rats are small mammals and only a small volume of blood can be drawn from them. Additionally, forensic toxicology laboratories are often specimen limited and analytical techniques which do not require a large sample volume are favored.

Materials and Methods

Chemicals and Reagents

U-47700, N-desmethyl-U-47700, N,N-didesmethyl-U-47700, and internal standard (ISTD), d6-U-47700, were purchased as methanolic solutions from Cayman Chemical (Ann Arbor, MI, US). Pooled human plasma with K2 EDTA anticoagulant was purchased from Innovative Research (Novi, MI) and stored in the freezer at -20 °C. Pooled rat plasma from drug-naïve rats was received from National Institute on Drug Abuse (Baltimore, MD, US). Solid phase extraction (SPE) was performed using PolyChrom Clin II (3 mL, 35 mg) SPE columns (SPEWare, Baldwin Park, CA) on a SPEWare System 48™ Pressure Processor. A BiotageTurboVap LV® (Charlotte, NC, US) equipped with nitrogen gas was used for solvent evaporation. Acetic acid (ACS reagent) was purchased from Mallinckrodt Pharmaceuticals (St. Louis, MO, US). Formic acid (LC-MS grade), dibasic sodium phosphate (ACS reagent), and monobasic sodium phosphate (ACS grade) were purchased from Sigma-Aldrich (St. Louis, MO, US). Deionized water was purified using a Millipore Direct-Q® 3UV system (Burlington, MA, US). Ammonium hydroxide (ACS reagent), hexane (analytical grade), methanol (LC-MS grade), and ethyl acetate (LC-MS grade) used in sample preparation and extraction were obtained from J.T. Baker (Center Valley, MA, US). LC-MS grade methanol used in the mobile phase was also purchased from J.T. Baker.

Preparation of Standard Solutions

Stock solutions of all analytes were prepared at 100,000 ng/mL (except ISTD at 10,000 ng/mL) in methanol. Mixed methanolic solutions for calibrators were prepared via serial dilution, resulting in concentrations of 500, 250, 125, 50, 12.5, 2.5, and 0.5 ng/mL, which produced the following concentrations when fortified in plasma: 100, 50, 25, 10, 2.5, 0.5, and 0.1 ng/mL. Mixed methanolic solutions for quality controls (QC) were prepared separately via serial dilution, resulting in concentrations of High 400, Medium 150, and Low 1.5 (7.5 for ddm-U-47700) ng/mL, which produced the following concentrations when fortified in plasma: 80, 30, and 0.3 (1.5 for ddm-U-47700) ng/mL. A methanolic ISTD solution was prepared at 100 ng/mL, resulting in 20 ng/mL when fortified in plasma. All solutions were stored in amber vials at -20°C.

Solid Phase Extraction

Plasma (100 µL) was fortified with 20 µL of appropriate calibrator or QC solution. Samples and negative controls were also fortified with 20 µL ISTD solution. To all samples, 900 µL of phosphate buffer (100 mM, pH 6.0) was added and vortexed to mix. The mixture was loaded to the SPE column and washed with 1 mL deionized water and 1 mL acetic acid (1 M). The column was dried at maximum pressure under nitrogen for 5 min and then washed with 1 mL each: hexane, ethyl acetate, and methanol. Analytes were eluted with 1 mL 80:20 mixture of dicholormethane:isopropanol (*v/v*) with 5% concentrated ammonium hydroxide. The elution solvent was evaporated to dryness under nitrogen at 50 °C and reconstituted in 50 µL 60:40 mobile phase A:B. The aqueous mobile phase (A) consisted of 5 mM ammonium formate with 0.05% formic acid in deionized water. The organic mobile phase (B) consisted of 0.1% formic acid in

methanol. Samples were centrifuged at 1276 g for 1 min before transferring to an autosampler vial. A total of 10 µL was injected onto the LC-MS/MS for analysis.

Instrumentation

Liquid Chromatography

Analysis was performed on an Agilent 1290 Infinity II Liquid Chromatograph coupled to an Agilent 6470 Triple Quadrupole Mass Spectrometer (Santa Clara, CA, US). Analyte separation occurred across an Agilent ZORBAX Eclipse Plus C18 column (1.8 µm, 2.1 x 50 mm) with a matching guard column. The column temperature was held at 30 °C. Separation was achieved using isocratic elution with mobile phase A:B at 60:40 at a 0.4 mL/min flow rate with a run time of 8 min.

Mass Spectrometry

Electrospray ionization was operated in positive mode. A multiple reaction monitoring (MRM) method was used to detect the analytes with one transition for quantification and one transition for qualification. Data acquisition and analysis was performed using Agilent MassHunter Workstation software (Santa Clara, CA, US). The gas temperature was set at 300°C with a gas flow of 13 L/min. The nebulizer was at 45 psi. The sheath gas was at 350°C with a flow of 12 L/min. The capillary voltage was set at 4500 V. The optimized MS/MS parameters are summarized in **Table 6.1**.

Table 6.1 Optimized mass spectrometry parameters; Q1 –quadrupole 1, Q3 – quadrupole 3, CE - collision energy, RT - retention time, ITSD – internal standard; quantifying transitions are italicized

Analyte	Q1 mass (*m/z*)	Q3 mass (*m/z*)	Fragmentor (V)	Dwell (ms)	CE (V)	RT (min)	ITSD
U-47700	*329.2*	*172.9*	108	20	32	2.380	U-47700-d_6
	329.2	144.9		20	56		
N-desmethyl -U-47700	*315.2*	*172.9*	108	20	32	2.572	U-47700-d_6
	315.2	144.9		20	52		
N,N-didesmethyl -U-47700	*301.1*	*189.9*	108	20	20	2.800	U-47700-d_6
	301.1	144.9		20	56		
U-47700-d_6	*335.2*	*172.9*	108	20	36	2.378	-
	335.2	144.9		20	60		

Method Validation

Method validation was carried out according to the standard practices for method validation of the Scientific Working Group for Forensic Toxicologists (SWGTOX) (29). Validation parameters evaluated included calibration models and linearity, limits of detection (LOD), limits of quantification (LOQ), bias, precision, ion suppression/enhancement, recovery, interference studies, carryover, and dilution integrity. Calibration models over a working range were determined using 7 non-zero calibrators over 5 days, except for ddm-U-47700 (6 non-zero calibrators). Linearity was achieved using the least squares model and considered acceptable when $R^2 > 0.99$. LODs and LOQs were established by evaluating decreasing analyte concentrations. Once established, LODs and LOQs were analyzed in triplicate over three days. LODs were considered acceptable with proper qualifying ion ratios (within 20% of calibrators),

signal to noise ratio ≥3, and retention time within ± 0.1 min. LOQs were considered acceptable by meeting LOD criteria as well as signal to noise ratio ≥10 and precision and bias within 20%.

Bias and precision were determined at three different QC concentrations in triplicate over 5 days. Bias (%) and precision (% coefficient of variation, CV) were considered acceptable within ±20%. Within-day and between-day precision were calculated at each QC concentration. Carryover was evaluated on three days by injecting an extracted negative sample (ISTD only) following the injection of the highest calibrator. Carryover was considered negligible if peaks fell below LOD criteria.

Endogenous interferences were evaluated by analyzing blank matrix with and without internal standard daily. Samples fortified with ISTD were examined for presence of non-deuterated analytes. Additionally, samples were prepared at 50 ng/mL in duplicate in hemolyzed plasma (5 - 40% hemolysis) to ensure proper quantification of analytes and to check for interferences. Ion suppression and enhancement, or matrix effects, were determined using post-extraction addition (30) over 4 days in triplicate at 50 ng/mL. Matrix effects were determined by taking the mean response of the post-extraction fortified samples divided by the mean response of the neat standards. The recovery was calculated by taking the mean response of the extracted samples over the mean response of the post-extraction fortified samples. Matrix effects were considered acceptable when they fell within ±25% with acceptable precision (<15% CV).

Exogenous interferences (10000 ng/mL) were fortified into low QC samples (n=3) and extracted as described above. The compounds evaluated included Δ^9-tetrahydrocannabinol, alprazolam, amobarbital, amphetamine, amitriptyline, butalbital,

caffeine, carbamazepine, carisoprodol, cocaine, codeine, cotinine, cyclobenzaprine, dextromethorphan, diazepam, diphenhydramine, hydrocodone, hydromorphone, ketamine, methadone, nicotine, nordiazepam, oxazepam, oxycodone, pentobarbital, phencyclidine, phenobarbital, propoxyphene, secobarbital, tetrahydrocannabinolic acid, tramadol, and zolpidem. Exogenous interferences were considered acceptable if there were no co-eluting peaks and if the QCs quantified within ±20% of target.

Dilution integrity was examined by performing a 1:10 dilution of a sample fortified at 2.5 times the highest QC (200 ng/mL) in triplicate. Stability was assessed in triplicate at low and high QC concentrations at room temperature (24 h, 20 ° C), refrigerated (72 h, 4 °C), and after three freeze/thaw cycles (-20 °C). Processed samples were also evaluated in the autosampler (72 hours, 4 °C). Dilution and stability were considered acceptable if bias was within ±20%.

Rat Plasma Cross-Validation

Cross-validation was performed in rat plasma using the optimized extraction and acquisition methods. QCs at low, medium, and high concentrations in were prepared in plasma from drug-naïve rats in triplicate and analyzed over three days against calibration curves prepared with human plasma. Precision, bias, matrix effects, and recovery were assessed as described above.

Authentic Samples

A total of 15 postmortem blood samples were received and analyzed as described above. However, an additional centrifugation step was added to sample preparation before loading onto the SPE columns to prevent clogging. QCs at low, medium, and high concentrations were prepared in blood in triplicate and analyzed over three days against

calibration curves prepared with plasma. Precision, bias, matrix effects, and recovery were assessed as described above.

Results and Discussion

Method Development

Solid phase extraction was optimized for a small sample size (100 µL) and low LOQ due to nature of future applications (controlled drug administration to rats). Extensive method development took place to minimize interferences and maximize instrument response for the metabolites. Chromatographic separation of analytes was initially investigated with mobile phase A as 5 mM ammonium formate with 0.05% formic acid in deionized water and mobile phase B with 0.01% formic acid in acetonitrile. Starting mobile phase composition and several different gradients were evaluated in order to achieve sufficient separation. Ultimately, isocratic elution was explored at several different mobile phase compositions (90:10, 80:20, and 60:40 A:B). The isocratic gradient at 60:40 resulted in the best resolution for all analytes. Despite chromatography optimization, there was a small unknown peak in ddm-U-47700's extracted ion chromatogram (**Figure 6.2**, left).

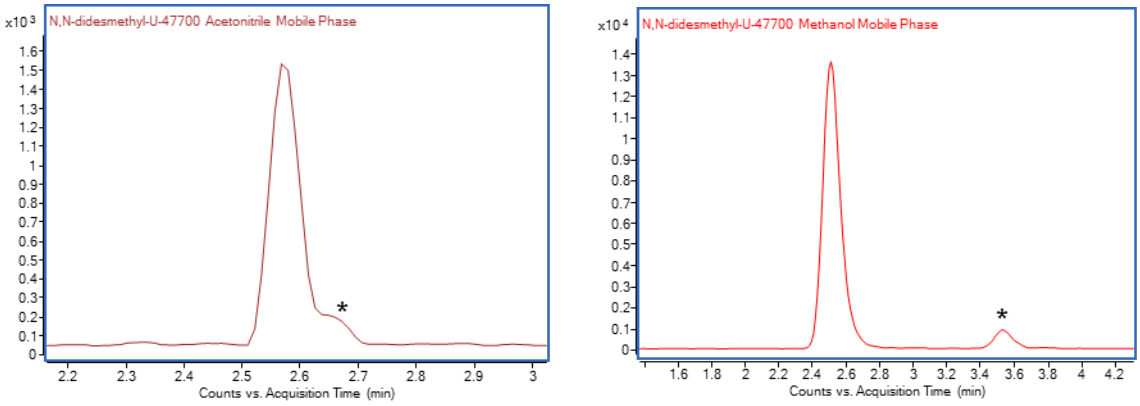

Figure 6.2 Comparison of ddm-U-47700 eluted with acetonitrile (left) and methanol (right) to separate interferant (*)

This shoulder was from an unknown source and reproducible in neat and extracted samples for both the quantifying and qualifying transitions. To eliminate this unknown shoulder, various columns were assessed including: Agilent ZORBAX Eclipse C18 and Agilent 120 Poroshell C18-EC. The ZORBAX column improved peak shape compared to the Poroshell but did not offer complete separation from the interference. Next, mobile phase B composition was switched from 0.1% formic acid in acetonitrile to 0.1% formic acid in methanol. Though acetonitrile is commonly used in laboratories, due to its ability to wash the column more thoroughly and lack of increased back-pressure, only methanol was able to separate the interference from the analyte peak of interest. The shoulder was separated from the analyte and eluted on its own approximately one minute later (**Figure 6.2**, right). This separation was achieved in all neat and extracted samples , therefore methanol was used in the final method. This interferant was also observed in an authentic specimen presented by Vogliardi (27). After separation, the peak was shown to share the same monitored transitions of ddm-U-47700 which could possible be attributed to related compounds, synthetic bioproducts, or metabolites.

Method Validation

Linearity was determined using a $1/x^2$ weighting. Least squares regression was used with 7 non-zero calibrators, except ddm-U-47700 (6 non-zero calibrators). Calibration curves resulted in R^2 values ≥ 0.996 for U-47700, dm-U-47700, and ddm-U-47700 from 6 days of validation experiments, as summarized in **Table 6.2**.

Table 6.2 Linearity, limit of detection (LOD), limit of quantification (LOQ) and linear range for U-47700 and metabolites in human plasma

Analyte	LOD (ng/mL)	LOQ (ng/mL)	Linear Range (ng/mL)	y-intercept (mean ± sd, *n*=6)	Slope (mean ± sd, *n*=6)	R^2 (range, *n*=6)
U-47700	0.05	0.1	0.1-100	0.0002 ± 0.0003	0.0545 ± 0.0014	0.997-0.999
N-desmethyl-U-47700	0.05	0.1	0.1-100	4.4E-5 ± 8.2E-5	0.03287 ± 0.0012	0.996-0.999
N,N-didesmethyl-U-47700	0.1	0.5	0.5-100	4.7E-5 ± 0.0004	0.0031 ± 0.0005	0.996-0.999

The LOD were 0.05 ng/mL for U-47700 and dm-U4, and 0.1 ng/mL for ddm-U4. The LOQ were found to be 0.1 for U-47700 and dm-U-47700 (Calibrator 1) and 0.5 ng/mL for ddm-U-47700 (Calibrator 2). Bias and precision at the LOQ were within -8.8 - 14.4% and 0.005 - 0.111 % CV, respectively. Analyte response was linear from the LOQ to 100 ng/mL, as summarized in **Table 6.2**. Extracted ion chromatogram for U-47700 and its metabolites at the LOQ are depicted in **Figure 6.3**. The limits of quantification for U-47700 are comparable to or better than those reported elsewhere: 0.3125 ng/mL and 1 ng/mL (14,21,23).

Bias and precision results for three QC concentrations prepared in human plasma are displayed in **Table 6.3**. For all analytes, bias ranged from -1.6 to -9.2%. Between-run precision was 2.9 - 7.6% CV. Maximum within-run precision was 2.8 - 13.6% CV. All of these data ranges within 15% indicate acceptable bias and precision for all analytes according to SWGTOX.

Table 6.3 Bias and Precision data for analytes in human plasma at three quality control (QC) concentrations over the linear range

Analyte	Bias (%, *n*=15)			Between-run imprecision (%CV, *n*=15)			Maximum within-run imprecision (%CV, *n*=5)		
	LQC[a]	MQC[b]	HQC[c]	LQC	MQC	HQC	LQC	MQC	HQC
U-47700	-6.0	-2.3	-2.7	6.5	5.2	3.1	13.6	8.6	2.8
N-desmethyl-U-47700	-9.2	-4.6	-5.2	7.6	4.5	2.9	12.3	6.9	5.0
N,N-didesmethyl-U-47700	-8.3	-4.8	-1.6	6.5	4.4	3.0	9.9	7.2	4.3

[a] Low QC concentration: 0.3 ng/mL (except 1.5 ng/mL for N,N-didesmethyl-U-47700)
[b] Medium QC concentration: 30 ng/mL for all analytes
[c] High concentration were 80 ng/mL for all analytes

Matrix effects were analyzed to determine ion suppression and enhancement using post-extraction addition. In the same set of samples, extraction recovery was also evaluated. Due to limited rat plasma supply, only one lot of pooled plasma was available. Samples were analyzed at 50 ng/mL for matrix effects and recovery, as summarized in **Table 6.4**.

Table 6.4 Extraction efficiency and matrix effects evaluated at 50 ng/mL in human plasma

	Pooled Plasma	
Analyte	**Extraction Efficiency (%, *n*=3)**	**Matrix Effects (%, *n*=3)**
U-47700	79.4	4.7
N-desmethyl-U-47700	79.9	0.2
N,N-didesmethyl-U-47700	84.5	3.9
U-47700-d_6	88.0	-5.4

Analyte recovery ranged from 79.4 - 88.0% and matrix effects were -5.4 - 4.7%, indicating minimal ion suppression or enhancement. The deuterated internal standard

sufficiently matched the parent drug and metabolites in terms of matrix effects and recovery. Matrix effects were within the ±25% acceptable SWGTOX guidelines. During this study, a second internal standard became commercially available, d_3-N-desmethyl-U-47700. This internal standard was evaluated as a possible addition to the assay. However, due to the location of the deuterium on the molecule, the product ions and matrix effects differed from the non-deuterated metabolite and did not offer improvement in calibration.

Endogenous interferences were evaluated in blank plasma and hemolyzed plasma. No interfering peaks were detected. In addition, QCs prepared in hemolyzed plasma quantified within ±7.2% of targeted concentration when analyzed against a non-hemolyzed plasma calibration curve. Negative samples (ISTD only) were evaluated for presence of non-deuterated analytes and no peaks were detected. Interferences of common drugs of abuse were evaluated by evaluating 32 basic, acidic and neutral drugs. Interferences were fortified at 10000 ng/mL into low QCs (n=4) and evaluated for bias. The QC samples containing interference mixtures quantified with ±16.7% of targeted concentration, and was considered acceptable.

Table 6.5 Fortified and processed sample stability at two quality control (QC) concentrations in human plasma stored in various conditions

Analyte	24 h 22°C (%, *n*=3)		72 h 4°C (%, *n*=3)		3 freeze/thaw cycles (%, *n*=3)		72 h autosampler (%, *n*=3)	
	LQC[a]	HQC[c]	LQC	HQC	LQC	HQC	LQC	HQC
U-47700	-5.0	-0.9	-2.7	-1.9	-2.9	-1.5	3.7	4.2
N-desemthyl -U-47700	-9.1	-3.4	-2.2	-2.2	-9.3	-2.0	-2.0	0.0
N,N-didesmethyl-U-47700	-10.6	-5.0	-5.6	-6.7	-5.7	-4.0	-3.1	-4.1

[a] Low QC concentration: 0.3 ng/mL (except 1.5 ng/mL for N,N-didesmethyl-U-47700)
[c] High concentration were 80 ng/mL for all analytes

When determining carryover, analytes in the negative were evaluated to determine if they met or exceeded LOD criteria in terms of peak area and ion ratio. No carryover was observed. For dilution integrity, bias was -3.0 - 2.6% and was considered acceptable. Results from the stability studies are summarized in **Table 6.5**. Bias for all the stability studies were calculated and ranged from -10.6 - 4.2% and was also considered acceptable.

Rat Plasma Cross-Validation

The cross validation results are shown in **Table 6.6** below**.** Precision and bias were evaluated and found acceptable. Matrix effects and extraction efficiency were also determined to be within SWGTOX criteria and comparable to human plasma data. These are the desired results to be applied towards a future animal model study to determine pharmacokinetic and pharmacodynamics properties of these analytes.

Table 6.6 Bias, precision, extraction efficiency and matrix effects for U-47700 and metabolites in rat plasma

Analyte	Bias (%, *n*=9)			Between-run imprecision (%CV, *n*=9)			Maximum within-run imprecision (%CV, *n*=3)			Extraction Efficiency (%, *n*=9)		Matrix Effect (%, *n*=9)	
	LQC[a]	MQC[b]	HQC[a]	LQC	MQC	HQC	LQC	MQC	HQC	LQC	HQC	LQC	HQC
U-47700	5.5	4.9	1.9	-10.8	-8.5	1.9	6.1	15.7	2.0	86.3	86.2	0.8	2.4
N-desmethyl-U-47700	6.0	5.9	2.8	-8.9	-8.8	2.8	5.6	14.4	4.5	90.7	86.4	0.2	3.5
N,N-didesmethyl-U-47700	6.6	6.2	2.1	-14.7	-10.1	2.1	7.3	14.7	1.7	87.9	82.4	-0.1	-1.6
U-47700-d6	-	-	-	-	-	-	-	-	-	87.6	85.1	1.2	-0.2

[a] Low QC concentration: 0.3 ng/mL (except 1.5 ng/mL for N,N-didesmethyl-U-47700)
[b] Medium QC concentration: 30 ng/mL for all analytes
[c] High concentration were 80 ng/mL for all analytes

Authentic Samples

A fit for purpose validation of blood was performed to ensure that quantifiable concentrations of these analytes could be obtained using the validated plasma method. Precision and bias were within acceptable criteria (range: -8.4 – 13.3 % CV), while matrix effects displayed slight enhancement (15 – 35 %) for all analytes. Postmortem blood samples (n=15) were analyzed and concentrations are shown in **Table 6.7**. Mean (range) concentrations were 232 (1.1-1367), 289 (4.0-1400), and 136 (0.3-658) ng/mL for U-47700, dm-U-47700, and ddm-U-47700, respectively.

Table 6.7 Post mortem blood concentrations for U-47700 and its dealkylated metabolites

Sample Number	Source (Blood)	U-47700 (ng/mL)	N-desmethyl-U-47700 (ng/mL)	N,N-didesmethyl-U-47700 (ng/mL)
1	Cardiac	1367	1177	658
4	Femoral	145	465	402
7	Peripheral	144	258	195
9	Not specified	208	109	8.1
11	Iliac	543	33.0	1.6
13	Iliac	38.5	4.0	0.3
17	Peripheral	1.1	ND	ND
18	Femoral	4.2	15.8	17.0
20	Cardiac	431	1400	361
22	Iliac	68.3	56.7	16.4
23	Femoral	114	8.0	0.7

Sample Number	Source (Blood)	U-47700 (ng/mL)	N-desmethyl-U-47700 (ng/mL)	N,N-didesmethyl-U-47700 (ng/mL)
25	Femoral	46.6	28.0	12.1
27	Femoral	50.7	14.1	12.5
28	Femoral	207	225	85.5
30	Iliac	126	253	129
	Mean	233	289	136
	Median	126	82.9	16.7
	Min	1.1	4.0	0.3
	Max	1367	1400	658

ND=Not Detected

Concentrations for U-47700 were comparable to previous literature ranges 17-1,460 ng/mL in blood (14,20,23). In Jones *et al*, urine and serum concentrations were reported for U-47700, dm-U-47700, and ddm-U-47700. The metabolite concentrations were approximated based on the response of U-47700. Although this was not a validated quantitative method, the concentration of dm-U-47700 in urine was almost 5 times higher than the parent drug [19]. This result is comparable to that of sample 20, which was a blood sample, in this study. In one study, U-47700 was detected at higher concentrations in blood compared to urine [21], while a different study found the opposite to be true [16]. However, without knowledge of dose or time of administration, data are difficult to interpret. Further, concentrations detected in postmortem cases can be challenging due to potential for postmortem redistribution. In McIntyre *et al*, U-47700 concentrations in various fluids and tissues from a single case were examined [26]. Comparison of the

central to peripheral blood concentrations as well as the liver to peripheral blood concentrations indicated that U-47700 may exhibit postmortem redistribution. For the present study, injection or consumption times of these drugs are unknown, therefore pharmacokinetic studies are needed to understand the parent-metabolite half-lives of these drugs. Though it is not uncommon for U-47700 to be detected with other drugs such as fentanyl, alprazolam, and other synthetics, for the scope of this study, we were only interested in quantifying U-47700 and its metabolites (17,26,32).

Conclusions

A method for the detection and quantification of U-47700 and its metabolites was developed and validated following SWGTOX guidelines using a low sample volume. The method validation parameters met acceptability criteria. This is the first method, to our knowledge, which quantifies two phase I metabolites of U-47700: N-desmethyl-U-47700 and N,N-didesmethyl-U-47700. The final method was sensitive and limits of quantification are sufficiently low to detect intoxicating or fatal concentrations of these analytes. Further studies need to be assessed to validate a quantitative method in blood, however when analyzed against plasma calibrators, this method was able to detect and quantify these analytes in authentic postmortem blood samples. The future goal of this study is to analyze rat plasma following administration of U-47700 in a controlled environment in order to assess PK and PD. Animal models are used to study PD/PK so scientists can better understand the drug without risking the well-being of humans. Animal models are crucial to studying novel psychoactive substances and can produce important pharmacological data.

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CHAPTER VII

Pharmacokinetics and Pharmacodynamics of U-47700 and its Metabolites

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This dissertation follows the style and format of *Journal of Analytical Toxicology*.

Abstract

Originally developed as a potential analgesic in the 1970's, U-47700 was the first U-series opioid to emerge on the illicit market. Metabolic studies using structural elucidation and human liver microsomes previously determined four major metabolites. Of these metabolites, only two are commercially available (N-desthmethyl-U-4700 and N,N-didesmethyl-U-47700). To date, there are no pharmacological studies in animal or human models for U-47700. This study's goal was to evaluate pharmacokinetics and pharmacodynamics of U-47700 through an animal model. Rats were injected with 0 (saline), 0.3, 1.0 or 3.0 mg/kg U-47700. Blood samples were collected at 15, 30, 60, 120, 240, 480 min after injection for quantification of U-47700 and its metabolites. Pharmacodynamic effects, including hot plate latency, catalepsy, and core temperature, were also assessed at the same time points. It was determined that doses of U-47700 had a positive correlation with the behaviors observed which further demonstrates the analgesic effects of this novel synthetic opioid.

Keywords: U-47700, Pharmacokinetics, Pharmacodynamics, NPS

Introduction

According to the National Forensic Laboratory Information System (NFLIS), there were over 180,000 reports of narcotic analgesics in United States in 2018 (1). The opioid epidemic that the United States is facing is all encompassing, which includes prescription and illicit drugs with a trend towards the use of novel synthetic opioids (NSO). U-47700 is one such NSO that emerged globally between 2015 and 2016 (2,3). Originally developed as a potential analgesic by the Upjohn Company in the 1970's, U-47700 was the first U-series opioid to emerge. It was determined to be a selective μ-receptor agonist and 7.5 times more potent than morphine (4). While pharmacological information is minimal, it is theorized that U-47700 is abused due to its euphoric and potential analgesic effects. Fatal intoxications are not uncommon with the abuse of U-47700. Blood concentrations found in fatal intoxication range from 7.8-3040 ng/mL (5-18), with most cases involving another illicit drug or other opioids. Acute intoxications are prevalent as well for U-47700 with blood concentrations ranging from 94-351 ng/mL (19,20).

Metabolism studies have been performed to determine metabolites for U-47700. Krotulski *et al* was the first to perform a metabolic study to identify four metabolites using human liver microsomes (HLMs) and verified those metabolites with authentic urine specimens (21). The major metabolite identified was N-desmethyl-U-47700. Richeval *et al* also conducted a metabolism study using HLMs and verified predicted metabolites in authentic specimens (11). The same metabolites predicted in Krotulski *et al* were predicted in Richeval *et al*, with an additional positional isomer of the hydroxylation on the cyclohexane ring metabolite. Other studies have used structure

elucidation on authentic samples to predict metabolites and have determined the same metabolites (9,22). There have been two quantitative methods developed for U-47700 and its metabolites (23,24). Rojek *et al* developed and validated a method to detect U-47700 and its metabolites in blood and applied the method to postmortem blood samples (N=12). Smith *et al* optimized, developed, and validated a sensitive method for the quantification of U-47700, N-desmethyl-U-47700, and N,N-didesmethyl-U-47700 in plasma utilizing liquid chromatography tandem mass spectrometry (LC-MS/MS) and cross validated in rat plasma (23). A major difference observed between the two methods was that during optimization, Smith *et al* was able to chromatographically separate a N,N-didesmethyl-U-47700 interferant.

In order to better understand the biological effects of U-47700 and other synthetic opioids, Baumann *et al* conducted a rat study to examine opioid receptor interaction and antinociceptive effects (25). It was determined that U-47700 was 31 times more potent than morphine in regards to the tail flick assay and confirmed that U-47700 was a selective μ agonist. It was also concluded that *in vivo* potency cannot be predicted by *in vitro* binding affinity. In order to understand the true pharmacology of U-47700, pharmacokinetic (PK) profiles are needed and metabolite bioactivity should be evaluated.

To date no studies have been conducted to fully determine the pharmacokinetic and pharmacodynamic properties of U-47700 in human or animal models. In order to better understand this compound, *in vivo* studies using animal models are necessary. In the present study, the analytical method developed by Smith *et al* (23) was used to quantify U-47700 and its metabolites in rat plasma following subcutaneous (sc) administration of U-47700 at three doses (0.3, 1.0, and 3.0 mg/kg). Plasma samples were

collected at several time points in conjunction with a battery of behavior and physiological measures in order to assess U-47700 pharmacodynamics and PK.

Materials and Methods

Reagents and Standards

Chemicals and reagents commonly used for extraction and LC-MS/MS were the highest purity available. U-47700, N-desmethyl-U-47700, N,N-didesmethyl-U-47700, and U-47700-d6 were purchased from Cayman Chemical Company (Ann Arbor, MI, USA) . Pooled blank male Sprague Dawley rat plasma preserved with sodium heparin was obtained from BioIVT (Medford, MA, USA).

Animals and Surgery

Sprague-Dawley (male) rats (250-400g) were housed (lights on for 12 hours) with free access to water and food, with controlled temperature (22±2 °C) and humidity (45%±5%). These experiments followed the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Study procedures were approved by NIDA IRP Animal Care and Use Committee (ACUC). Two weeks were allowed for acclimation prior to surgeries and experiments.

Surgeries for PK experiments consisted of implantation of IPTT-300 transponder (Bio Medic Data Systems, Seaford, DE, USA) for monitoring body temperature and a catheter in the right jugular vein. Rats were allowed a week for recovery.

PK Experiments

Rats were moved from their acclimated environments to the testing room and given 1 h to acclimate. In order to facilitate blood collection through the catheters, 0.3 mL of 48 IU/mL heparin saline was flushed through. Rats (n=6) were grouped and

received subcutaneous (sc) of 0 (saline), 0.3, 1.0, or 3.0 mg/kg of U-47700. Blood (0.30 mL) was collected before injection and at 15, 30, 60, 120, 240, and 480 min subsequently. Equal amounts of saline were infused after each blood collection in order to maintain osmotic homeostasis and volume. Blood was transferred to 1.5 mL tubes fortified with 5 µL of 250 nM sodium metabisulfite and 5 µL of 1000 IU heparin and then centrifuged at 3000 rpm for 10 min. After centrifugation, plasma was decanted and stored at -80 °C and shipped frozen for analysis.

Behavior assessments (90 seconds) were accompanied with blood collections at 15, 30, 60, 120, 240, and 480 min. Catalepsy was evaluated by placing the rats in an uncomfortable position and recording the amount of time it took for the rat to return to a normal position. Catalepsy was scored similar to published methods (26). Briefly, three endpoints were considered at each time point: 1) immobility, 2) flattened body posture, and 3) splayed limbs; each of these endpoints was scored as either absent "1" or present "2" at each time point prior to blood withdrawal, so the minimum summed score for no symptoms is "3" and the maximum score for the all symptoms present is "6". Analgesic effects were evaluated by a hot plate latency study. Rats were placed on hot plate and time was recorded as to when the rat tried to jump off plate or exhibit behaviors such as licking its paws. The maximum allotted time allowed for this study was 45 seconds. After behavior observations, core temperatures were recorded at each time point.

Quantification of U-47700 and its Metabolites in Plasma

Plasma was analyzed by a previously validated method (Smith *et al*) (23). Briefly, internal standard was added to plasma (100 µL) and buffered before loading on solid phase extraction cartridges. Analytes were eluted with dichloromethane:isopropyl alcohol

(80:20, v/v) with 5% ammonium hydroxide, then dried under nitrogen and reconstituted in 50 µL of 5 mM ammonium formate with 0.05% formic acid in water: 0.1% formic acid in methanol (60:40, v/v).

Samples were analyzed on an Agilent 1290 Infinity II Liquid Chromatograph system equipped with an Agilent 6470 Triple Quadrupole Mass Spectrometer (Santa Clara, CA, USA). Agilent MassHunter Software was used for data acquisition and analysis of U-47700, N-desmethyl-U-47700, and N,N-didesmethyl-U-47700. Linear ranges for U-47700 and N-desmethyl-U-47700 were 0.1-100 ng/mL and 0.5-100 ng/mL for N,N-didesmethyl-U-47700. The limit of detection were 0.05 ng/mL for U-47700 and N-desmethyl-U-47700 and 0.1 ng/mL for N,N-didesmethyl-U-47700.

Data Analysis

Plasma PK data were analyzed using APL Pharmacokinetic Modeling Program (PKMP) to determine non-compartmental PK parameters such as half-life, area under the curve, and C_{max}. Possible non-linearity was evaluated by calculating expected values for area under the curve (AUC) values and comparing with observed values. The 0.3 mg/kg values were multiplied by 1/3 and 10 in order to calculate the expected AUC values for the 1 and 3 mg/kg doses, respectively. The data from observed and expected AUC were compared using nonparametric t-tests and results were evaluated using two-way ANOVA. Behavioral data versus U-47700 concentrations were evaluated using hysteresis graphs.

Results and Discussion

PKs of U-47700 and its Metabolites

Time-concentration profiles for plasma U-47700, N-desmethyl-U-47700, and N,N-didesmethyl-U-47700 after sc injection of 0.3, 1.0, and 3.0 mg/kg U-47700 are shown in **Figure 7.1** below.

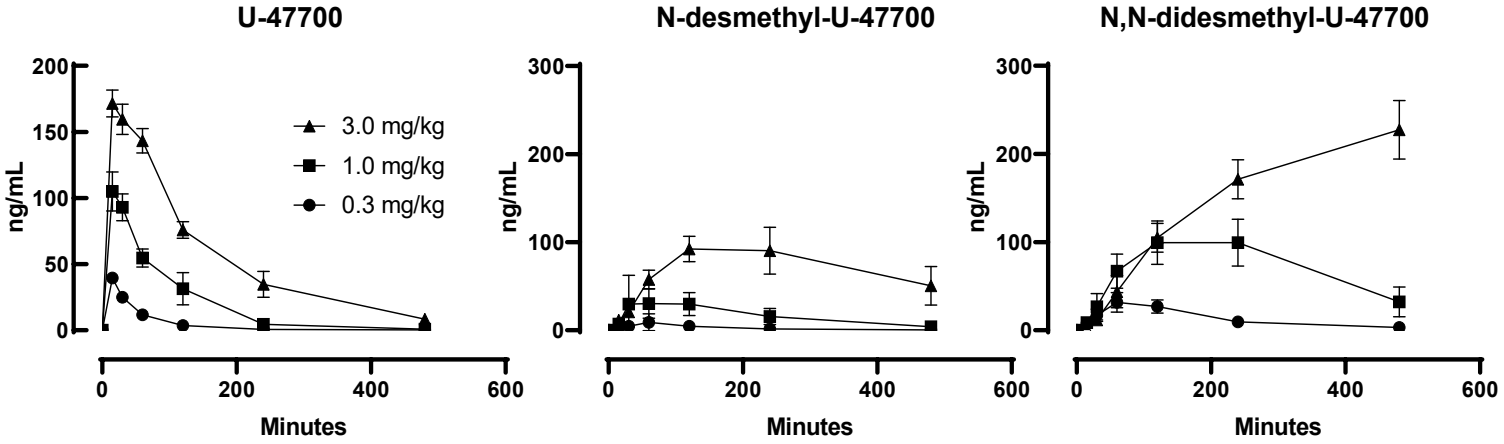

Figure 7.1. Concentration-time profiles for U-47700, N-desmethyl-U-47700, and N,N-didesmethyl-U-47700 after sc injection of U-47700. Data are mean ± SEM for n=5-6 rats per group.

Data depicted in **Figure 7.1** were used to derive PK constants presented in **Table 7.1**.

Table 7.1 Pharmacokinetic constants (mean±SEM) for plasma U-47700, N-desmethyl-U-47700, and N,N-didesmethyl-U-47700 after subcutaneous U-47700 administration at 0.3, 1.0, and 3.0 mg/kg to rats (N=5-6 per group)

Analytes	Dose (mg/kg)	C_{max} (ng/mL)	T_{max} (min)	AUC (min*ng/mL)	$t_{1/2}$ (min)	C_{last} (ng/mL)
U-47700	0.3	40±3	15	2169±164	82±9	0.27±0.09
	1.0	110±13	38±17	9872±1063	68±4	0.84±0.3
	3.0	173±9	24±9	26692±2861	102±16	8±4
N-desmethyl-U-47700	0.3	9±4	80±13	1289±306	110±7	0.46±0.10
	1.0	46±11	75±15	8158±1526	136±17	4±1
	3.0	102±23	168±29	33881±8794	-	76±31
N,N-didesmethyl-U-47700	0.3	32±11	90±13	6427±1890	126±10	3±1
	1.0	108±26	160±25	34463±9920	301±84	32±17
	3.0	236±31	384±59	69834±5492	-	191±46

U-47700 plasma concentrations increased in a dose-related way. The C_{max} values were 40, 110, and 173 ng/mL for the 0.3, 1.0, and 3.0 mg/kg U-47700 doses and the corresponding AUC values were 2169, 9872, and 26692 min x ng/mL, respectively. Antemortem U-47700 concentrations have been reported between 7.6-251 ng/mL in blood and serum (19,20,22,27,28). Of these concentrations, Jones *et al* was the only to report a concentration of U-47700 (228 ng/mL in serum) that did not have any other illicit drugs of abuse identified (22). Given that interspecies extrapolation is difficult to determine and the blood to plasma ratios for U-47700 are unknown, the determined C_{max} values for U-47700 (40-173 ng/mL) are feasible concentrations applicable to the forensic casework. T_{max} for U-47700 was observed within the first 60 min with values of 15, 38, and 24 min and $t_{1/2}$ values of 82, 68, and 102 for the corresponding doses (0.3, 1.0, and

3.0 mg/kg U-47700). Koch *et al* determined the half-life of U-47700 in a patient, prior to death, to be 6 hours (15). Half-life was calculated by taking several blood serum samples during hospitalization and plotting concentration-time curve. The proposed half-life from Koch *et al* and the current study do not align, but the differences could be attributed to the limited sample size and lack of sufficient descending data points in PK curve. N-desmethyl-U-47700 C_{max} values were a third of those of U-47700, while N,N-didesmethyl-U-47700 C_{max} values were comparable to those of U-47700. T_{max} values for N-desmethyl-U-47700 were 80, 75, and 168 min for the respective doses 0.3, 1.0, and 3.0 mg/kg U-47700. For the 3.0 mg/kg dose, there were not enough descending data after T_{max} as seen in **Figure 7.1**. Due to this, $t_{1/2}$ could not be determined for 3.0 mg/kg. However, $t_{1/2}$ values of N-desmethyl-U-47700 were 110 and 136 min for 0.3 and 1.0 mg/kg U-47700. AUC values for N-desmethyl-U-47700 were 1289, 8158, and 33,881 for 0.3, 1.0, and 3.0 mg/kg U-47700, respectively. C_{max} values for N,N-didesmethyl-U-47700 were comparable to that of U-47700. Similarly to N-desmethyl-U-47700, N,N-didesmethyl-U-47700 does not have enough data to determine $t_{1/2}$ for 3.0 mg/kg U-47700 due to increasing concentrations over the 480 min. Values for $t_{1/2}$ for N,N-didesmethyl-U-47700 were 126 and 301 min for 0.3 and 1.0 mg/kg U-47700. N,N-didesmethyl-U-47700 displayed the largest AUC for all three analytes with values of 6427, 34,463, and 69,834 min x ng/mL for 0.3, 1.0 and 3.0 mg/kg U-47700. Pharmacokinetic plots represent free, non-conjugated analytes as hydrolysis was not performed.

By comparing the expected AUC to the observed AUC, the possibility of a non-linear accumulation of U-47700 and metabolites was evaluated. This data is shown in

Figure 7.2 for U-47700, N-desmethyl-U-47700, and N,N-didesmethyl-U-47700. The only analyte with observed AUC values greater than expected AUC was N-desmethyl-U-47700, but the difference was only statistically significant for the 3.0 mg/kg U-47700 dose ($T(9)=2.433$, $p=0.038$). Although there was a significant difference observed at 3.0 mg/kg for N-desmethyl-U-47700, this difference is due to incomplete elimination of the metabolite over the examined timeframe of the experiment.

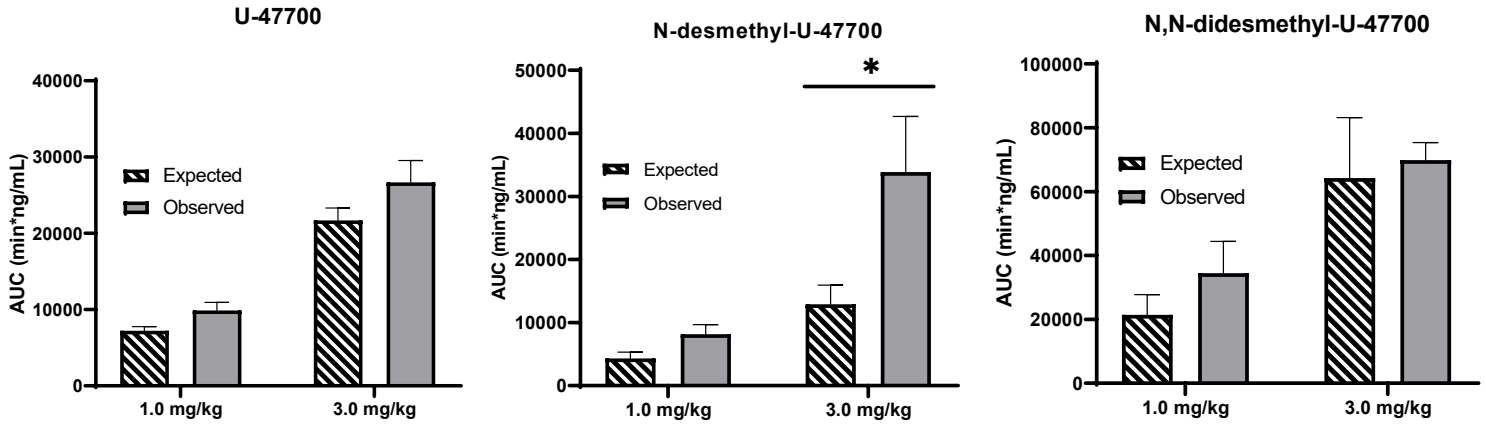

Figure 7.2 Comparison of expected vs observed AUC for U-47700, N-desmethyl-U-47700, and N,N-didesmethyl-U-47700 after subcutaneous U-47700 injection at 0.3, 1.0, and 3.0 mg/kg to rats (N=5-6 per group). Data are mean ± SEM.

Pharmacodynamic Effects of U-47700

The PK values shown in **Figure 7.1** correspond to the pharmacodynamic data shown in **Figure 7.3**. Rats that received saline (0 mg/kg U-47700) scored a time of approximately 12 seconds during the hot plate latency study. Rats demonstrated increased hot plate latency 15-30 mins after sc injection at all three doses. Rats that received 1.0 and 3.0 mg/kg U-47700 reached the appointed ceiling of 45 seconds at 30 mins post sc injection. Core body temperatures for rats that received saline, 0.3 and 1.0 mg/kg U-47700 were between 36.8-38.3°C. Rats that received the 3.0 mg/kg U-47700 dose exhibited lowered body temperatures that lasted 120 mins after sc injection. In a human, lower body temperature is an indicator of being under the influence of narcotic analgesics used by drug recognition evaluators. Similar to the hot plate latency study, rats scored the highest (4-6) 15-30 min after sc injection during the catalepsy assessment. Rats that received the 3.0 mg/kg U-47700 injection reached the appointed ceiling score of 6 at the 30 min time point.

To correlate the PK data with the pharmacodynamic studies, hysteresis plots were formed between the observed behavioral or physiological measures and the corresponding plasma U-47700 concentrations. These graphs are shown in **Figure 7.4** below. At the 1.0 and 3.0 mg/kg doses, the hysteresis progression was determined to be counter clockwise for hot plate latencyand catalepsy. The highest plasma concentrations of U-47700 at these doses was achieved at 30 min and this corresponded to the highest score for the catalepsy study and the longest amount of time in the hot plate latency study. In Crugten *et al*, it was determined that the characterization of morphine and morphine-6-glucuronide plasma concentration and antinociceptive effects produced a

counter clockwise hysteresis as well. It was concluded that the lag between concentrations and effects reflected time to cross the blood-brain barrier (29). The counter clockwise relationship observed in the current study could also be due to crossing the blood-brain barrier. Another possibility could be that one of the metabolites is pharmacologically active and could be competing for the receptor or contributing to the pharmacodynamics effect.

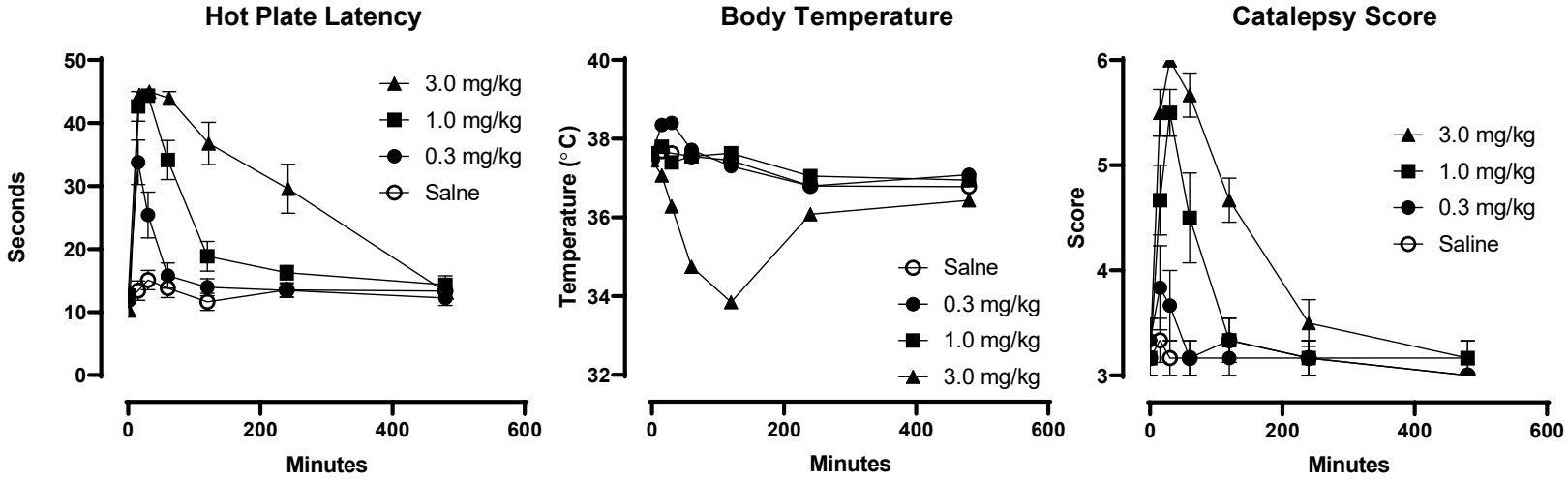

Figure 7.3 Pharmacodynamic data for rats receiving saline or subcutaneous U-47700 injection of 0.3, 1.0, and 3.0 mg/kg (N=5-6 per group). Data are mean ± SEM.

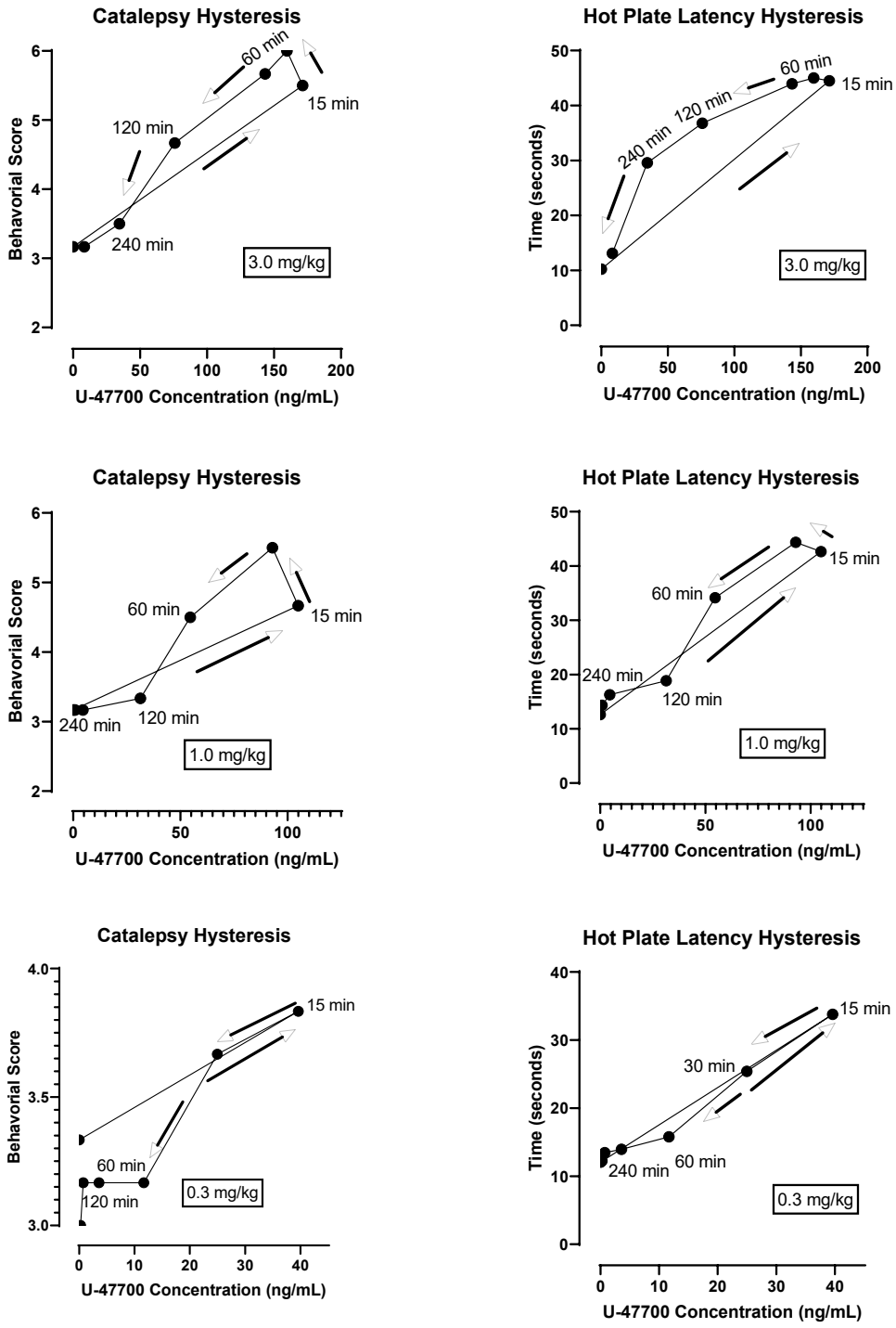

Figure 7.4 Relationship between values from catalepsy and hot plate latency studies (mean, n=5-6) and mean plasma U-47700 concentrations in rats after sc U-47700 injection at 0.3, 1.0, and 3.0 mg/kg doses. Arrows represent hysteresis curve progression over time for 480 min.

Conclusion

To the author's knowledge, this is the first pharmacokinetic study for U-47700 and its metabolites in rats. All analytes were determined to have linear accumulation, with the exception of N-desmethyl-U-47700 after the high dose of U-47700. It is hypothesized that additional time points beyond 480 min could possibly give a more defined determination of this metabolites' linearity of accumulation. Correlations between PK and pharmacodynamic data concluded that U-47700 exhibits a counter clockwise hysteresis progression over time. Further studies testing the bioactivity of the metabolites are needed to evaluate what causes the delay of action observed by the counter clockwise hysteresis. Peak plasma concentrations for U-47700 were achieved within the first hour and had positive correlations with the observed behaviors. These positive correlations reinforce the theory that U-47700 is a potent analgesic. Knowledge of these PK profiles is beneficial to the forensic community for improved interpretation of U-47700 concentrations in toxicological investigations of potential intoxications.

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CHAPTER VIII

5F-MDMB-PICA Metabolite Identification and Cannabinoid Receptor Activity[1]

This dissertation follows the style and format of *Journal of Analytical Toxicology*.

[1]Truver M.T., Wantanabe, S., Åstrand A., Vikingsson S., Green H., Swortwood M.J., and Kronstrand R. (2019) 5F-MDMB-PICA Metabolite Identification and Cannabinoid Receptor Activity. *Drug Testing and Analysis*, DOI:10.1002/dta.2688

Abstract

According to EMCDDA, there were 179 different synthetic cannabinoids reported as of 2017. In the US, 5F-MDMB-PINACA, or 5F-ADB, accounted for 28% of cannabinoid seizures 2016-2018. The synthetic cannabinoid, 5F-MDMB-PICA, is structurally similar to 5F-MDMB-PINACA with an indole group replacing the indazole. Limited data exist from *in vivo* or *in vitro* metabolic studies of these synthetic cannabinoids, so potential metabolites to identify use may be missed. The goals of this study were to 1) investigate 5F-MDMB-PICA and 5F-MDMB-PINACA *in vitro* metabolism utilizing human hepatocytes; 2) verify *in vitro* metabolites by analyzing authentic case specimens; and 3) identify the potency and efficacy of 5F-MDMB-PICA and 5F-MDMB-PINACA by examining activity at the CB_1 receptor. Biotransformations found in this study included phase I transformations and phase II transformations. A total of 22 5F-MDMB-PICA metabolites (A1 to A22) were identified. From hepatocyte incubations and urine samples, 21 metabolites (B1 to B21) were identified with 3 compounds unique to urine specimens for 5F-MDMB-PINACA. Phase II glucuronides were identified in 5F-MDMB-PICA (n=3) and 5F-MDMB-PINACA (n=5). For both compounds, ester hydrolysis and ester hydrolysis in combination with oxidative defluorination were the most prevalent metabolites produced *in vitro*. Additionally, the conversion of ester hydrolysis with oxidative defluorination to pentanoic acid for the first time was identified for 5F-MDMB-PICA. Therefore, these metabolites would be potentially good biomarkers for screening urine of suspected intoxication of 5F-MDMB-PICA or 5F-MDMB-PINACA. Both 5F-MDMB-PICA and 5F-MDMB-PINACA were

acting as full agonists at the CB_1 receptor with higher efficacy and similar potency as JWH-018.

Keywords: 5F-MDMB-PICA, 5F-ADB, MDMB-2201, Hepatocyte Metabolism, Synthetic Cannabinoids, CB_1 activity

Introduction

The emergence of novel psychoactive substances (NPS) has had a detrimental global impact. Synthetic cannabinoids are some of the first NPS that increased dramatically in the early century and started the epidemic seen today. Substances such as "Spice" or "K2" were commonly found at gas stations or smoke shops across the United States (1) and in vending machines in Japan (2). These substances were frequently found to contain the "JWH" series of synthetic cannabinoids with JWH-018 being the most predominant. Since their arrival, the number of synthetic cannabinoids has grown rapidly. According to the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA), as of 2017 there had been 179 different synthetic cannabinoids reported to them (3). In the United States, there were 24,501 identifications of synthetic cannabinoids reported to National Forensic Laboratory Information System (NFLIS) in 2017 (4). The synthetic cannabinoid, 5F-MDMB-PINACA or 5F-ADB, accounted for 28% of those identifications in 2017 (4).

The synthetic cannabinoid, 5F-MDMB-PICA , first identified in herbal incense packages by Risseeuw *et al* (5), is structurally similar to 5F-MDMB-PINACA but the indazole has been replaced by an indole group. It has gained popularity in recreational use and between 2016 and 2018, there have been 340 reports containing 5F-MDMB-PICA according to NFLIS (6). In 2018, it was placed as a schedule I substance in the United States (7). The fluoropentyl side chain of 5F-MDMB-PICA, shared with 5F-MDMB-PINACA, indicates high potency at the CB_1 receptor (8). This was verified by *Banister et al* using the FLIPR^{TM} assay (9). 5F-MDMB-PICA was shown to be a full agonist at the CB1 receptor with an EC_{50} concentration of 0.45 nM, similar to 5F-

MDMB-PINACA (0.59 nM). In contrast, Noble *et al* showed 5F-MDMB-PICA to have an EC_{50} value of 3.3 nM and an efficacy three times that of the full agonist JWH-018 using a β-arrestin based assay (8). This study aligns with previous studies, using a complimentary aequorin-luminescence based approach measuring the intracellular Ca^{2+} changes.

Parent structures of synthetic cannabinoids are often difficult to detect in biological matrices, so metabolism studies are needed to improve detection of newly emerging synthetics. Mogler *et al* used pooled human liver microsome assays to identify phase I metabolites of 5F-MDMB-PICA. Authentic urine samples (n=24) were also analyzed and 12 phase I metabolites were identified with the ester hydrolysis metabolite being the most abundant (10).

Although there is information available involving *in vivo* and i*n vitro* metabolism studies of 5F-MDMB-PICA and 5F-MDMB-PINACA, potential metabolites have not been identified using human hepatocytes. While human liver microsomes are beneficial for discovering metabolites, they are unable to produce some phase II metabolites, unlike human hepatocytes which can help characterize both phase I and phase II metabolites (11). Also metabolites produced by human hepatocyte incubations tend to have concentrations more similar to those observed *in vivo* (11).

The specific goals of this study were to 1) investigate the metabolism of 5F-MDMB-PICA and confirm 5F-MDMB-PINACA metabolism utilizing human hepatocyte incubations to identify *in vitro* metabolites; 2) examine authentic case specimens that involved these synthetic cannabinoids in order to verify metabolites identified by

hepatocyte incubations; and 3) identify the potency and efficacy of 5F-MDMB-PICA and 5F-MDMB-PINACA on the activation of the CB_1 receptor.

Materials and Methods

Chemicals and Reagents

A MilliQ Gradient 10 production unit from Millipore (Billerica, MA, USA) was used for in-house ultra-pure water production. 5F-MDMB-PICA and 5F-MDMB-PINACA were purchased from Chiron (Trondheim, Norway). JWH-018 was purchased from THC Pharm (Frankfurt am Maine, Germany). InVitro Gro HT and cryopreserved mixed gender human hepatocytes LiverPool™ (20-donor) were acquired from Bioreclamation IVT (Brussels, Belgium). HEPES buffer, L-glutamine, DMEM/HAM's F12, and Williams E buffer were acquired from ThermoFisher (Gothenburg, Sweden). Coelenterazine h was obtained from Nanolight® Technology (Pinetop, AZ, USA). Acetonitrile, formic acid, methanol, and water liquid chromatography mass spectrometry (LC-MS) grade from Fisher Scientific (Gothenburg, Sweden). Ammonium formate, digitonin, protease-free BSA, and ATP were purchased from Fluka (Sigma-Aldrich, Stockholm, Sweden). The β-glucuronidase/arylsulfatase (*Helix pomatia*) mixture was from Roche (Mannheim, Germany).

Human Hepatocytes Incubation

Thawed cryopreserved human hepatocytes were added into InVitro Gro HT Medium. The cell solution was centrifuged at 100 g for 5 min at room temperature. The supernatant was discarded and the pellet was re-suspended in a mixture of 2 mM Williams E medium supplemented with L-glutamine and 20 mM HEPES buffer. An additional washing was performed. The Trypan Blue (0.4% v/v) exclusion method was

utilized for the cell viability (91%) determination. 5F-MDMB-PICA and 5F-MDMB-PINACA were incubated with hepatocytes in 96-well-plates (10^5 cells/0.1 mL/well) with a final concentration of 5 μmol/L. Ice cold acetonitrile was used to end incubations after 1, 3, and 5 h. Baseline (t_0) samples contained hepatocytes and acetonitrile before drugs were added. Positive controls consisted of CYP substrates: caffeine, bupropion, diclofenac, omeprazole, dextromethorphan, chlorzoxazone, and midazolam. Negative controls contained incubations without drug standards. Degradation controls were incubations without addition of hepatocytes.

Authentic Urine Sample Preparation

Postmortem (n=3) and antemortem (n=1) urine samples were analyzed with and without hydrolysis. Urine (100 μL) with 300 μL of 1 M sodium acetate buffer (pH 5) was incubated for 2 h at 40 °C with 10 μL of glucuronidase/arylsulfatase (4.5 U/mL and 14 U/mL, respectively) in autosampler vials for hydrolysis. For non-hydrolyzed samples, 10 μL of the sodium acetate buffer was added instead of enzyme. After incubation, unopened vials were placed in the autosampler for analysis.

Authentic Urine Samples

Urine from three cases that presented with positive results for 5F-MDMB-PICA in blood were analyzed for metabolites to corroborate the hepatocyte findings. Two cases were autopsy cases that are described below and the third was from a suspected DUID (no case history available). In addition an autopsy case positive for 5F-MDMB-PINACA was analyzed. The study was approved by the regional ethics committee in Linköping (2018-186/31).

Case 1

A 47 year old male with a history of drug abuse and diagnosed with type I diabetes was found lying on the floor of his bathroom. The post-mortem examination revealed pulmonary edema (combined weight 1745 g), fatty liver and underweight with a BMI of 15.9. Toxicological analysis showed no ethanol but 0.45 part per thousand of acetone in femoral blood as well as 0.60 part per thousand in urine. Vitreous glucose was 78.4 mmol/L and the femoral blood had an elevated BHB at >1000 µg/g. The only exogenous compound found was 0.28 ng/g 5F-MDMB-PICA in femoral blood. The investigation concluded that the cause of death was diabetic ketoacidosis.

Case 2

A 49 year old male with a history of alcohol and drug abuse was found dead outside. The post-mortem examination was unremarkable and no underlying pathology was found. The toxicological analysis revealed no ethanol but 0.13 part per thousand of acetone in femoral blood as well as 0.74 part per thousand in urine. Glucose was negative but the femoral blood had an elevated BHB at >1000 µg/g. The only exogenous compound found was 0.32 ng 5F-MDMB-PICA/g femoral blood. The investigation concluded that the cause of death was ketoacidosis possibly with a contribution from his drug use.

Liquid Chromatography and High Resolution Mass-Spectrometry for Metabolite Identification

An Agilent 1290 infinity ultra-high performance liquid chromatography system coupled with an Agilent 6550 iFunnel quadrupole time-of-flight (QTOF) mass spectrometer equipped with a Dual Agilent Jet Stream electrospray ionization source

(Santa Clara, CA, USA) was used for analysis. Agilent MassHunter software was used for data acquisition and analysis.

Chromatographic separation was achieved using an Acquity HSS T3 column (150 mm x 2.1 mm, 1.8µm) from Waters (Sollentuna, Sweden). Column temperature was maintained at 60 °C. Injection volume was 5 µL. Mobile phases were 0.05% formic acid in 10 mM ammonium formate (mobile phase A) and 0.05% formic acid in acetonitrile (mobile phase B). Gradient elution at 0.5 mL/min was initiated with 99% A and a 0.6 min hold, switched to 80% A at 0.7 minutes, then ramped to 20% A over 13 minutes followed by a 3 minute rinse of high organic (5:95, A:B) and a 2 minute re-equilibration (99:1, A:B) for a total runtime of 19 min.

Auto tandem mass spectrometry (MS/MS) acquisition was performed in positive mode with the following parameters: MS range 100 to 950 *m/z*, threshold of 5000 for precursor selection, drying gas temperature at 150 °C, drying gas flow at 18 L/min, sheath gas temperature at 375 °C, sheath gas flow at 11 L/min, nebulizer at 50 psi, and fragmentor at 380 V. During acquisition, automated calibration was in place.

Metabolite Identification

Data from hepatocyte incubations and urine samples was processed using a Personal Compound Database and Library (PCDL) generated in-house using MassHunter PCDL Manager. Potential metabolites for 5F-MDMB-PICA and 5F-MDMB-PINACA were predicted using the following biotransformations either alone or in combination: phase I transformations (amide hydrolysis, butanoic acid formation at the indole/indazole side chain, carboxylation, dehydrogenation, dihydrodiol formation, dihydroxylation, ester hydrolysis, ketone formation, N-dealkylation, hydroxylation, oxidative defluorination,

oxidative defluorination to pentanoic acid, propionic acid formation at the indole/indazole side chain) and phase II transformations (glucuronidation and sulfation). Criteria for metabolite identification were as follows: mass error of less than ± 5 ppm for protonated molecule, MS/MS spectra showing plausible product ions, retention time feasible for the structures, appropriate peak shape and absence in negative controls.

Receptor Activation

Analysis of receptor activation was carried out on AequoScreen recombinant CHO-K1 cell lines purchased from Perkin Elmer (Groningen, Netherlands) expressing the human CB_1 receptor (ES-110-A) according to the manufacturer specifications. Specifically, cells have been quickly thawed in BSA medium that was previously warmed to 37 °C. Cells were centrifuged at 150 g for 5 min at room temperature and the cells were re-suspended with BSA medium (1 mL) in order to have $3x10^5$ cells/mL in a 20 mL tube. Coelenterazine was added to achieve a final concentration of 2.5 µM in BSA medium. The cells were incubated at room temperature in the dark during rotation for three hours. 5F-MDMB-PICA and 5F-MDMB-PINACA were prepared in 96-well-plates at descending concentrations using serial dilutions. The concentrations were 20,000, 4,000, 800, 160, 32, 6.4, 1.28, 0.256, 0.0512, 0.01024 ng/mL in each well. JWH-018 was also analyzed as a reference agonist for the CB_1 receptor. Digitonin was used as a positive control for the coelenterazine cell loading and blank wells with no drug used as negative controls. The activation at each drug concentration was determined using a Tecan Spark™ 10M (Switzerland) as 50 µL cell suspension was added to the well (15000 cells). The Spark 10M reading protocol was set to 200 luminosity readings and the cells were added to each well at reading cycle #10 (baseline) and luminescence registration

was conducted for ~25 s. Data was fitted to the Hill equation using GraphPad Software, Prism version 8. Statistical differences were investigated using one-way ANOVA.

Results

Metabolic Profile of 5F-MDMB-PICA

From hepatocyte incubations and urine samples, 22 metabolites (A1 to A22) were identified for 5F-MDMB-PICA in this study with mass errors within ±3.27 ppm (**Figure 8.1 1a and 1b**).

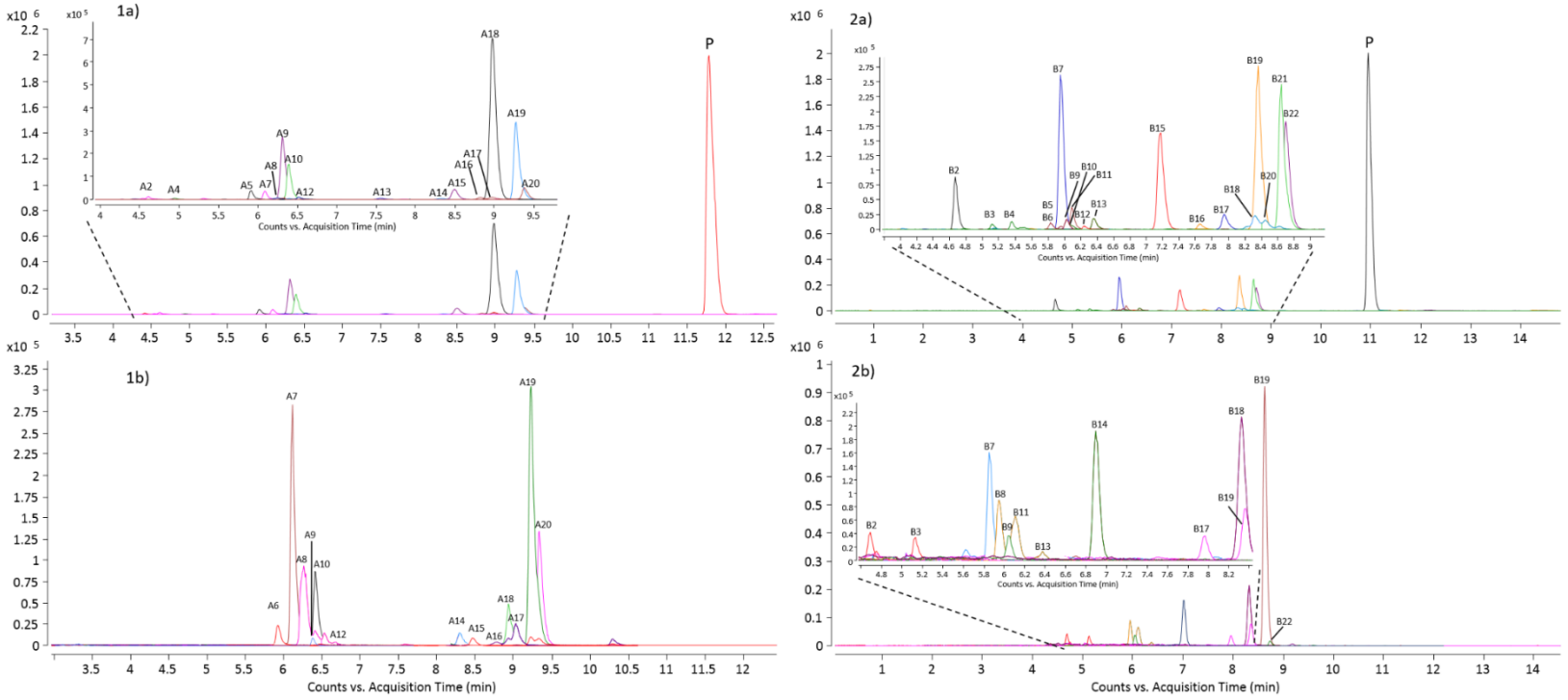

Figure 8.1 Extracted Ion Chromatograms (EIC) of hepatocyte incubations and urine samples yielding the most metabolites: 1a) first hour incubation of 5F-MDMB-PICA with hepatocytes, 1b) metabolites found in antemortem case 4 urine, 2a) first hour incubation of 5F-MDMB-PINACA with hepatocytes, 2b) metabolites identified in case 3 urine

These metabolites are summarized in **Table 8.1** in retention time order. Metabolites were formed via carboxylation (A16) or hydroxylation on the aliphatic chain (A20) or aromatic ring (A18) followed by glucuronidation (A4); N-dealkylation (A15) followed by hydroxylation on the aromatic ring (A3); oxidative defluorination (A19) followed by conversion to pentanoic acid (A17); oxidative defluorination with conversion to propionic acid (A14). Oxidative defluorination pathway also subsequently underwent hydroxylation on the aromatic ring (A5, A9) followed by N-dealkylation (A3) or glucuronidation (A1). The ester hydrolysis (A21) metabolite was further metabolized via hydroxylation (A8, A10, A13), glucuronidation (A12), or dehydrogenation (A22) followed by N-dealkylation (A2), or ester hydrolysis with oxidative defluorination (A11) followed by dehydrogenation (A7), or conversion to pentanoic acid (A6). The proposed metabolic pathway is depicted in **Figure 8.2**.

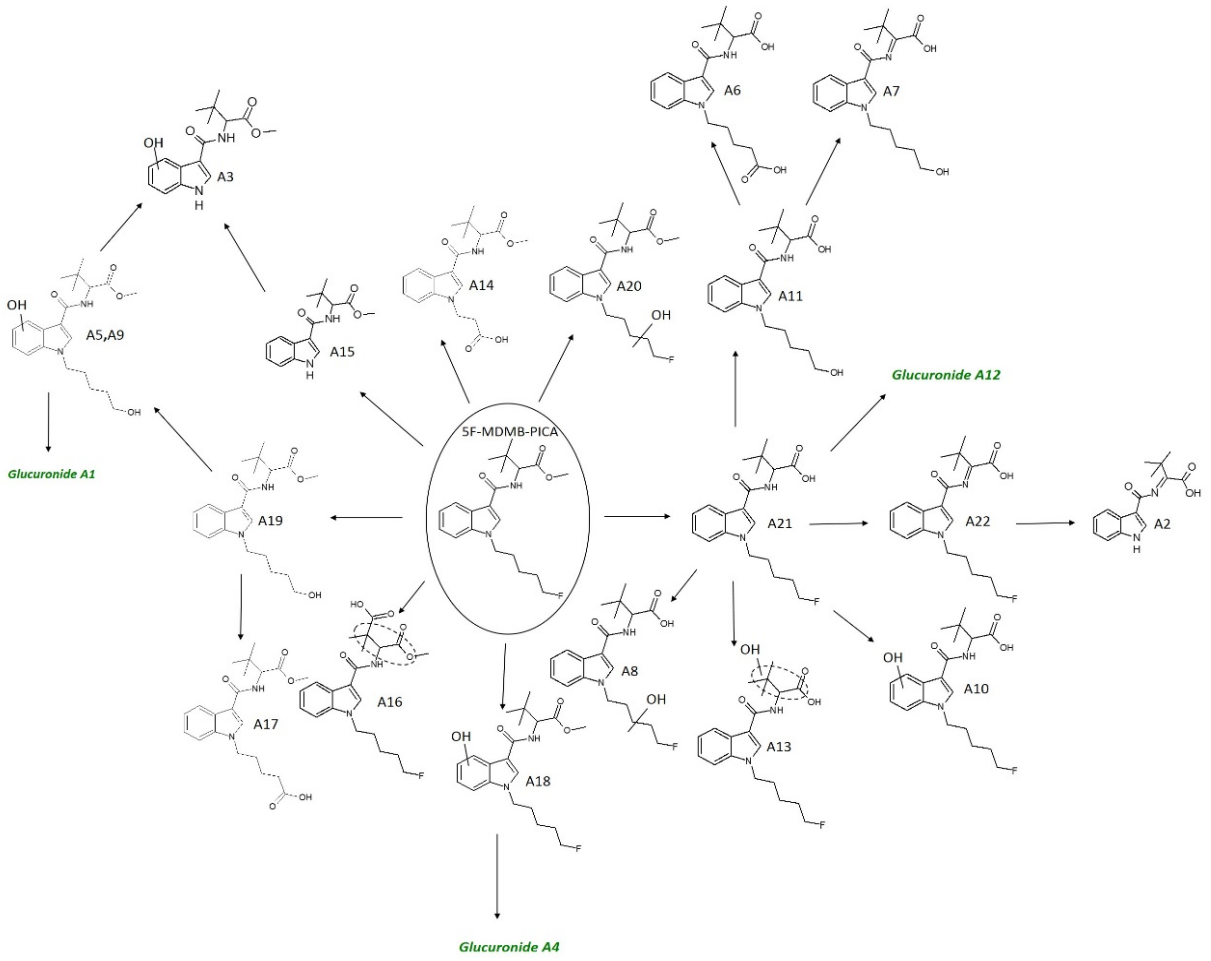

Figure 8.2 Proposed metabolic pathway for 5F-MDMB-PICA

Table 8.1 5F-MDMB-PICA metabolite identification in order of retention time

					Peak Area (Hepatocyte Samples)				Peak Area (Urine Samples)				
Metabolite ID	Biotransformation	Retention Time (min)	Molecular Weight (Formula)	Mass Error (ppm)	1 Hour Rep 1 Rep 2	3 Hour Rep 1 Rep 2	5 Hour Rep 1 Rep 2	Hepatocyte Rank	Case 1 Hydrolyzed Non	Case 2 Hydrolyzed Non	Case 4 Hydrolyzed Non	Characteristic Fragments	Urine Rank
A1	Oxidative defluorination+ hydroxylation on aromatic ring+ glucuronidation	4.06	566.2476 ($C_{27}H_{38}N_2O_{11}$)	-3.07	ND 12166	21085 25779	22977 24950	15	ND ND	ND ND	ND ND	246.1118 160.0380	NA
A2	Ester hydrolysis+ N-dealkylation+ dehydrogenation	4.66	272.1161 ($C_{15}H_{16}N_2O_3$)	1.44	329267 270496	188724 182229	51056 77301	4	ND ND	ND ND	166054 68307	144.0447	NA
A3	N-dealkylation+ hydroxylation on aromatic ring	5.11	304.1423 ($C_{16}H_{20}N_2O_4$)	-3.27	32728 35826	ND ND	ND ND	NA	ND ND	ND ND	136805 ND	160.0394	NA
A4	Hydroxylation on aromatic ring+ glucuronidation	5.35	568.2432 ($C_{27}H_{37}FN_2O_{10}$)	-2.62	63869 33500	79243 79586	67131 98346	8	ND ND	ND ND	ND ND	248.1076 160.0388	NA
A5	Oxidative defluorination+ hydroxylation on aromatic ring	5.82	390.2155 ($C_{21}H_{30}N_2O_5$)	-2.69	42277 30001	ND ND	ND ND	NA	ND ND	ND ND	ND ND	246.1112 160.0397	NA
A6	Ester hydrolysis+ oxidative defluorination to pentanoic acid	5.83	374.1842 ($C_{20}H_{26}N_2O_5$)	-1.28	22031 ND	40523 93826	83386 67635	10	64579 36601	ND ND	610619 352689	244.0965 144.0440	2
A7	Ester hydrolysis+ oxidative defluorination+ dehydrogenation	5.95	358.1893 ($C_{20}H_{26}N_2O_4$)	1.14	1172121 979283	462102 488663	197327 142542	2	ND ND	ND ND	ND ND	230.1170 144.0440	NA
A8	Ester hydrolysis+ hydroxylation on aliphatic chain	5.95	378.1955 ($C_{20}H_{27}FN_2O_4$)	1.59	ND ND	ND ND	ND ND	NA	ND ND	ND ND	356827 132741	248.1073 144.0438	NA
A9	Oxidative defluorination+ hydroxylation on aromatic ring	6.02	390.2155 ($C_{21}H_{30}N_2O_5$)	-2.52	67389 65233	ND 6074	ND ND	16	ND ND	ND ND	200209 ND	246.1113 160.0398	NA
A10	Ester hydrolysis+ hydroxylation on aromatic ring	6.06	378.1955 ($C_{20}H_{27}FN_2O_4$)	-2.74	42571 37835	44148 58706	39946 36634	12	ND ND	ND ND	354931 160387	248.1072 160.0389	NA
A11	Ester hydrolysis+ oxidative defluorination	6.08	360.2049 ($C_{20}H_{28}N_2O_4$)	-0.12	137432 178387	177364 310742	319107 224584	3	ND ND	ND ND	ND ND	230.1170 144.0438	NA
A12	Ester hydrolysis+ glucuronidation	6.23	538.2327 ($C_{26}H_{35}FN_2O_9$)	-2.36	22909 40467	51126 96821	50924 69951	9	ND ND	ND ND	ND 722762	232.1126 144.0422	NA
A13	Ester hydrolysis+ hydroxylation at t-butyl	6.34	378.1955 ($C_{20}H_{27}FN_2O_4$)	-1.32	84663 87274	90405 133794	65296 102267	6	ND ND	ND ND	59106 32601	232.1121 144.0435	NA
A14	Propionic acid	6.90	360.1685 ($C_{19}H_{24}N_2O_5$)	1.47	ND ND	ND ND	ND ND	NA	ND ND	ND ND	889309 593451	216.0656 144.0443	NA
A15	N-dealkylation	7.17	288.1474 ($C_{16}H_{20}N_2O_3$)	1.02	926331 1040733	ND 25064	ND 6784	14	ND ND	ND ND	ND ND	144.0443	NA
A16	Carboxylation at t-butyl	7.66	406.1904 ($C_{21}H_{27}FN_2O_5$)	-2.47	44054 45585	25726 32371	9325	13	ND ND	ND ND	ND ND	232.1114 144.0442	NA
A17	Oxidative defluorination to pentanoic acid	7.94	388.1998 ($C_{21}H_{28}N_2O_5$)	-0.86	133783 142291	148533 180157	146763 144844	5	ND ND	ND ND	183439 58011	244.0959 144.0441	NA

					Peak Area (Hepatocyte Samples)				Peak Area (Urine Samples)				
Metabolite ID	Biotransformation	Retention Time (min)	Molecular Weight (Formula)	Mass Error (ppm)	1 Hour Rep 1 Rep 2	3 Hour Rep 1 Rep 2	5 Hour Rep 1 Rep 2	Hepatocyte Rank	Case 1 Hydrolyzed Non	Case 2 Hydrolyzed Non	Case 4 Hydrolyzed Non	Characteristic Fragments	Urine Rank
A18	Hydroxylation on aromatic ring	8.32	392.2111 ($C_{21}H_{29}FN_2O_4$)	-1.97	155367 28698	ND ND	ND ND	NA	ND ND	ND ND	1179888 36683	248.1076 160.0387	NA
A19	Oxidative defluorination	8.36	374.2206 ($C_{21}H_{30}N_2O_4$)	0.59	1478954 1734475	75913 94696	136918 32006	7	ND ND	ND ND	448349 ND	230.1167 144.0433	NA
A20	Hydroxylation on aliphatic chain	8.44	392.2111 ($C_{21}H_{29}FN_2O_4$)	-0.99	116739 ND	ND ND	ND ND	NA	ND ND	ND ND	ND ND	248.1077 144.0445	NA
A21	Ester hydrolysis	8.64	362.2006 ($C_{20}H_{27}FN_2O_3$)	0.59	1305438 1359268	1186575 1460964	974983 1044946	1	30647 21306	37661 ND	4658623 426210	232.1133 144.0442	1
A22	Ester hydrolysis+ dehydrogenation	8.70	360.1849 ($C_{20}H_{25}FN_2O_3$)	2.06	1032988 1184191	46628 67374	ND 17238	11	ND ND	ND ND	71015 ND	232.1117 144.0444	NA
P	5F-MDMB-PICA	10.94	376.2162 ($C_{21}H_{29}FN_2O_3$)	0.58	13050788 16050804	1872788 1776511	535212 773432		ND ND	ND ND	ND ND	232.1134 144.0445	NA

The two most abundant metabolites after the 5 h incubation with hepatocytes were ester hydrolysis (A21) and ester hydrolysis with oxidative defluorination (A11). The Case 4 antemortem urine had the most metabolites observed (A2, A3, A6, A8-10, A12-A14, A17-A19, and A21-A22), while case 1 had a couple metabolites (A6 and A21) and case 2 only had a single metabolite identified (A21). The ester hydrolysis product was the only metabolite present in all incubation time points and authentic urine specimens. 5F-MDMB-PICA was not detected in any of the urine specimens, but was dominant in all hepatocyte incubations. The mass spectra of 5F-MDMB-PICA and the 9 most abundant metabolites present in urine from Case 4 are shown in Figure S3 in the Supporting Information. 5F-MDMB-PINACA tentatively identified metabolites (Table S1), proposed metabolic pathway (Figure S1), and mass spectra from metabolites found in authentic (Figure S3) are provided in the Supporting Information.

Receptor Activation

Dose response curves for the full concentration range used are shown in **Figure 8.3** after normalization to the digitonin response. Both 5F-MDMB-PICA and 5F-MDMB-PINACA are full agonists reaching 83% and 88% of the digitonin signal, respectively. Compared to JWH-018 they were significantly more effective in activating the CB_1 receptor, reaching an efficacy of 129 % (5F-MDMB-PICA, $p<0.05$) and 136% (5F-MDMB-PINACA, $p=0.02$) of JWH-018.

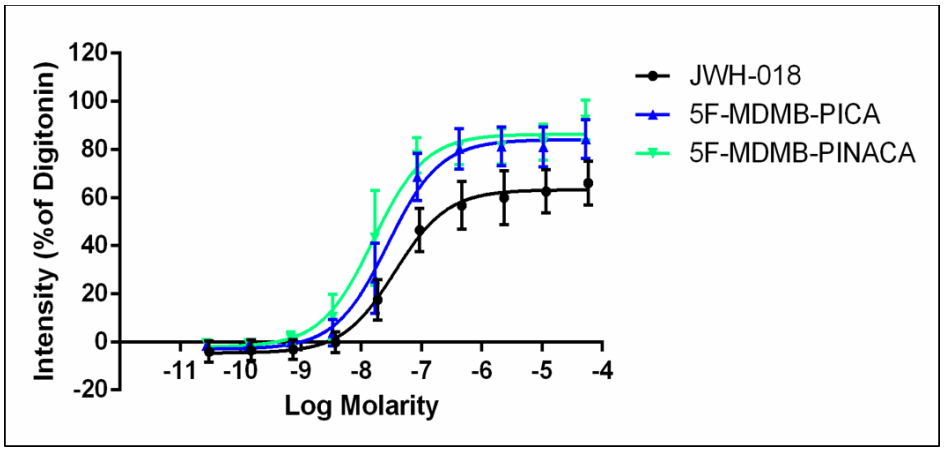

Figure 8.3 Receptor curves normalized to digitonin for JWH-018, 5F-MDMB-PICA, and 5F-MDMB-PINACA, error bars signify standard deviation

In **Table 8.2**, the effective concentration values (EC) expressed both in ng/ml and M including the 95% confidence intervals are shown for JWH-018, 5F-MDMB-PICA, and 5F-MDMB-PINACA. There was no significant difference in comparison of EC50 values of 5F-MDMB-PICA, 5F-MDMB-PINACA and JWH-018 using one-way ANOVA, p=0.05.

Table 8.2 Effective concentrations for synthetic cannabinoids

Compound	EC_{50}		95% Confidence Intervals	
	Molarity (nM)	ng/mL	Molarity (nM)	ng/mL
JWH-018	36.6	12.48	27.2 to 49.1	9.29 to 16.77
5F-MDMB-PINACA	15.7	5.94	12.0 to 21.6	4.53 to 8.15
5F-MDMB-PICA	27.6	10.37	21.7 to 35.0	8.17 to 13.18

Discussion

As seen in **Figure 8.3**, both compounds have comparable potency to JWH-018, but both have slightly higher efficacy. The EC_{50} for 5F-MDMB-PINACA and 5F-MDMB-PICA are comparable to each other which was also reported by Banister *et al*

(9). However, our EC_{50} values are around 60x higher than those reported by Banister *et al (9)* and *Noble et al* report an EC50 value for 5F-MDMB-PICA 8x lower than in the present study (8). Noble *et al* also report 5F-MDMB-PICA to be 13x more potent than JWH-018 while our results indicate similar potency. In our study, 5F-MDMB-PICA was also found to have an E_{max} 30 % higher than that of JWH-018. This result differ from what has been reported by Noble *et al*, with an E_{max} 3 times higher than that of JWH-018. The differences between our results and those reported by Noble *et al* could be attributed to the different techniques and intracellular pathways used to determine receptor activity between this study and Noble *et al,* which monitored the interaction between βarr2 and CB_1 and CB_2 receptors (8). This study is aequorin-luminescence based on Ca^{2+} flux. This method is based on the activation of the calcium sensitive apoaequorin/aequorin system where coelenterazine is converted by the aequorin upon activation. After the G-protein activation, the second messenger phospholipase C is activated, with succeeding diacylglycerol and inositoltriphosphate production and intracellular calcium ions release (12). The calcium release activates the apoaequorin/aequorin system and with coelenterazine as substrate luminescence is produced. That said, substantial differences were also seen when comparing our results to those of Banister *et al* even though they also used an assay based on Ca^{2+} flux (FLIPR^{TM}). Taken together, our results as well as those reported by Banister *et al (9)* and Noble *et al* (8) illustrate that efficacy and potency estimates are method specific and highlight the need to use several different assays to describe the potency of a CB_1 agonist.

Although the cause of death in both Case 1 and 2 was attributed to diabetic ketoacidosis, the presence of synthetic cannabinoids cannot be excluded as a contributing

or underlying factor. Hess *et al* presented a fatality that involved several synthetic cannabinoids where cause of the death was also assumed to be from diabetic ketoacidosis(13). The decedent in their study and from the present study (Case 1) had a history of diabetes. However, the decedent in Case 2 had no history of diabetes which is similar to what was reported by Demirci *et al* (14). 5F-MDMB-PICA is a full agonist to the CB_1 receptor and may produce severe adverse side effects such as hyperglycemia(13). In cases of ketoacidosis with or without history of diabetes, comprehensive toxicology screens may be valuable in determining root cause.

For both compounds, ester hydrolysis and ester hydrolysis in combination with oxidative defluorination were the most prevalent metabolites produced in vitro. At least one of these biotransformations were present in each of the case samples presented.

Authentic Urines

In the postmortem urine samples, a total of 2 metabolites were identified (A6 and A21). The ester hydrolysis (A21) was identified in the case 2 sample following hydrolysis, indicating cleavage of the glucuronide. In the antemortem urine sample, 14 metabolites (A2, A3, A6, A8-A10, A12-A14, A17-A19, and A21-A22) were found, with 2 metabolites (A8 and A14) not produced *in vitro*. Variation in the numbers of metabolites in the 3 different case samples is difficult to interpret as time of administration is unknown but the low number of metabolites in the post mortem samples suggest administration shortly prior to death. Evidence of phase II hydrolytic cleavage was evident in most metabolites, especially with metabolites A3, A9, A19, and A22 as they were identified only after hydrolysis. In all 3 cases, ester hydrolysis was the most abundant metabolite, which is consistent with the study by Mogler *et al (10)*. Further, the

metabolites produced in these *in vitro* and *in vivo* samples aligned with the 12 identified by Mogler *et al* (10), with the exception that the amide hydrolysis was not detected in the present study . However, A6 was not identified by Mogler *et al (10)*. This metabolite was present in two of the three urine cases presented, with being the most abundant metabolite found in Case 1. The corresponding metabolite for 5F-MDMB-PINACA (B7), was found in all incubations of the present study and was the second most abundant metabolite found in Case 3. Yeter *et al* ranked this metabolite second most abundant metabolite found in both hydrolyzed and non-hydrolyzed authentic urine samples (n=30) (16). Based on the structural similarity to 5F-MDMB-PINACA and urine findings of the present study, A6 is likely to be a major metabolite in urine for 5F-MDMB-PICA. To the author's knowledge, this is the first report identifying A6 as a suitable marker for screening.

The ranking for hepatocyte incubations and urine samples share the same metabolite for the top ranking (A21). Although A6 is not in the top 3–5 metabolites in hepatocyte incubations, due to its abundance in two of the three urine specimens and previous data about the corresponding metabolite for 5F-MDMB-PINACA, it is given the second top ranking for urine samples.

Conclusion

Based on the aequorin-luminescence method used for this study, the EC_{50} at the CB1 receptor for 5F-MDMB-PICA and 5F-MDMB-PINACA were found to be comparable to each other and JWH-018. The EC_{50}-value of 5F-MDMB-PICA was found to be 27.6 nM, 8 to 60 times lower than indicated by other assays, indicating that 5F-MDMB-PICA might be less potent than previously believed. For both compounds, ester

hydrolysis and ester hydrolysis in combination with oxidative defluorination were the most abundant metabolites produced *in vitro*. Additionally, we described the conversion of ester hydrolysis with oxidative defluorination to pentanoic acid (A6) for the first time for 5F-MDMB-PICA. At least one of these biotransformations were present in each of the case samples presented and are in agreement with previous literature. Therefore, these metabolites would be potentially good biomarkers for screening urine of suspected intoxication of 5F-MDMB-PICA or 5F-MDMB-PINACA. However, these metabolites are not specific to the consumption of 5F-MDMB-PICA or 5F-MDMB-PINACA, i.e., they can be formed from drugs of similar structures. Therefore, based on the hepatocyte incubation abundance, ester hydrolysis (A21), and ester hydrolysis with oxidative defluorination to pentanoic acid (A6), based on authentic urine data, are recommended to be monitored for the purpose of screening/confirmation. As parent drug was not present in the urine samples analyzed, metabolites may be of utmost importance when determining drug intake.

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CHAPTER IX

Conclusions

The age of NPS has no immediate end in sight. The only way to overcome the emergence of NPS is by identifying obstacles that can help alleviate the difficulties that arise when analyzing samples that possibly contain NPS. Prevalence plays key role in the emergence of NPS as the illicit drug community and drug market are constantly changing which new high is sought after. Although the oral fluid method that was developed to help determine prevalence of NSO in certain communities (such as prison detainees) did not detect such compounds in a limited sample size, valuable information was obtained during these collections. The evaluator opinion of drug class was confirmed in oral fluid 90% of time and in urine 85% of the time in reference to scope of testing. Data indicate that oral fluid may be a viable source for confirming driving under the influence of drugs (DUID). This information is important to the forensic community as some states, such as Alabama, have started to incorporate oral fluid as a matrix of interest within their analytical testing.

Knowledge of drug stability is necessary when dealing with compounds such as NPS. Toxicologists need to know if compounds are not detected because they are truly absent from the sample or if the compounds have degraded. Through the 36-week study that was conducted, the stability of seven NSO was determined. Even at elevated temperatures, all analytes were deemed stable (within 20% of target concentration) for at least two weeks at low concentrations. For blood samples that may contain these NSO, it is still recommended that specimens be stored refrigerated or frozen, when possible, in

order to preserve analyte stability. Additionally, samples should be analyzed within a month in order to determine accurate concentration of the synthetic opioids.

Understanding the pharmacology of a NPS is essential to properly interpret impairment of individuals under the influence of a possible NPS. It was determined that doses of U-47700 in an animal model had a positive correlation with observed behaviors, further demonstrating the analgesic effects of this novel synthetic opioid. Knowing the possible pharmacological effects of U-47700 as well as the pharmacokinetic profiles of the drug and its metabolites will allow forensic toxicologists to better interpret toxicological data for possible intoxications.

Metabolism studies are essential for determining NPS use, especially when synthetic cannabinoids are suspected as parent drug may no longer be detectable. Using human liver microsomes, there were 22 metabolites identified for 5F-MDMB-PICA and 21 metabolites identified for 5F-MDMB-PINACA. For both compounds, ester hydrolysis and ester hydrolysis in combination with oxidative defluorination were the most abundant metabolites *in vitro* and *in vivo*. In the authentic urine samples, parent compounds were not identified, further demonstrating the need for metabolic studies to identify potential biomarkers. Without identification of metabolites, synthetic cannabinoids could go undetected in toxicological examinations.

Using any of the previously described approaches (evaluating prevalence, determining instability, characterizing pharmacokinetics, or identifying potential biomarkers) will allow the forensic community to develop useful processes for ensuring proper detection and interpretation of emerging synthetic compounds in toxicological investigations.

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APPENDIX

Supplemental Information for Chapter VIII

S1. Materials and Methods

S1.1 Case 3

A 51 year old male was found unconscious in his apartment. After cardiac arrest resuscitation was unsuccessful and he was pronounced dead at the arrival to hospital. In the apartment there were signs of drug use as well as suspected drugs. The post-mortem examination revealed a fatty liver, arteriosclerosis, enlarged heart and pulmonary embolism. The toxicological analysis showed a number of medications including mirtazapine, clomipramine, and diazepam, and new psychoactive substances including dibutylone, diclazepam, N-ethyl-4-methylnorpentedrone, norfludiazepam, and 5F-MDMB-PINACA. The cause of death was attributed to pulmonary embolism with a possible contribution from drug use.

S2. Results

S2.1 Metabolic Profile of 5F-MDMB-PINACA

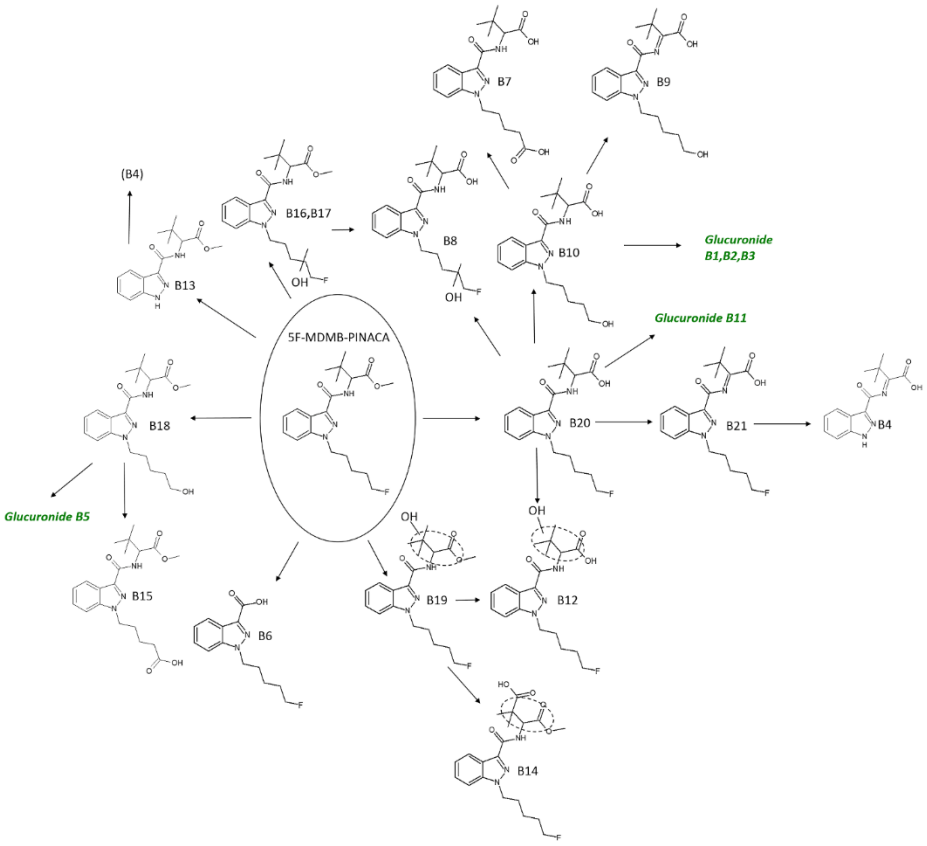
A total of 21 5F-MDMB-PINACA metabolites (B1 to B21) were identified with low mass error (within ±3.33 ppm), with 3 compounds unique to urine specimens. Extracted ion chromatograms of the hepatocyte incubation yielding the most metabolites (**2a**) and case 3 hydrolyzed urine (**2b**) are shown in **Figure 2**. Metabolites (in retention time order) and rankings are shown in **Supplemental Table 1**. The rank was determined by the 3 hr incubation abundance of the metabolites and occurrence of the metabolite in the authentic sample.

Supplemental Table 1. 5F-MDMB-PINACA metabolite identification in order of retention time

					Peak Area (Hepatocyte Samples)				Peak Area (Urine Sample)		
Metabolite ID	Biotransformation	Retention Time (min)	*Molecular Weight* (Formula)	Mass Error (ppm)	1 Hour Rep 1 Rep 2	3 Hour Rep 1 Rep 2	5 Hour Rep 1 Rep 2	Hepatocyte Rank	Case 3 Hydrolyzed Non-hydrolyzed	Characteristic Fragments	Rank
B1	Ester hydrolysis+ oxidative defluorination+ glucuronidation	4.53	537.2322 ($C_{25}H_{35}N_3O_{10}$)	-2.38	ND ND	31567 43405	64894 84203	10	ND ND	231.112 213.1020 145.0937	NA
B2	Ester hydrolysis+ oxidative defluorination+ glucuronidation	4.62	537.2322 ($C_{25}H_{35}N_3O_{10}$)	-2.60	68758 88271	46811 54655	ND ND	8	ND ND	231.113, 213.1016 145.0389	NA
B3	Ester hydrolysis+ oxidative defluorination+ glucuronidation	4.74	537.2322 ($C_{25}H_{35}N_3O_{10}$)	-3.19	ND ND	34600 38837	64954 88127	11	ND ND	231.1114 213.1003 145.0371	NA
B4	Ester hydrolysis+ N-dealkylation+ dehydrogenation	4.96	273.1113 ($C_{14}H_{15}N_3O_3$)	-3.00	24900 21898	ND ND	ND ND	NA	ND ND	145.0399	NA
B5	Oxidative defluorination+ glucuronidation	5.92	551.2479 ($C_{26}H_{37}N_3O_{10}$)	-3.04	152441 184443	72994 78467	23604 30095	5	ND ND	231.1113 213.1020	NA
B6	Amide hydrolysis	5.93	250.1118 ($C_{13}H_{15}FN_2O_2$)	-1.83	ND ND	ND ND	ND ND	NA	112570 109160	233.1049 145.0385	9
B7	Ester hydrolysis+ oxidative defluorination to pentanoic acid	6.09	375.1794 ($C_{19}H_{25}N_3O_5$)	1.65	142042 181838	209514 268761	291719 554731	3	1186331 1011040	245.0892 217.0966 145.0388	1
B8	Ester hydrolysis+ hydroxylation on aliphatic chain	6.26	379.1907 ($C_{19}H_{26}FN_3O_4$)	-3.00	32200 46325	45338 54851	44543 70631	9	684660 226170	249.1027 231.0909 145.0396	3
B9	Ester hydrolysis+ oxidative defluorination+ dehydrogenation	6.32	359.1845 ($C_{19}H_{25}N_3O_4$)	-1.85	1272919 1246396	211707 233902	41376 40983	4	ND ND	231.1157 213.1010 145.0385	NA
B10	Ester hydrolysis+ oxidative defluorination	6.39	361.2002 ($C_{19}H_{27}N_3O_4$)	0.86	680485 823487	961704 1131647	986929 1335568	2	401782 38025	231.1117 213.1008 145.0393	6
B11	Ester hydrolysis+ glucuronidation	6.45	539.2279 ($C_{25}H_{34}FN_3O_9$)	-0.23	ND ND	ND ND	ND ND	NA	ND 112608	233.1073 145.0396	8
B12	Ester hydrolysis+ hydroxylation at t-butyl	6.53	379.1907 ($C_{19}H_{26}FN_3O_4$)	-3.33	42810 52441	64079 63696	44590 55193	7	83273 61329	233.1083 213.1005 145.0386	10
B13	N-dealkylation	7.56	289.1426 ($C_{15}H_{19}N_3O_3$)	-2.15	39096 39401	ND ND	ND ND	NA	ND ND	230.1288 145.0393	NA
B14	Carboxylation at t-butyl	8.32	407.1857 ($C_{20}H_{26}FN_3O_5$)	-2.11	23783 27213	ND ND	ND ND	NA	81036 22575	233.1075 213.1001 145.0382	11
B15	Oxidative defluorination to pentanoic acid	8.51	389.1951 ($C_{20}H_{27}N_3O_5$)	-2.75	257333 289164	67809 76152	26373 32773	6	54061 40915	245.0920 217.0962 145.0378	12
B16	Hydroxylation at aliphatic chain	8.85	393.2064 ($C_{20}H_{28}FN_3O_4$)	-2.03	41395 53637	ND ND	ND ND	NA	ND ND	249.1025 177.0460 145.0392	NA
B17	Hydroxylation at aliphatic chain	8.98	393.2064 ($C_{20}H_{28}FN_3O_4$)	-1.83	59651 78002	ND ND	ND ND	NA	ND ND	249.1030 231.0916 145.0396	NA
B18	Oxidative defluorination	9.00	375.2158 ($C_{20}H_{29}N_3O_4$)	-2.80	4152647 4690259	22331 26323	ND ND	12	239969 ND	231.1082 213.1015 145.0392	5
B19	Hydroxylation at t-butyl	9.03	393.2064 ($C_{20}H_{28}FN_3O_4$)	0.21	ND ND	ND ND	ND ND	NA	187358 ND	233.1078 177.0444, 145.0390	7
B20	Ester hydrolysis	9.31	363.1958 ($C_{19}H_{26}FN_3O_3$)	1.03	1765730 1763407	1206366 1251346	787034 975701	1	1684045 222137	233.1082 213.1013 145.0392	2

					Peak Area (Hepatocyte Samples)				Peak Area (Urine Sample)		
Metabolite ID	Biotransformation	Retention Time (min)	*Molecular Weight* (Formula)	Mass Error (ppm)	1 Hour Rep 1 Rep 2	3 Hour Rep 1 Rep 2	5 Hour Rep 1 Rep 2	Hepatocyte Rank	Case 3 Hydrolyzed Non-hydrolyzed	Characteristic Fragments	Rank
B21	Ester hydrolysis+ dehydrogenation	9.41	361.1802 ($C_{19}H_{24}FN_3O_3$)	0.13	276539 316708	ND ND	ND ND	NA	704598 22462	233.1083 213.1018 145.0393	4
P	5F-MDMB-PINACA	11.83	377.2115 ($C_{20}H_{28}FN_3O_3$)	1.94	13514687 12671740	16215063 4629113	943624 1691085	NA	ND ND	233.1088 213.1025 145.0397	NA

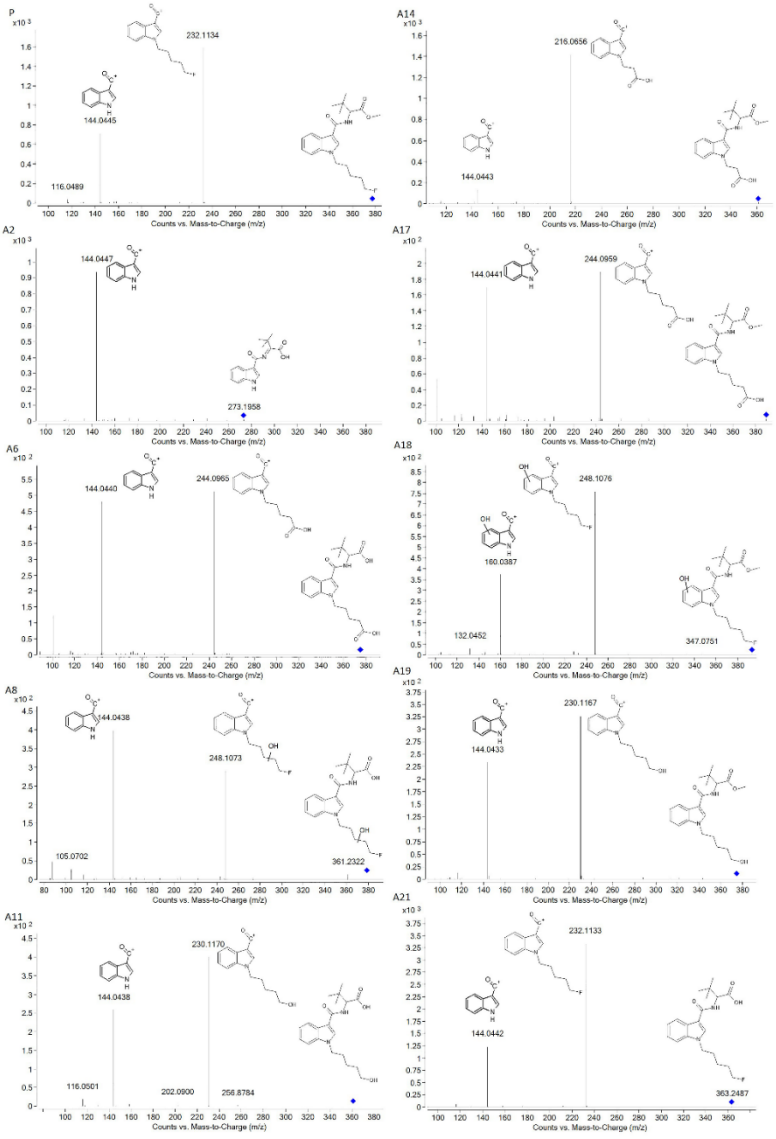
The biotransformations that were observed were similar to those of 5F-MDMB-PICA and the proposed metabolic pathway is depicted in **Supplemental Figure 1**.



Supplemental Figure 1. Proposed metabolic pathway for 5F-MDMB-PINACA

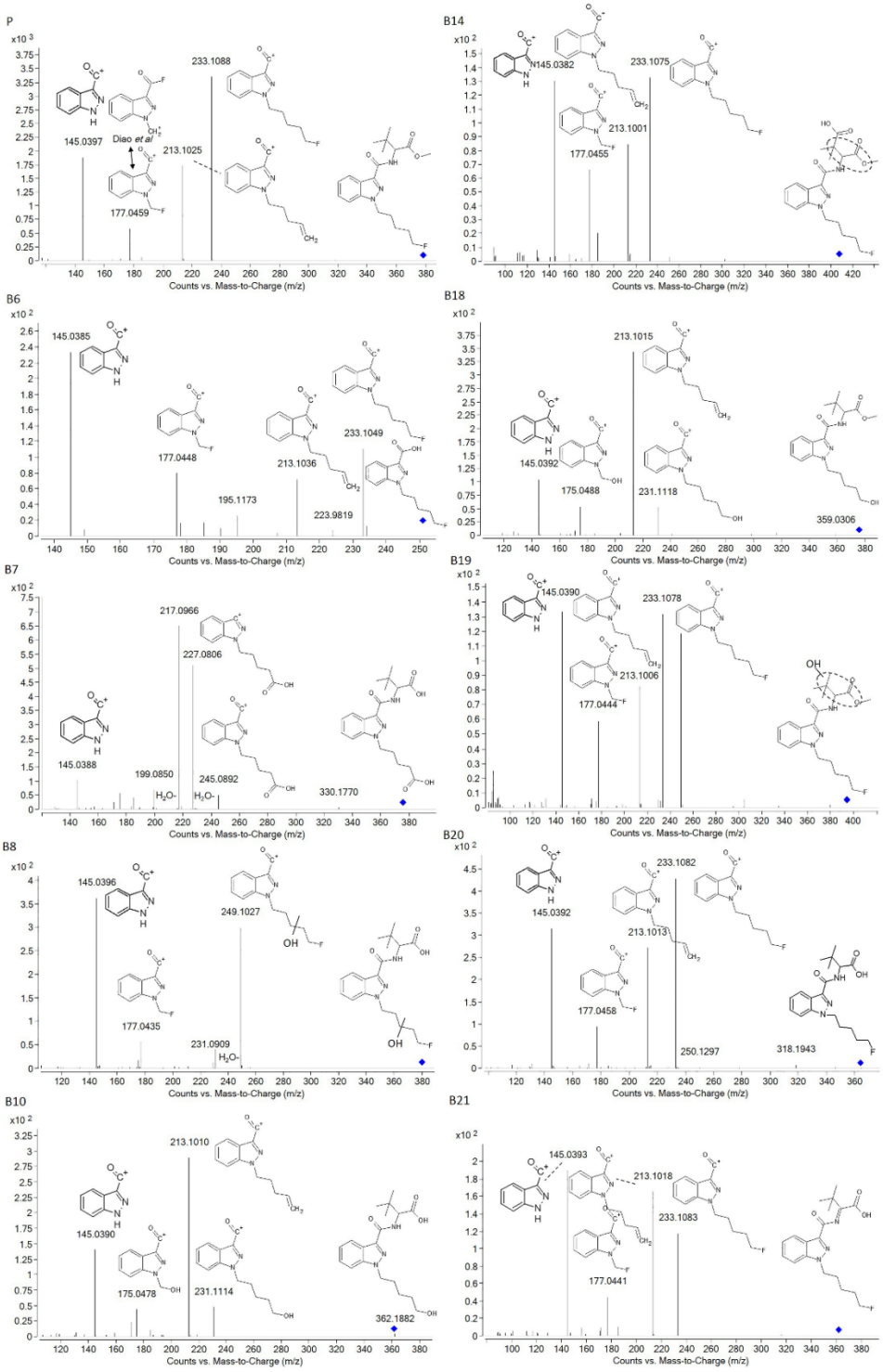
S2.2 Mass fragmentation of 5F-MDMB-PICA and 5F-MDMB-PINACA

The two main fragments used to characterize 5F-MDMB-PICA were *m/z* 232.1132 (containing the indole with the fluoropentyl side chain) and 144.0444 (the indole group) (**Supplemental Figure 2**). The mass spectra of 5F-MDMB-PICA and 9 most abundant metabolites identified in case 4 are shown in **Supplemental Figure 2**.



Supplemental Figure 2. Mass spectra of 5F-MDMB-PICA and 9 most abundant metabolites found in case 4 urine

The following were used as characteristic fragments of the 5F-MDMB-PINACA: *m/z* 233.1085 (containing the indazole and fluoropentyl side chain), 213.1022 (the indazole and side chain without the fluorine), 177.0459 (containing the indazole group with fluoromethyl group) and 145.0396 (containing the indazole group) (**Supplemental Figure 3**). There are two structures shown for the fragment 177.0459 in Supplemental Figure 2. One was proposed by Diao *et al* when characterizing the metabolism of THJ-2201 (a fluorinated analogue of THJ-018) [11] and the other is the proposed structure containing the fluoromethyl group from this study . Changes to these fragments helped identify where biotransformations took place. The mass spectra of 5F-MDMB-PINACA and the 9 most abundant metabolites found in the urine sample from case 3 are shown in **Supplemental Figure 3**.



Supplemental Figure 3. Mass spectra of 5F-MDMB-PINACA and 9 most abundant metabolites found in case 3 urine

S3. Discussion

S3.1 5F-MDMB-PICA Hydroxylations

5F-MDMB-PICA produced many hydroxylated metabolites. As mentioned before, fragments *m/z* 232 (containing the indole group with fluoropentyl chain) and 144 (containing the indole group) were used to characterize 5F-MDMB-PICA. Modifications of +15.995 Da to either of these fragments were indicative of hydroxylation. The metabolite A20 had hydroxylation on the fluoropentyl side chain due to the conservation of *m/z* 144 and shift of *m/z* 248. A18 produced shifts of both characteristic fragments to *m/z* 248 and 160, concluding that the hydroxylation occurred on the aromatic ring. These fragments were present in A4 as well and a shift of +176.0309 Da in the *m/z* of the protonated molecule due to glucuronidation. A10 hydroxylation on the indole ring also had fragments *m/z* 248 and 160 present, but the ester hydrolysis shifted the molecular mass by -14.016 Da. A8 was produced by hydroxylation and ester hydrolysis as well. While the *m/z* 144 fragment was conserved, a shift of +15.995 Da on the *m/z* 232 fragment to m/z 248 indicated hydroxylation on the aliphatic side chain. A13 was another metabolite produced by the combination of ester hydrolysis and hydroxylation, but presence of both m/z 232 and 144 indicated that hydroxylation occurred on the *tert*-leucinate group. The fragment *m/z* 160 was used to characterize A3 due to *N*-dealkylation occurring with the hydroxylation. With the presence of the *m/z* 160, hydroxylation was on the indole group.

S3.2 Oxidative Defluorination and Carboxylation of 5F-MDMB-PICA

Oxidative defluorination is characterized by the shift -1.996 Da with the fragment *m/z* 230. A19 contains fragments *m/z* 230 and 144 which are indicative of oxidative

defluorination. A11 also possesses these fragments, but shifts -14.016 Da due to additional ester hydrolysis. Another shift of -2.016 Da, A7 contains dehydrogenation with oxidative defluorination and ester hydrolysis. A5 and A9 possessed fragments *m/z* 246 (indicating both oxidative defluorination and a hydroxylation) and 160. With a shift on both fragments of +15.995 Da, the hydroxylation occurred on the aromatic ring of the indole. The same hydroxylation was present in A1, but in combination with glucuronidation with a shift +176.0309 Da.

The hydroxyl formed by oxidative defluorination is further converted into a pentanoic acid and shifts +13.980 Da. This is characterized with fragment *m/z* 244 as produced by A6 and A17. Another carboxylation observed was a conversion to propionic acid, A14. This metabolite was determined using the characteristic fragments *m/z* 216 (containing the indole with propionic acid) and 144. This metabolite was also tentatively identified in Mogler *et al* [10]. A16 retained both *m/z* 232 and 144 which would indicate carboxylation occurring at the t-butyl group. Although there was not an intermediate metabolite with hydroxylation observed, there was a similar biotransformation found with 5F-MDMB-PINACA (B19).

S3.3 Ester Hydrolysis and Other Biotransformations

Ester hydrolysis appeared in 10 of the 22 metabolites tentatively identified. Shifts to the molecular mass were used to identify this biotransformation. A21 contained fragments *m/z* 232 and 144 with a shift of -14.016 Da to the molecular mass indicative of ester hydrolysis. An additional shift of -2.016 Da to the molecular mass of A22 was indicative of ester hydrolysis in combination with dehydrogenation. N-dealkylation had the characteristic fragment *m/z* 144 which was produced by both A2 and A15. A12

contained fragments *m/z* 232 and 144 with an exact mass of 566.2476 due to glucuronidation.

S3.4 Hydroxylations of 5F-MDMB-PINACA

B16, B17, and B19 were formed as a result of 5F-MDMB-PINACA hydroxylation. For B16 and B17, the fragments *m/z* 145 and 177, which contain the indazole group, were conserved, while the other fragments had an addition of +15.995 Da, indicating that the hydroxylation was on the pentyl chain. For B19, the fragments *m/z* 233 and 213 containing the intact fluoropentyl indazole group was observed, suggesting the hydroxylation on the *tert*-leucinate group.

S3.5 5F-MDMB-PINACA Oxidative Defluorination

The fragments *m/z* 231 indicating hydroxypentyl side chain and the *m/z* 213 indicating an indazole with the side chain without the fluorine were used as characteristic fragments for the identification of B9, B10, and B18 as metabolites with oxidative defluorination. Other biotransformations found in association with oxidative defluorination were conversion to pentanoic acid (B7, B15) and glucuronidation (B1-B3, B5). The fragments that were indicative of conversion to pentanoic acid were *m/z* 245, 227, and 217. B5 conserved the same fragments used to characterize B18 (231, 213, 175, and 145), but had a *m/z* of 553 which indicates that glucuronidation had occurred. The same can be applied to B1-B3, but these metabolites had a protonated molecule mass of 538.2389 due to the ester hydrolysis.

S3.6 Hydrolytic Biotransformations and N-dealkylation of 5F-MDMB-PINACA

Amide hydrolysis (B6) at m/z 251 and ester hydrolysis (B20) at m/z 364 shared the same characteristic fragments *m/z* 233, 213, 177, and 145 which represent

conservation of the indazole and fluoropentyl chain. Ester hydrolysis in combination with dehydrogenation (B21) or glucuronidation (B11) also share these fragments, but are differentiated with their m/z at 362 and 540. *N*-dealkylation (B13) had two fragments used to indicate its presence (230 and 145). Ester hydrolysis with *N*-dealkylation and dehydrogenation (B4) was characterized only by *m/z* 145.

S3.7 Authentic Urine

There were 12 metabolites (B6-B8, B10-B12, B14, B15, B18-B21) tentatively identified in the Case 3 sample and of those, 3 metabolites (B6, B11, and B19) were not produced *in vitro*. B18 and B19 were found in the hydrolyzed sample and not in the non-hydrolyzed sample, indicating that hydrolysis of the phase II conjugation had occurred. The glucuronidated product of B18 (B5) was not detected *in vivo*. Further, no conjugated metabolites of B19 were observed *in vitro* or *in vivo*, probably due to low abundance in the urine. The most abundant metabolite found in this sample was ester hydrolysis (B20), which was already previously identified in literature [15-17]. Of the 12 metabolites, 2 metabolites (B6 and B14) were not identified in another study.

S4. References

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9. Funayama M, Usui K, Fujita Y, Kamijo Y, Kokaji T. Identification of 5-Fluoro ADB in Human Whole Blood in Four Death Cases. *J Anal Toxicol.* 2017;42(2):e21-e25.

10. Yeter O, Ozturk YE. Metabolic profiling of synthetic cannabinoid 5F-ADB by human liver microsome incubations and urine samples using high-resolution mass spectrometry. *Drug Test Anal.* 2019;0(0).

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IRB Consent forms

Sam Houston State University

A Member of The Texas State University System

COLLEGE OF CRIMINAL JUSTICE

DEPARTMENT OF FORENSIC SCIENCE

Quantification of Novel Psychoactive Substances in Oral Fluid Survey

In providing a saliva (oral fluid) sample, you are indicating that you are an adult over the age of 18 years who is not currently using opiates/opioids for medicinal or recreational purposes, including but not limited to morphine, codeine, heroin, AH-7921, MT-45, U-47700, and W-18.

Please check or fill in the appropriate response.

Have you brushed your teeth, chewed gum, eaten, drank, or smoked in the last 30 minutes?

No ❑ Yes ❑

When was the last time you had any oral consumption, including water?

Time: _____ Date: _____

Product/type: _____

Do you currently smoke? No ❑ Yes ❑

This information is confidential and all responses are anonymous.

Questionnaires and saliva (oral fluid) samples cannot be used to identify a participant in the study.

IRB

Sam Houston State University
A Member of The Texas State University System

Subject Information Sheet

My name is Dr. Madeleine Swortwood, and I am an Assistant Professor of the Department of Forensic Science at Sam Houston State University. I would like to invite you to participate in a research study to examine synthetic opioid prevalence in oral fluid (saliva). We hope that data from this research will inform us about drug use trends. You have been asked to participate in the research because we would like to study synthetic drug use in populations that may be subjected to routine drug testing.

The research is relatively straightforward, and we do not expect the research to pose any risk to any of the volunteer participants. If you would like to participate in this research, you will be asked to swab your mouth with a small pad to collect oral fluid (saliva). Any data obtained from you will only be used for demographic purposes and generalizing drug trends. Under no circumstances will you or any other participants who participated in this research be identified. In addition, your data will remain confidential. This research will require about 30 minutes of your time. Participants will not be paid or otherwise compensated for their participation in this project. Drug testing results cannot be linked to you in any way.

Participation is voluntary. If you decide to not participate in this research, your decision will not affect your future relations with Sam Houston State University. Also, if at any point during the research you decide to withdraw, or do not wish to, participate in the remainder of the research you are free to withdraw your permission and to discontinue participation at any time without affecting that relationship. If you have any questions, please feel free to ask me using the contact information below. If you are interested, the results of this study will be available at the conclusion of the project.

If you have any questions about this research, please feel free to contact me, Dr. Madeleine Swortwood, using our contact information below.

Dr. Madeleine Swortwood
Department of Forensic Science
Sam Houston State University
Huntsville, TX 77341
Phone: (936) 294-*4319*
E-mail: mjs079@shsu.edu

☐ I understand the above and would like to participate.

☐ I do not wish to participate in the current study.

A copy of this permission form is available for your records.

Version 1.0 / November 27, 2017
SHSU IRB # 2017-11-37550 Approved: 2/23/2018 Expiration Date: 2/23/2019

VITA

Michael T Truver

EDUCATION

- Sam Houston State University Huntsville, Texas
  Doctor of Philosophy Science in Forensic Science
  - Expected Graduation: December 2019
  Bachelor of Science
  - Major: Chemistry; Concentration in Professional Chemist
  - Minors: Biology
  - Graduation: May 2015

RELEVENT WORK EXPERIENCE

- **Sam Houston State University** Huntsville, Texas
  August 2015-current
  Graduate Assistant
  - Assist the lab supervisor, maintain labs, managing excel databases
- **Harris County Institute of Forensic Sciences** Houston, Texas
  May 2016-March 2017
  Intern/Toxicology Method Development Validation and Research Analyst
  - Perform liquid/liquid and solid phase extractions for method development and validation activities
  - Prepare laboratory reagents and maintain supplies according to laboratory procedures
  - Assist in ensuring the laboratory conforms to ABFT, ASCLD/LAB-*International* and ISO 17025 accreditation standards
- **Texas Research Institute for Environmental Studies** Huntsville, Texas
  July 2014-May 2015
  Laboratory Assistant
  - Assist the lab supervisor, maintain lab, preform tests on soils and waters

RESEARCH EXPERIENCE

- **Sam Houston State University** Huntsville, Texas
  June 2016-Present
  - Method development and validation of novel psychoactive substances in various matrices
  - Metabolic profiling using high resolution mass spectrometry
- **Sam Houston State University** Huntsville, Texas
  August 2016-May 2017
  - Optimize an extraction method of GHB in hair using LCMSMS

- Working with Mass Hunter on a LCMSMS
- **Sam Houston State University** Huntsville, Texas
  June 2014-May 2015
  - Collected volatile organic compounds from cadavers at Southeast Texas Applied Forensic Science facility
  - Used SPME fibers to collect compounds and ran them on GC/MS to identify volatile organic compounds

SCHOLARY PRODUCTS
Peer-Reviewed Publications

- Truver M.T., Wantanabe, S., Åstrand A., Vikingsson S., Green H., Swortwood M.J., and Kronstrand R. (2019) 5F-MDMB-PICA Metabolite Identification and Cannabinoid Receptor Activity. *Drug Testing and Analysis*, DOI:10.1002/dta.2688

- Truver M.T., Palmquist K. B., Swortwood M.J. (2019) Oral Fluid and Drug Impairment: Pairing Toxicology with Drug Recognition Expert Observations. *Journal of Analytical Toxicology,* **43**, 637-643.

- Lowry J., Truver M.T., Swortwood M.J. (2019) Quantification of seven synthetic opioids in blood using LC-MS/MS. *Forensic Toxicology*, **37**, 215-223.

- Smith C.R., Truver M.T., Swortwood M.J. (2019) Quantification of U-47700 and its metabolites in plasma by LC-MS/MS. *Journal of Chromatography B*, **1112**, 41-47.

- Truver M. T. and Swortwood M. J. (2018) Quantitative Analysis of Novel Synthetic Opioids, Morphine and Buprenorphine in Oral Fluid by LC–MS-MS. *Journal of Analytical Toxicology*, **42**, 554-561.

Peer-Reviewed Presentations

- M.T. Truver*, S. Watanabe, A. Åstrand, S. Vikingsson, H. Green, M.J. Swortwood, and R. Kronstrand. 5F-MDMB-PINACA and 5F-MDMB-PICA Metabolite Identification and Cannabinoid Receptor Activity. ORAL PRESENTATION. Society of Forensic Toxicologists Annual Meeting. San Antonio, TX. October 2019.

- M.T. Truver*, A. Gilbert, M.J. Swortwood. Stability of Novel Synthetic Opioids in Blood. San Antonio, TX. POSTER. Society of Forensic Toxicologists Annual Meeting. October 2019.

- M.T Truver, M.J. Swortwood. Drug impairment: Pairing toxicology with drug recognition expert observations. POSTER. PITTCON NIJ Poster Symposium. Philadelphia, PA. March 2019.

- M.T. Truver*, K.B. Palmquist*. Detection and quantification of synthetic opioids in oral fluid. ORAL PRESENTATION. American Academy of Forensic Science NIJ Symposium. Baltimore, MD. February 2019.

- C.R. Smith*, M.T. Truver, M.J. Swortwood. Quantification of novel synthetic opioids in blood using LC-MS/MS. ORAL PRESENTATION. Society of Forensic Toxicologists Annual Meeting. Minneapolis, MN. October 2018.

- J. Lowry*, M.T. Truver, M.J. Swortwood. Quantification of novel synthetic opioids in blood using LC-MS/MS. ORAL PRESENTATION. Society of Forensic Toxicologists Annual Meeting. Minneapolis, MN. October 2018.

- M.T. Truver*, M.J. Swortwood. Quantitative analysis of novel synthetic opioids, morphine, and buprenorphine in oral fluid by LC-MS/MS. POSTER. Society of Forensic Toxicologists Annual Meeting. Minneapolis, MN. October 2018.

- M.T. Truver*, S. Kerrigan. Optimized digestion and extraction of endogenous gamma hydroxybutyrate (GHB) in human hair. POSTER. Society of Forensic Toxicologists Annual Meeting. Boca Raton, FL. January 2018.

AWARDS

- Educational Research Award; Society of Forensic Toxicologists (2019)

GRANTS

- Swortwood MJ. Prevalence of Novel Psychoactive Substances in Oral Fluid. National Institute of Justice Research and Development in Forensic Science for Criminal Justice Purposes. Federal Grant Number 2017-R2-CX-0019.Role: Graduate Assistant.

PROFESSIONAL MEMBERSHIPS

- American Academy of Forensic Sciences-Student Affiliate (2016-Present)
- Society of Forensic Toxicologists- Student Affiliate (2017-Present)