METABOLITE PROFILING OF SYNTHETIC CANNABINOIDs AND IDENTIFICATION IN HUMAN BLOOD VIA HUMAN LIVER MICROSOME INCUBATION AND HIGH RESOLUTION TANDEM MASS SPECTROMETRY

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by
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ABSTRACT

Synthetic cannabinoids are recreational drugs designed to mimic the effects of Δ⁹-tetrahydrocannabinol (THC), the main psychoactive component present in cannabis. These drugs exhibit severe toxic effects upon consumption due to their high binding affinity and potency at the cannabinoid receptors (CB₁ and CB₂). Synthetic cannabinoids have proliferated over the last decade and become a major public health and analytical challenge, critically impacting the clinical and forensic communities. Indazole carboxamide and indole carboxamide class synthetic cannabinoids have been particularly rampant, and are the compound classes most frequently reported to governmental agencies worldwide. However, the metabolic and pharmacological properties of many of these compounds remains unknown. Elucidating these characteristics allows members of the clinical and forensic communities to identify causative agents in patient samples, as well as render conclusions regarding their toxic effects.

The aim of this research study was to assess the in vitro Phase I metabolic profile of five synthetic cannabinoids and report the major metabolites identified; compounds evaluated included MDMB-CHNINACA; APP-CHMINACA (PX-3); 5F-APP-PICA (PX-1); 5F-MDMB-PINACA (5F-ADB); and FUB-AMB. These analytes were incubated for 120 minutes with human liver microsomes, followed by analysis of the extracts via ultra high performance liquid chromatography – tandem mass spectrometry (UHPLC-MS/MS). The high-resolution mass spectrometry tool utilized (quadrupole-time of flight mass spectrometry, QTOF) allowed for a thorough characterization of the metabolites, including the assignment of a chemical formula and structure, and accurate mass.
The metabolic stability and kinetic profiles of 5F-ADB and FUB-AMB were evaluated by aliquoting the incubation samples at various time points throughout the procedure. It was observed that these compounds were metabolized rapidly, resulting in short half-lives and relatively elevated metabolic clearances.

A variety of metabolites were identified for most of the species studied, and this was dependent on the chemical structure of the parent molecule. The major metabolites identified overall for the species were products of amide or ester hydrolysis; hydroxylation (including polyhydroxylation) of the pentyl side chain or cyclohexylmethyl moiety; and oxidative defluorination. It is proposed that these metabolites (especially analyte-specific metabolite) be included in laboratory assay panels to facilitate unequivocal identification of the synthetic cannabinoid agent of interest.

For select compounds (5F-ADB and FUB-AMB), authentic forensic human blood samples which screened positive for these analytes were provided by a renowned forensic toxicology laboratory. These samples were tested to verify that the major metabolites identified in the in vitro studies were also present in blood in vivo; the resultant data from the 5F-ADB and FUB-AMB samples showed that the major hydroxylated and hydrolysis metabolite, respectively, were present in greater abundance than the parent molecule, which was most often absent or not present in an appreciable quantity. Additionally, it was observed in the time studies of 5F-ADB and FUB-AMB that the metabolites containing carboxylic acid functional groups were detected in incubation samples longer than the hydroxylated metabolites, potentially indicative of longer detection windows in human samples. These findings have important toxicological implications; many
synthetic cannabinoid metabolites, including those identified in this study may have pharmacological activity and contribute to a drug user’s overall impairment profile; identifying them in blood in the absence of parent compound can point to the causative agent. The results demonstrate that it is imperative that synthetic cannabinoid assays screen for known pharmacologically active metabolites; this is particularly important for drugs with short half-lives.

The results of this research can be applied to the prediction of metabolic pathways for synthetic cannabinoids as well as non-drug substances with similar structural elements whose metabolic profile has not yet been elucidated, and whose pharmacological activity is currently unknown. Additionally, the results provide reference standard manufacturers and research scientists with further insight into the metabolic products of synthetic cannabinoids and related compounds for the synthesis of materials for the development of laboratory assays.
I dedicate this project to my children (Breyon, Bryce and Brayden and any to come in the future); and to my wife Alayshia, with whom I have had the pleasure of walking with throughout this journey. Thank you for all of your support. This is your success, too!
One of my favorite passages of scripture is Ecclesiastes 7:8 (ESV): “Better is the end of a thing than its beginning”. What a journey this has been, and I am glad that the end is finally here. Thank you Jesus for giving me the strength to finish what I started.

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you first invited me to co-author a review article on my current research topic. You asked for a writing sample and I gave you a very amateur undergraduate laboratory report on GC-MS; I know now that it was not exactly what you had in mind, but thank you for helping me get from that stage to becoming a published author of multiple manuscripts. Thank you for providing me with many professional development opportunities and for taking time to thoroughly review every one of my publications line by line over the years. Your attention to detail and professionalism will always be remembered.

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to press on, even on my worst days. Your reminders that it is all going to be worth it in the end kept me going. Thank you for your love, your prayers and encouragement.

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CHAPTER 1
INTRODUCTION

Synthetic Cannabinoids Overview

For more than a decade, synthetic cannabinoids (also termed synthetic cannabinoid receptor agonists) have been a large part of the worldwide recreational drug landscape, affecting the clinical, forensic and public health and safety communities.\(^1,2\)

Many synthetic cannabinoids were originally studied by pharmaceutical scientists as potential therapies for an array of diseases and as an alternative to natural cannabinoid \(\Delta^9\)-tetrahydrocannabinol (THC) (Figure 1.1), but have been appropriated into the illicit drug market over the years; numerous patents contain compounds reported in the forensic literature that have appeared in seized drug materials and biological specimens submitted to forensic laboratories.\(^3-9\)

Figure 1.1 Chemical structures of synthetic cannabinoids tested in this study: (a) MDMB-CHMINACA; (b) APP-CHMINACA (PX-3); (c) 5F-APP-PICA; (d) 5F-MDMB-PINACA (5F-ADB); (e) FUB-AMB; and (f) THC
Synthetic cannabinoids bind to and activate cannabinoid receptor type-1 (CB₁) and type-2 (CB₂), producing effects that often mirror those of THC\(^{10-13}\). Synthetic cannabinoids elicit effects more severe than those triggered by THC; for example, the consumption of synthetic cannabinoids has been attributed to acute kidney injury, seizures, coma, cardiovascular abnormalities, hallucinations, nausea, psychosis and death.\(^5\) Many of these pharmacological effects are the result of the manner in which the molecules interact with the CB₁/CB₂ receptors, as determined by medicinal chemists via structure-activity relationship (SAR) studies.\(^{13-15}\) Synthetic cannabinoids have been implicated in severe outbreaks resulting in mass hospitalizations \(^{16-18}\).

The indazole and indazole carboxamide subclass of synthetic cannabinoids are the most commonly reported group of chemicals, and have been involved in more drug seizures, intoxications and fatalities than any other subclass \(^{13,14,19-26}\). After their emergence in 2008, synthetic cannabinoids risen to become the largest class of abused chemicals in the recreational drug community, with over 250 individual substances reported to the United Nations Office on Drugs and Crime (UNODC) as of 2018.\(^2,26\)

Synthetic cannabinoids are classified based on their chemical structure. For example, compounds containing an indazole ring and connecting to another moiety via a carboxamide bond are termed “indazole carboxamides”. Similar reasoning is applied to indole carboxamide compounds. Many synthetic cannabinoids are named by taking portions of the formal International Union of Pure and Applied Chemistry (IUPAC) nomenclature, creating an abbreviated common name. For example, \(N\)-(1-amino-3,3-dimethyl-1-oxobutan-2-yl)-1-(4-fluorobenzyl)-1H-indazole-3-carboxamide is commonly
referred to as ADB-FUBINACA, an indazole carboxamide compound. AKB48, another indazole carboxamide, is also termed APINACA based on the contraction of its IUPAC name, N-(1-adamantyl)-1-pentyl-1H-indazole-3-carboxamide.

Some examples of drugs of the indazole and indole carboxamide chemical classes include those evaluated in this research project, namely: MDMB-CHMINACA, APP-CHMINACA (PX-3); 5F-APP-PICA (PX-1); 5F-MDMB-PINACA (5F-ADB); and FUB-AMB (Figure 1.1).

The compounds are synthesized in clandestine laboratories, dissolved in organic solvents and sprayed onto inert (non-psychoactive) botanical material; after drying, the material is then packaged and sold online and in smoke shops around the world as “herbal incense”, “fake weed”, and “synthetic marijuana”. Manufacturers of products containing synthetic cannabinoids often target younger populations. The packaging, which is often colorful in appearance and has familiar characters (cartoons, movies, etc.) is designed to attract teens and young adults, and is presented as a legal alternative to marijuana. Synthetic cannabinoids have also been distributed in powder and liquid form, in capsules and also on infused papers.

In the United States, synthetic cannabinoids are often banned by the Drug Enforcement Administration via the Controlled Substances Act. After evaluating their impact on public health, they are controlled under Schedule I, which indicates that the substances have no known accepted medicinal use, have a high potential for abuse by users, and exhibit a threat to public safety. Two compounds tested in this study, 5F-ADB and FUB-AMB are DEA Schedule I substances. Internationally, many synthetic
cannabinoids have been monitored over the years, and some placed under international control by the UNODC.\textsuperscript{38}

The binding affinity ($K_i$) of a synthetic cannabinoid to the CB receptors is often measured (relative to THC) to determine the degree to which it interacts with the receptor. A variety of techniques have been used to measure the affinity of a ligand for a specific receptor. Most studies on synthetic cannabinoid binding affinity are based on the competitive binding assay described by Compton et. al.\textsuperscript{39} This process involves incubation of isolated CB\textsubscript{1} and/or CB\textsubscript{2} receptors with a predetermined concentration of radiolabeled CP 55,490. The test compound (a synthetic cannabinoid) is then added at increasing concentrations and allowed to compete with the radioligand for binding. As the concentration of unlabeled ligand is increased, the amount of radioligand that binds to the receptor decreases. IC\textsubscript{50} (inhibitory concentration) is the concentration of unlabeled ligand necessary to displace 50\% of the radiolabeled CP 55,490. Once IC\textsubscript{50} is determined for a specific drug its $K_i$ can be calculated. $K_i$ is calculated using the Chen-Prousoff equation below:

$$K_i = \frac{IC_{50}}{1 + \frac{[L]}{K_d}}$$

where [L] is the concentration of radioactive ligand used in the experiment and $K_d$ is the dissociation constant for the ligand\textsuperscript{40}. The lower the $K_i$ of a synthetic cannabinoid, the greater its ability to bind to the receptor.
It is also necessary to evaluate the efficacy and potency at the CB receptors. Efficacy is the maximum biological effect a drug can have based on its receptor binding, and potency is a measure of the amount of drug needed to achieve a pre-defined biological effect. Functional assays designed to measure the efficacy and potency of a receptor ligand are used to determine if the substance is an agonist or antagonist and are a means of evaluating the likelihood a compound will be used for its pharmacological effect.

The most common approach used to determine if a specific analyte is an agonist for the CB$_1$ receptor is to assess its binding affinity and to evaluate guanosine 5'-O-[gamma-thio]triphosphate (GTP$\gamma$S) binding as an indicator of signal transduction. Agonists of CB$_1$ receptors cause a conformational change that results in the G-protein release of guanasine diphosphate (GDP) and binding of guanasine triphosphate (GTP). Use of GTP$\gamma$S, a radiolabelled GTP analog allows for the measurement of activation of the receptor. Stimulation is measured by determining the EC$_{50}$, i.e. the concentration of the candidate compound at which the response is 50% of the response of the normalization compound. Frequently $E_{\text{max}}$, the maximum response (relative to a normalization compound) that can be achieved by the compound being tested, is also determined in this way.$^{5,12,34}$

To bring these values into perspective, consider the $K_i$ and EC$_{50}$ values for two compounds tested in this study, MDMB-CHMINACA and 5F-ADB (Figure 1.1). For MDMB-CHMINACA, Schoeder et al.$^{13}$ reported $K_i$ values of 0.135 nM (at CB$_1$), demonstrating high affinity at this receptor. THC was also evaluated in the Schoeder et
al. study, and MDMB-CHMINACA bound to CB₁ with approximately 30-times greater affinity (THC Kᵢ: 3.87 nM at CB₁). Banister et al.¹⁵ reported EC₅₀ values of 10 nM (at CB₁) for MDMB-CHMINACA. Banister et al. also tested the potency of THC at CB₁ in their study; MDMB-CHMINACA was found to be approximately 17-times more potent than THC (EC₅₀ of 171 nM at CB₁).

In the same study by Schoeder et al.¹³, for 5F-ADB, a Kᵢ value of 23.3 nM at was reported, demonstrating that 5F-ADB does not bind to the receptor as strongly as MDMB-CHMINACA. 5F-ADB bound to CB₁ approximately 6-times less strongly than THC, Kᵢ: 3.87 nM at CB₁. Banister et al. reported an EC₅₀ value of 0.59 nM (at CB₁) for 5F-ADB, showing approximately 290-times more potent than THC. These results show that though 5F-ADB did not bind to the receptor as strongly as the other test compounds, it activated the receptor significantly more, and may have greater pharmacological effects in users.

**Synthetic Cannabinoid Metabolism**

Metabolism of ingested drugs proceeds via two enzymatic pathways known as Phase I and Phase II biotransformation. In Phase I, the drug molecule undergoes structural modifications, commonly via oxidation, commonly resulting in a hydroxylated or carboxylated metabolite (Figure 1.2). In the subsequent Phase II, the oxidized molecule is often conjugated with glucuronic acid, generally to the site of the newly introduced hydroxyl or carboxyl groups. The resulting conjugates are more water-soluble, usually exhibit reduced or negligible pharmacological activity and are readily-eliminated
via urine or bile. Liver has the highest drug-metabolizing activity, but some activity is also present in other organs, specifically the intestine, kidney and lung.

![Figure 1.2 Phase I metabolic pathway of 5F-ADB, showing hydroxylated and carboxylated metabolites](image)

Most synthetic cannabinoid metabolism studies have been conducted via in vitro human liver microsome (HLM) or human hepatocyte incubations. HLM are subcellular liver cell fractions which are rich in drug-metabolizing enzymes such as the Phase I Cytochrome P450 (CYP450) enzymes, carboxylesterase (CES) enzymes and Phase II Uridine 5'-diphospho-glucuronosyltransferase (UGT) enzymes. In general, there are inter-individual variations in the presence and activity of human liver enzymes in vivo; in laboratory experiments, this issue is accounted for through the use of pooled HLM which are typically composed of 50 or more donors. While HLM are an effective matrix for
metabolism determination studies, human hepatocytes are superior to HLM, allowing for better simulation of the human liver environment. Some hepatocyte studies were accompanied by HLM incubation to determine metabolic clearance of the analytes of interest as well as application of the method to authentic human urine samples for determination of the most appropriate biomarkers for analytical drug screening.43–46

In general, there are two primary Phase I metabolic pathways observed for synthetic cannabinoids. The first is hydroxylation (primarily monohydroxylation) that may occur at various sites on the molecule, but predominantly along the aliphatic side chains; this hydroxylation is often followed by oxidation to the corresponding carboxylic acid. CYP enzymes are typically responsible for these types of biotransformations, as demonstrated in numerous studies.25,41,47,48 Figure 1.2 provides a scheme of the major biotransformation pathways of 5F-ADB, including major hydroxylated and carboxylated metabolites, as described later in Chapter 6. Fluorinated synthetic cannabinoids commonly undergo oxidative defluorination during metabolism; however, ones that are substituted with fluorobenzyl (e.g. FUB-AMB, Figure 1.1) in the core structure do not commonly undergo biotransformation on that site of the molecule, but on other portions of the molecule.49,50

The second major metabolic route is via hydrolysis of compounds with amide or ester functional groups, to carboxylic acid metabolites (Figure 1.3). This process of metabolism is typically facilitated by various hydrolases, including CES enzymes and/or amidase enzymes.49,51–53 In these specific biotransformations, CYP enzymes are not the primary agents involved with the production of the major metabolites. An example of this
biotransformation occurs with the metabolism of FUB-AMB and AB-FUBINACA (Figure 1.3). These synthetic cannabinoids share a hydrolysis metabolite (Figure 1.3c), which is a product of ester hydrolysis and amide hydrolysis, respectively. It should be noted that care must be taken when adding metabolites to the scope of laboratory tests, as some compounds (such as FUB-AMB and AB-FUBINACA) have common metabolites. When this occurs, laboratories must be careful to acknowledge that the metabolite cannot be attributed to a single species unless the parent compound is also identified in the same matrix or in a related sample (for example: metabolite identified in urine; parent compound found in blood).

**Figure 1.3** Chemical structures of (a) FUB-AMB; (b) AB-FUBINACA; (c) the shared Phase I hydrolysis metabolite of FUB-AMB and AB-FUBINACA

Many hydroxylated metabolites of SCs exhibit pharmacological activity, binding to and activating the CB\textsubscript{1} receptor to produce a biological effect more strongly than that of Δ\textsuperscript{9}-THC, as demonstrated in multiple studies by Brents et al. and Gamage et al.\textsuperscript{54–56} These findings have toxicological relevance, as the contribution of pharmacologically active metabolites to the drug user impairment profile cannot be discounted, especially when the parent compound is not identified in the sample.

A study that demonstrates the importance of this argument involved the analysis of over 600 blood samples that were screened for the presence of JWH-018 and one
hydroxylated (JWH-018 N-(5-hydroxypentyl)) and one carboxylated metabolite (JWH-018 N-pentanoic acid) via ultra high performance liquid chromatography – tandem mass spectrometry (UHPLC-MS/MS). JWH-018 was identified in only 3 of the 600 samples at a low concentration range of 0.3 – 0.8 ng/mL; JWH-018 N-(5-hydroxypentyl) (a known pharmacologically active metabolite) was detected in 92 samples (concentration range: 0.3 – 22.7 ng/mL) while JWH-018 N-pentanoic acid was detected in 145 samples (concentration range: 0.3 – 63.5 ng/mL). It is common for synthetic cannabinoid metabolites to be present in blood samples more frequently than the parent molecules and also at higher concentrations, as observed in this research project (see Chapters 6 and 7). The number of published studies in which blood was tested is limited and only few describe the identification and quantitation of SC metabolites in blood. The presence of metabolites may also extend the detection window since the parent compound is rapidly eliminated from the blood.

Another consideration of metabolic processes is the determination of a compound’s metabolic stability and kinetic properties, namely the microsomal half-life ($T_{1/2}$), intrinsic clearance ($CL_{int}$), human hepatic clearance ($CL_{H}$) and extraction ratio (ER). The half-life of a drug is the amount of time required to eliminate 50% of the drug from the body. This value is computed by plotting the log of the concentration versus time; the slope of this value gives the rate ($k$) of the reaction, and the half-life is given by the following equation:

$$T_{1/2} = \frac{\ln(2)}{k}$$
Once the half-life is known, the intrinsic clearance can be determined. The intrinsic clearance is a measure of the ability of the liver to metabolize a drug in the absence of limitations of flow binding to cells or proteins in the blood. The units of are mL min\(^{-1}\) kg\(^{-1}\), and it is given by the following equation\(^{66,67}\):

\[
CL_{\text{int}} = \frac{\ln(2)}{T_{1/2}} \times \frac{\text{mL incubation}}{\text{mg microsomes}} \times \frac{45 \text{ mg microsomes}}{g \text{ liver}} \times \frac{20 \text{ g liver}}{kg \text{ body weight}}
\]

The hepatic clearance (CLH) is a measure of the blood flowing through the liver that is cleared of drug per unit of time, with units of mL min\(^{-1}\) kg\(^{-1}\). It is dependent on the hepatic blood flow (Q\(_H\)) which is commonly 20 mL min\(^{-1}\) kg\(^{-1}\) for humans. The equation is given by:

\[
CL_H = \frac{Q_H \times CL_{\text{int}}}{Q_H + CL_{\text{int}}}
\]

The extraction ratio (ER) is a measure of the liver’s efficiency in eliminating a drug over a single pass through the organ. It is a ratio of the hepatic clearance relative to the hepatic blood flow given by the equation below; a value close to 1 demonstrates the liver’s ability to efficiently clear the drug in a single pass.

\[
ER = \frac{CL_H}{Q_H}
\]
Examples of these determinations from the current study include data generated while evaluating 5F-ADB; the half-life was 3.1 min ± 0.14 min (mean ± standard deviation; n=3). The intrinsic clearance 256.2 mL min⁻¹ kg⁻¹. The predicted CL_H was 18.6 mL min⁻¹ kg⁻¹ with an extraction ratio (ER) of 0.93, demonstrating that this compound is rapidly metabolized.

In comparison, the half-lives, CL_H, and ER of two other 5-fluoropentyl synthetic cannabinoids, 5F-AMB and 5F-CUMYL-PICA (Figure 6.1), have been reported.⁶⁸,⁶⁹ 5F-AMB differs from 5F-ADB in that it possesses an isopropyl group instead of a tert-butyl group adjacent to the ester group. 5F-CUMYL-PICA is an indole carboxamide compound with a cumyl group connected at the carboxamide nitrogen. 5F-AMB data are as follows: T_{1/2}, 1.0 ± 0.2; CL_H, 19.4; and ER, 1.00. 5F-CUMYL-PICA data are as follows: T_{1/2}, 1.77; CL_H, 19.15; and ER, 0.96. Overall, these values are comparable to 5F-ADB (and within the same order of magnitude), demonstrating that some other 5-fluoropentyl synthetic cannabinoids are also rapidly metabolized.

A gap still exists in the field of synthetic cannabinoids and drug metabolism, as the metabolic profiles of many synthetic cannabinoids identified in non-biological materials have yet to be determined. More resources need to be allocated to research laboratories to investigate this valuable information, which will empower the clinical and forensic communities to identify causative agents in casework, and better understand this ever-growing subfield of drug testing.
UHPLC-QTOF Analysis of Synthetic Cannabinoids and Metabolites

The ability to detect and quantify synthetic cannabinoids and their metabolites in human biological samples is essential in the fields of clinical and forensic toxicology. Common forensic analyses include testing samples collected from patients in hospitals after intoxication events and death, workplace drug testing, probation and parole drug testing, forensic psychiatry facilities, and samples collected from those driving under the influence of drugs (DUID) and post-mortem evaluations.\textsuperscript{14}

Selection of a biological matrix for toxicological analysis is driven by several factors, the most important being the purpose of the testing. Blood, serum and plasma can provide information about the circulating drug concentrations at the time of sample collection. Synthetic cannabinoids are readily detectable in blood both as the parent compound and as metabolites. Previously, the pharmacokinetics and biological activity of most synthetic cannabinoid metabolites was unknown, and it was generally sufficient to detect the parent drug in blood; however, with the increasing knowledge of synthetic cannabinoid metabolic profiles, more sophisticated analytical approaches are being undertaken.

Instrumentation that possesses adequate sensitivity and selectivity is crucial for the unequivocal identification of drugs and their metabolites. This is particularly important for synthetic cannabinoids blood drug testing, as synthetic cannabinoid parent compounds are frequently present at sub-nanogram concentrations in authentic biological samples\textsuperscript{25}; typical concentrations of parent compounds in blood are 0.1 – 10 ng/mL, with the concentrations generally on the lower end of this range.\textsuperscript{25,57-60}
Quantitative analysis is typically conducted via LC-MS/MS; however, the use of ultra high performance liquid chromatography – tandem mass spectrometry (UHPLC-MS/MS) for the separation, identification and quantitation of analytes of interest is becoming more prevalent. Higher throughput is more readily achievable using UHPLC-MS/MS with shorter run times and targeted analyses. High resolution mass analyzers including time-of-flight (TOF) instruments are being used more regularly in laboratories for synthetic cannabinoids analysis, particularly in determining metabolite profiles.\textsuperscript{25,70–72}

The use of these mass analyzers is essential due to the diversity of SCs and the large number of isomeric compounds within this class that exist; it is important to have sufficient chromatographic separation as well as detection method to ensure proper identification.\textsuperscript{49,73,74}

QTOF tandem mass spectrometry, operates by filtering a selected ion’s mass (generally a charged parent molecule), known as the precursor mass, allowing it to then pass through a collision cell where it is bombarded with high energy argon atoms and fragmented. These fragments (known as product ions) then pass into a flight tube under high energy where the time of the ion’s trajectory through the tube and to the detector is measured. Ion masses are differentiated based on their “flight time”. The equation used to calculate the mass of the ion is provided below:

\[
\frac{m}{z} = \frac{2Vt^2}{L^2}
\]

where \(m\) and \(z\) are the mass and charge of the ion, respectively; \(V\) is the system voltage, \(t\) is the flight time, and \(L\) is the length of the flight tube.
QTOF mass spectrometry is particularly beneficial as it allows for the determination of a high-resolution accurate mass (typically to four decimal places) and the elemental composition of the molecules being analyzed. This allows for greater selectivity of the analysis, and facilitating differentiation of one chemical species from another. An example of an output of the QTOF instrument used in this research is presented as Figure 1.4, the product ion mass spectrum of 5F-ADB; of note is the accurate mass of the precursor (m/z 378.2197) and product ions (six different ions), given to 4 decimal places.

![Product ion spectrum (MS/MS) and structure of 5F-ADB](image)

**Figure 1.4** Product ion spectrum (MS/MS) and structure of 5F-ADB (top left)

This type of data is useful for the analysis of synthetic cannabinoids and their metabolites and provides a greater degree of confidence, particularly when analyzing molecules for the first time, and when considering isobaric species.
CHAPTER 2
MATERIALS AND METHODS

Chemicals and Reagents

Reference standards of MDMB-CHMINACA, APP-CHMINACA, PX-1, (S)-5F-ADB, 5F-ADB Metabolite 2 (5-hydroxypentyl), 5F-ADB Metabolite 7 (3,3-dimethylbutanoic acid), FUB-AMB (as “MMB-FUBINACA”) and its ester hydrolysis 3-methylbutanoic metabolite (as “AB-FUBINACA Metabolite 3”), each at ≥ 98% purity, were purchased from Cayman Chemical (Ann Arbor, MI, USA). Nicotinamide adenine dinucleotide phosphate (NADPH) sodium salt (≥ 95% purity) was also purchased from Cayman. Diazepam certified reference material (99.9% purity) was obtained from Cerilliant® Corporation (Round Rock, TX, USA). Pooled human liver microsomes (Gibco®, 50-donors, 20 mg/mL protein concentration), and LC-MS grade formic acid (Pierce™, >99% purity) were sourced from Thermo Fisher Scientific (Waltham, MA, USA). Sodium phosphate monobasic (monohydrate), sodium phosphate dibasic (anhydrous), magnesium chloride (hexahydrate) (ACROS Organics) and Fisherbrand™ microcentrifuge tubes (1.5 mL capacity) were purchased from Fisher Scientific (Waltham, MA, USA). LC-MS grade water and acetonitrile were obtained from Honeywell (Morris Plains, NJ, USA). Ammonium formate (Alfa Aesar), aqueous sodium hydroxide (VWR Chemicals, BDH®, 10 N), MTBE (J.T. Baker), TRIS free base (ultrapure; VWR Life Science, Amresco), concentrated hydrochloric acid (VWR Chemicals, BDH®), and Costar® Spin-X® centrifuge tube filters (Corning®, 0.45 µm pore
size) were purchased from VWR™ International (Radnor, PA, USA). Human whole blood preserved with sodium fluoride and potassium oxalate (used as a blank control) was sourced from BioIVT (Westbury, NY, USA).

**In Vitro Human Liver Microsome Incubations**

The in vitro Phase I metabolic profile of each synthetic cannabinoid was elucidated via incubation with HLM over a period of 120 minutes. A stock standard solution of the analyte of interest was prepared at a concentration of 1 mg/mL from reference material. (Note: For 5F-ADB and FUB-AMB the 1 mg/mL stock solution was further diluted to a concentration of 0.05 mg/mL, which was used as a working standard). Additional reagents, including 100 mM phosphate buffer (pH 7.4) with 10 mM MgCl₂, and 10 mM NADPH solution, were prepared in LC-MS grade water. A diazepam incubation set was run in parallel with each synthetic cannabinoid sample set as an in-process control to verify the enzymatic activity of the microsomes. Representative diazepam metabolites (nordiazepam, oxazepam, and temazepam) were monitored to confirm that metabolism occurred during the incubation process. Each incubation was performed in duplicate. HLM were kept frozen (-80 °C) prior to use in experiments; they were subsequently thawed for immediate sample preparation. Synthetic cannabinoid and diazepam standards were dried down under nitrogen using a Turbovap® (Zymark) and reconstituted in a 1:1 (v/v) mixture of phosphate buffer:acetonitrile.

For MDMB-CHMINACA, APP-CHMINACA and PX-1, drug reaction mixtures were comprised of drug standard (5 µL) (final concentration of 21 µM), phosphate buffer
(520 µL), microsomes (25 µL) and NADPH solution (50 µL); each incubate had a final volume of 600 µL. All samples were prepared in glass test tubes. Samples were vortex mixed and placed into a shaking water bath (Bellco Glass Co, model 7746-22110) set to 37 °C for 120 minutes. Two control samples were analyzed with each drug incubation set. The first was a mixture of drug standard (5 µL) and phosphate buffer (595 µL), without the addition of NADPH solution nor microsomes; this control sample was designed to exclude the possibility of hydrolysis facilitated by the buffer solution. The second included drug standard (5 µL), phosphate buffer (570 µL) and microsomes (25 µL), but no NADPH solution; this sample was implemented to evaluate the production of any metabolites in the presence of enzymes, but absence of NADPH.

Incubations were terminated by adding 500 µL of LC-MS grade acetonitrile followed by vortex mixing. Samples were then transferred to microcentrifuge tubes and centrifuged at 10,000 x g for 5 minutes. The supernatants were transferred to test tubes and partially dried down (to approximately 50% of the original volume) under nitrogen using a Turbovap®. The samples were subsequently transferred to Costar® Spin-X® tubes and centrifuged at 10,000 x g for 5 minutes. As a final step, samples were transferred to autosampler vials for UHPLC-MS/MS analysis.

Overall sample preparation for 5F-ADB and FUB-AMB was similar to the other drugs, but with a few modifications. The drug reaction mixtures were comprised of drug standard (10 µL) (final concentration of 1 µM), phosphate buffer (1,040 µL), microsomes (50 µL; equating to 1 mg HLM) and NADPH solution (100 µL); each incubate had a final volume of 1,200 µL (1.2 mL). Samples were gently vortexed and placed into a
shaking water bath (Stuart™, model SBS40) set to 37 °C and sampled over a period of 120 minutes as follows: 0, 5, 10, 15, 30, 60 and 120 minutes. Two control samples were analyzed with each drug incubation set. The first was a mixture of 5F-ADB standard (10 µL) and phosphate buffer (1,190 µL), without the addition of NADPH solution nor microsomes; the second included 5F-ADB standard (10 µL), phosphate buffer (1,140 µL) and microsomes (50 µL), but no NADPH solution.

Aliquots of 100 µL were sampled at each time point into microcentrifuge tubes, to which 100 µL of ice-cold LC-MS grade acetonitrile was added to terminate the reaction, followed by vortex mixing. (Note: For the diazepam control set, samples were only collected at 120 minutes). Samples were then centrifuged twice and loaded onto the instrument as in the case of the other synthetic cannabinoids.

**Extraction of Human Blood Samples**

To assess the presence of its metabolites in human blood, authentic forensic case samples provided by NMS Labs (Horsham, PA, USA) that initially screened positive for 5F-ADB (nine) and FUB-AMB (twelve) were extracted and analyzed via UHPLC-MS/MS. Case types included driving under the influence of drugs (DUID) and postmortem, collected from male and female patients aged 24-51. Upon receipt from NMS, samples were frozen at -20 °C until analysis.

Samples were prepared by aliquoting 0.5 mL of blood into a 13x100 mm glass test tube followed by the addition of 0.5 mL of TRIS buffer (1 M, pH 10.2), and brief vortex mixing. Next, 3 mL of MTBE were added, and the samples were vortex mixed
again. The tubes were then capped and rotomixed for approximately 15 minutes. Upon completion, the samples were centrifuged (Eppendorf®, model 5810) at 3500 revolutions per minute for 10 minutes. After placing the samples into the -80 °C freezer until the bottom aqueous layer was frozen, the top organic layer was collected in a new, clean 13x100 mm test tube. The samples were then evaporated to dryness at 30±5 °C in a Turbovap® for approximately 15 minutes. The dried samples were then reconstituted with 200 µL of 95:5 ammonium formate (10 mM, pH 3) in LC-MS grade water and 0.1% formic acid in 50:50 methanol:acetonitrile and analyzed by UHPLC-MS/MS. As a final step, samples were transferred to autosampler vials with conical inserts for analysis.

A blank human blood (no drug) control and a positive blood control were simultaneously analyzed using the same procedure outlined above. The positive control consisted of multiple synthetic cannabinoids of varying chemical structure spiked into blank blood (final concentration of 20 ng/mL blood) including: 5F-PB-22, UR-144, XLR-11, ADBICA, AB-PINACA, ADB-PINACA, PB-22, AM-2201, AKB48. Two 5F-ADB metabolites and one FUB-AMB metabolite (termed “5F-ADB Metabolite 2”; “5F-ADB Metabolite 7”; and “AB-FUBINACA Metabolite 3” by Cayman Chemical) were purchased and analyzed; these data were used to verify the identity of these metabolites in the blood via retention time and product ion mass spectra.
Instrument Parameters

Incubation samples and blood extracts were analyzed chromatographically on a Shimadzu (Kyoto, Japan) Nexera XR UHPLC interfaced with a Sciex (Framingham, MA, USA) TripleTOF® 5600+ QTOF mass spectrometer. Separation was achieved using a Kinetex C18 column, 50 x 3.0 mm, 2.6 µm (Phenomenex®, Torrance, CA, USA) with a column temperature of 30°C. Mobile phase A (MPA) consisted of 10 mM ammonium formate (pH 3) in water; Mobile phase B (MPB) was composed of 0.1% formic acid in 50:50 methanol:acetonitrile. A linear gradient of 95:5 MPA:MPB to 5:95 (after 13 minutes) and back to the original 95:5 ratio was applied. A flow rate of 0.4 mL/min and injection volume of 10 µL were used, with a total run time of 15.5 minutes.

The QTOF mass spectrometer was operated in positive electrospray ionization mode (+ESI). The +ESI source parameters were as follows: ion spray voltage, 2500 V; source temperature, 600 °C; declustering potential, 80 V; curtain gas, 30 psi; gas 1, 50 psi; gas 2, 50 psi. A TOF-MS scan (m/z 100 – 1000) was utilized to acquire accurate mass measurements of precursor ions; subsequently, precursor ions were fragmented using a collision energy spread of 35 ± 15 eV (scan range of m/z 40 – 1000), to generate product ions. Mass spectral data were acquired in Information-Dependent Acquisition (IDA) mode using Analyst® TF software (Sciex, Version 1.7). In IDA mode, the 16 most abundant ions were isolated per cycle with a minimum of 100 counts per second (cps); these ions were selected for dependent MS/MS scan. A calibration solution was injected throughout the run to maintain mass axis calibration and ensure accuracy of generated data.
MetabolitePilot™ (Sciex, Version 1.5; Version 2.0.3 for 5F-ADB and FUB-AMB) was used to characterize the chemical structures of the parent synthetic cannabinoids and their metabolites generated during incubations. PeakView® (Sciex, Version 2.2) was used to verify the presence of the species in samples and to generate extracted ion chromatograms (XIC) and product ion (MS/MS) spectra. MetabolitePilot™ data were generated based on a number of programmed parameters: mass defect filtering (50 mDa); isotope pattern (intensity tolerance of 20%; MS m/z tolerance of 3 mDa); characteristic product ions and neutral losses (minimum of 1 each); common and analyte-specific biotransformations (e.g. monohydroxylation, hydrolysis, etc.); number of allowed bond breakages (up to 2); background subtraction; chromatographic data (minimum peak intensity of 500 cps); MS m/z tolerance (20 ppm; minimum intensity of 500 cps); MS/MS tolerance (20 ppm; minimum intensity of 100 cps). All data generated were manually reviewed and interpreted with care. After data review, all accurate masses, ppm error, retention time, and formulae were tabulated.
CHAPTER 3
METABOLISM OF MDMB-CHMINACA

Background

MDMB-CHMINACA (Figure 3.1), methyl 2-[1-(cyclohexylmethyl)-1H-indazole-3-carboxamido]-3,3-dimethylbutanoate, is a synthetic cannabinoid of the indazole carboxamide class. It is classified based on its chemical structure, which contains an indazole ring, and a linking group, carboxamide, which connects the indazole ring to the methyl 3,3-dimethylbutanoate (MDMB) moiety. The compound also has a cyclohexylmethyl (CHM) substituent attached to the indazole ring. Indazole carboxamide synthetic cannabinoids are the most abundant and prevalent class of synthetic cannabinoids, and many compounds of this class are still emerging.\textsuperscript{25,75,76}

\textbf{Figure 3.1} Chemical structures of (a) MDMB-CHMINACA; (b) MDMB-CHMICA; (c) MAB-CHMINACA; (d) AB-CHMINACA; (e) MO-CHMINACA; (f) APP-CHMINACA (PX-3); (g) 4F-MDMB-BINACA; and (h) 5F-EDMB-PINACA

MDMB-CHMINACA was identified in 2014 along with four other indazole carboxamides in forensic chemistry casework samples (powders and botanical matrices) in several cities in Russia and Belarus.\textsuperscript{77} The chemical structure was characterized via gas
chromatography-mass spectrometry (GC-MS), ultra-high performance liquid chromatography-high resolution tandem mass spectrometry (UHPLC-HRMS) and $^1$H and $^{13}$C Nuclear Magnetic Resonance (NMR) spectroscopy.

MDMB-CHMINACA was originally presented in a patent produced by Pfizer in 2009 (see Example 129 in patent) to study the effects of compounds exhibiting CB$_1$ binding activity.$^{78}$ Multiple studies to determine the CB$_1$ binding affinity (K$_i$) and receptor activation were previously conducted by researchers.$^{13,15,78}$ Binding affinity was assessed through competitive binding assays with [$^3$H]CP 55,940, a cyclohexylphenol class synthetic cannabinoid; receptor activation was tested via a fluorometric imaging plate reader (FLIPR) assay.$^{78}$ The concentration of MDMB-CHMINACA at which the activation response was 50% of the response of the normalization compound (EC$_{50}$), was determined through this assay.$^{78}$ A K$_i$ value of 0.0944 nM (at CB$_1$) was reported by Buchler et al.$^{78}$; Schoeder et al.$^{13}$ reported K$_i$ values of 0.135 nM (at CB$_1$), and 0.222 nM (at CB$_2$), demonstrating high affinity for both receptors. THC was also evaluated in the Schoeder et al. study, and MDMB-CHMINACA bound to CB$_1$ with approximately 30-times greater affinity (THC K$_i$: 3.87 nM at CB$_1$). Banister et al. reported EC$_{50}$ values of 10 nM (at CB$_1$) and 128 nM (at CB$_2$) for MDMB-CHMINACA, demonstrating preference for activation of the CB$_1$ receptor. Banister et al.$^{15}$ also tested the potency of THC at CB$_1$ in their study; MDMB-CHMINACA was found to be approximately 17-times more potent than THC (EC$_{50}$ of 171 nM at CB$_1$). MDMB-CHMINACA was also identified in an activity-based bioassay screening procedure which tested authentic serum
samples for synthetic cannabinoids that activated CB receptors; its identity was confirmed via LC-MS/MS.  

Additional studies have been conducted on MDMB-CHMINACA, including its mass spectral characteristics in electrospray and electron ionization sources, as well as its stability in serum extracts under various laboratory storage conditions. Two MDMB-CHMINACA has also been included in the testing scope of various laboratories in serum and whole blood. Two other synthetic cannabinoids, MDMB-CHMICA and MAB-CHMINACA (ADB-CHMINACA) (Figure 3.1), are structurally-related to MDMB-CHMINACA and have been identified internationally and reported in peer-reviewed literature. MDMB-CHMICA is an indole carboxamide, differing from MDMB-CHMINACA only in that the core ring is indole instead of indazole. MAB-CHMINACA, an indazole carboxamide, has a terminal amide instead of an ester group. These compounds have been identified in herbal blends, and were associated with a number of intoxications and fatalities; both are Drug Enforcement Administration (DEA) Schedule I substances. MDMB-CHMICA has also been placed under international control by the International Narcotics Control Board (INCB).

The in-vitro and in-vivo metabolic profiles of several indazole carboxamide synthetic cannabinoids have been reported. The aim of the current study was to elucidate the in-vitro metabolic profile of indazole carboxamide synthetic cannabinoid, MDMB-CHMINACA. As described in Chapter 2, this was achieved through incubation of the compound with human liver microsomes (HLM), and subsequently analyzing the incubates via UHPLC-HRMS. Quadrupole-time of flight
mass spectrometry (QTOF-MS) was utilized to produce an accurate mass and molecular formula for each metabolite generated.

**Results**

*Mass Spectral Analysis of MDMB-CHMINACA*

MDMB-CHMINACA eluted at 10.62 minutes. The elemental composition generated by mass spectral analysis was C\(_{22}\)H\(_{31}\)N\(_3\)O\(_3\) with a precursor accurate mass of m/z 386.2420 ([M+H]\(^+\)) observed. The product ion spectrum and chemical structure with fragmentation details are provided in Figure 3.2. The base peak at m/z 241 represents cleavage of the amide bond; m/z 145 represents the indazole acylium ion (further loss of cyclohexylmethyl (CHM)); m/z 326 represents loss of the methyl ester group; m/z 354 represents the loss of methoxy from the methyl ester group.

![Figure 3.2 Product ion spectrum (MS/MS) and structures of MDMB-CHMINACA (top right) and fragments](image)

**Figure 3.2** Product ion spectrum (MS/MS) and structures of MDMB-CHMINACA (top right) and fragments
**Characterization of MDMB-CHMINACA Metabolites**

A total of twenty-seven (27) metabolites were identified during the study, encompassing twelve (12) different metabolite types. The major biotransformations that took place were monohydroxylation and methyl ester hydrolysis to carboxylic acid; hydrolyzed MDMB-CHMINACA subsequently underwent additional biotransformations including hydroxylation and dehydrogenation steps. Less prevalent metabolite classes were also observed.

A summary of the results of metabolite elucidation is reported in Table 3.1. During the study, the identity of all postulated metabolites was performed by evaluating the following: elemental composition and product ion spectra relative to the parent molecule, MDMB-CHMINACA; accurate mass; mass error (ppm); retention time. Consistency of the data between each replicate was verified in order to make a positive identification.

All metabolites eluted prior to MDMB-CHMINACA, consistent with retention order and method design based on molecular features and stationary phase polarity. The rank (relative abundance) was determined by dividing the raw peak areas of individual metabolites by the peak area of the most abundant metabolite identified. No metabolites were identified in the control sample containing only MDMB-CHMINACA and buffer solution, demonstrating that metabolism was enzyme-dependent.

For the purposes of this chapter, data for the most relevant metabolites will be highlighted. An extracted ion chromatogram (XIC) of the most prominent metabolites is provided in Figure 3.3. Representative product ion spectra for metabolites recommended
for inclusion in drug screening panels are provided in Figures 3.4 – 3.5. The product ion spectra of other metabolites are presented in Figures 3.S1 – 3.S10. In the in-text description of metabolites, the nominal mass of precursor and fragment ions will be used. The proposed metabolic pathways of MDMB-CHMINACA are presented in Figure 3.6. For metabolite isomers, the exact site of biotransformation on the molecule was not determined in this study and is represented by Markush bonds. In order to verify the specific site, each proposed metabolite and its positional isomers would need to be synthesized and analyzed, comparing the retention, mass spectral, and NMR profiles of the reference material to the data presented in the current study.

Figure 3.3 Extracted ion chromatogram (XIC) of the most prevalent metabolites identified: Class M1, M2, M8, M10, and M11
<table>
<thead>
<tr>
<th>Metabolite ID</th>
<th>Biotransformation</th>
<th>Retention Time (min)</th>
<th>Elemental Composition</th>
<th>Measured Accurate Mass (m/z)</th>
<th>Mass Error (ppm)</th>
<th>Diagnostic Product Ions</th>
<th>Rank</th>
</tr>
</thead>
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<tr>
<td>M1*</td>
<td>Ester Hydrolysis</td>
<td>10.00</td>
<td>C$<em>{2}$H$</em>{2}$N$<em>{2}$O$</em>{3}$</td>
<td>372.2283</td>
<td>0.3</td>
<td>145, 241, 326</td>
<td>2</td>
</tr>
<tr>
<td>M2.1</td>
<td>Monohydroxylation</td>
<td>9.37</td>
<td>C$<em>{2}$H$</em>{2}$N$<em>{2}$O$</em>{4}$</td>
<td>402.2390</td>
<td>0.6</td>
<td>145, 257, 342</td>
<td>1</td>
</tr>
<tr>
<td>M2.2</td>
<td>Monohydroxylation</td>
<td>9.19</td>
<td>C$<em>{2}$H$</em>{2}$N$<em>{2}$O$</em>{4}$</td>
<td>402.2387</td>
<td>-0.2</td>
<td>145, 257, 342</td>
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<td>M2.3</td>
<td>Monohydroxylation</td>
<td>8.96</td>
<td>C$<em>{2}$H$</em>{2}$N$<em>{2}$O$</em>{4}$</td>
<td>402.2388</td>
<td>0.3</td>
<td>145, 257, 342</td>
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<td>M2.4</td>
<td>Monohydroxylation</td>
<td>8.82</td>
<td>C$<em>{2}$H$</em>{2}$N$<em>{2}$O$</em>{4}$</td>
<td>402.2392</td>
<td>1.1</td>
<td>145, 257, 342</td>
<td>11</td>
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<td>M2.5</td>
<td>Monohydroxylation</td>
<td>8.67</td>
<td>C$<em>{2}$H$</em>{2}$N$<em>{2}$O$</em>{4}$</td>
<td>402.2390</td>
<td>0.6</td>
<td>145, 257, 342</td>
<td>13</td>
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<td>M3</td>
<td>Ketone Formation</td>
<td>8.77</td>
<td>C$<em>{2}$H$</em>{2}$N$<em>{2}$O$</em>{4}$</td>
<td>400.2228</td>
<td>-0.6</td>
<td>145, 255, 340</td>
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<td>M4</td>
<td>Dihydroxylation</td>
<td>8.14</td>
<td>C$<em>{2}$H$</em>{1}$N$<em>{2}$O$</em>{4}$</td>
<td>418.2336</td>
<td>-0.1</td>
<td>145, 273, 358</td>
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<td>M5</td>
<td>Dihydroxylation and Dehydrogenation</td>
<td>9.51</td>
<td>C$<em>{2}$H$</em>{1}$N$<em>{2}$O$</em>{4}$</td>
<td>416.2176</td>
<td>-0.9</td>
<td>145, 241, 398</td>
<td>25</td>
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<td>M6</td>
<td>Trihydroxylation and Dehydrogenation</td>
<td>7.45</td>
<td>C$<em>{2}$H$</em>{1}$N$<em>{2}$O$</em>{4}$</td>
<td>432.2133</td>
<td>0.9</td>
<td>145, 257, 386</td>
<td>24</td>
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<td>M7.1</td>
<td>Ester Hydrolysis and Monohydroxylation</td>
<td>8.27</td>
<td>C$<em>{2}$H$</em>{2}$N$<em>{2}$O$</em>{4}$</td>
<td>388.2235</td>
<td>1.1</td>
<td>145, 257, 342</td>
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<td>M7.2</td>
<td>Ester Hydrolysis and Monohydroxylation</td>
<td>8.65</td>
<td>C$<em>{2}$H$</em>{2}$N$<em>{2}$O$</em>{4}$</td>
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<td>145, 257, 342</td>
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<td>145, 241, 370</td>
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<td>M9.1</td>
<td>Ester Hydrolysis and Monohydroxylation and Dehydrogenation</td>
<td>7.84</td>
<td>C$<em>{2}$H$</em>{2}$N$<em>{2}$O$</em>{4}$</td>
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<td>145, 257, 368</td>
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<td>Ester Hydrolysis and Monohydroxylation and Dehydrogenation</td>
<td>8.23</td>
<td>C$<em>{2}$H$</em>{2}$N$<em>{2}$O$</em>{4}$</td>
<td>386.2075</td>
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<td>145, 257, 368</td>
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<td>M9.3</td>
<td>Ester Hydrolysis and Monohydroxylation and Dehydrogenation</td>
<td>7.65</td>
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<td>386.2076</td>
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<td>145, 257, 368</td>
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<td>M9.4</td>
<td>Ester Hydrolysis and Monohydroxylation and Dehydrogenation</td>
<td>7.47</td>
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<td>386.2075</td>
<td>0.1</td>
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<td>14</td>
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<tr>
<td>M9.5</td>
<td>Ester Hydrolysis and Monohydroxylation and Dehydrogenation</td>
<td>8.12</td>
<td>C$<em>{2}$H$</em>{2}$N$<em>{2}$O$</em>{4}$</td>
<td>386.2075</td>
<td>0.3</td>
<td>145, 257, 368</td>
<td>19</td>
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<tr>
<td>M10.1</td>
<td>Ester Hydrolysis and Ketone Formation and Dehydrogenation</td>
<td>7.67</td>
<td>C$<em>{2}$H$</em>{2}$N$<em>{2}$O$</em>{4}$</td>
<td>384.1921</td>
<td>0.8</td>
<td>145, 255, 384</td>
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<tr>
<td>M10.2</td>
<td>Ester Hydrolysis and Ketone Formation and Dehydrogenation</td>
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<td>C$<em>{2}$H$</em>{2}$N$<em>{2}$O$</em>{4}$</td>
<td>384.1919</td>
<td>0.4</td>
<td>145, 255, 384</td>
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Table 3.1. Table of metabolites identified and associated biotransformations, average retention time (min), elemental composition, measured accurate mass (m/z), mass error (ppm), diagnostic product ions, and rank (based on relative peak area percent). The metabolite marked with an asterisk represents that it was also identified in the control sample to which no NADPH was added.

Table 3.1 continued on next page
Table 3.1. Continued

Methyl ester hydrolysis metabolite M1 was observed at 10.00 minutes, with a nominal precursor mass of m/z 372. Product ions observed were identical to MDMB-CHMINACA except for the absence of m/z 354, indicating a change in the structure. Non-fragmented m/z 372 in the spectrum represents the presence of an ester hydrolysis product, converting the methyl ester to a carboxylic acid. The ion m/z 326 represents loss of the carboxylic acid group (See Figure 3.4). This metabolite was also identified in the control sample to which no NADPH was added (drug + buffer + HLM only); it is therefore a CYP-independent biotransformation. M1 was the second-most abundant metabolite identified.
Five monohydroxylated metabolites, M2.1 – M2.5 with precursor mass m/z 402, were identified at the following retention times: 8.67, 8.82, 8.96, 9.19 and 9.37 minutes. The metabolite at 9.37 minutes was the most abundant metabolite identified in the current study. As a class, monohydroxylated metabolites were the most prevalent. Hydroxylation took place on the indazole side of the molecule, as demonstrated by the presence of the following ions: m/z 145, representing intact indazole; m/z 257, an m/z increase of 16 (gain of O) relative to m/z 241 ion of MDMB-CHMINACA, indicating hydroxylation on the CHM moiety. This was further substantiated by the presence of m/z 342, an m/z increase of 16 relative to m/z 326 (loss of methyl ester) present in the product ion spectrum of MDMB-CHMINACA (See Figure 3.5).
Ketone product M3 was identified at 8.77 minutes, with a precursor mass of m/z 400, an m/z decrease of 2 from monohydroxylation (m/z 402). The presence of m/z 145 in the product ion spectrum was indicative of intact indazole; m/z 255 was the result of ketone formation on CHM, an m/z increase of 14 from m/z 241 ion of MDMB-CHMINACA (gain of O, loss of H₂). Loss of methyl ester, represented by m/z 340 was also present in the product ion spectrum (See Figure 3.S1).

Metabolite M4, a minor dihydroxylation product was identified at 8.14 minutes with a precursor mass of m/z 418. The presence of m/z 145 represented intact indazole; m/z 273 represented dihydroxylation of the CHM moiety, an m/z increase of 32 (gain of 2O) relative to m/z 241 ion of MDMB-CHMINACA and increase of 16 relative to m/z 257 present for M2 (See Figure 3.S2).
Metabolite M5 is a minor dihydroxylation product that also underwent dehydrogenation; its precursor mass is m/z 416, a decrease in mass of 2 m/z units relative to M4. Hydroxylation of this metabolite occurred on the methyl 3,3-dimethylbutanoate side of the molecule (tert-butyl), as indicated by the presence of m/z 145 and m/z 241 in the product ion spectrum, representing intact indazole and CHM rings, respectively (See Figure 3.S3).

Metabolite M6 is a minor product of trihydroxylation and dehydrogenation, identified at 7.45 minutes, with a precursor mass of m/z 432. It is dihydroxylated on the tert-butyl group, and monohydroxylated on the CHM group. The presence of m/z 145 and m/z 257 are consistent with M2 and MDMB-CHMINACA and represent intact indazole and monohydroxylated CHM, respectively (See Figure 3.S4).

Three minor ester hydrolysis and monohydroxylation metabolites, M7.1 – M7.3 with precursor mass m/z 388, were identified at the following retention times: 7.95, 8.27 (most abundant) and 8.65 minutes. After ester hydrolysis, monohydroxylation took place on the indazole side of the molecule, as demonstrated by the presence of the following ions: m/z 145, representing intact indazole; m/z 257 and m/z 342, an m/z increase of 16 (gain of O) relative to the m/z 241 and 326 ions of MDMB-CHMINACA, indicating monohydroxylation on the CHM moiety (consistent with M2) (See Figure 3.S5).

One ester hydrolysis and dehydrogenation metabolite, M8, was identified at 9.66 minutes. Its precursor mass is m/z 370. This metabolite generated product ions consistent with M1 except for the absence of m/z 326. The decrease in mass of 2 m/z units relative to M1 indicates the loss of 2H (dehydrogenation) (See Figure 3.S6).
Five minor ester hydrolysis and monohydroxylation and dehydrogenation metabolites, M9.1 – M9.5, precursor mass m/z 386, were identified at the following retention times: 7.47, 7.65, 7.84 (most abundant), 8.12 and 8.23 minutes. Product ions at m/z 145 and m/z 257 are indicative of intact indazole and monohydroxylation on the CHM ring, respectively, as observed for M7. The precursor mass of m/z 386 is consistent with dehydrogenation (loss of 2H). The ion present at m/z 368 represents loss of H₂O (See Figure 3.S7).

Two metabolites, M10.1 – M10.2, were products of ester hydrolysis and ketone formation and dehydrogenation. The metabolites were identified at 7.56 and 7.67 (most abundant) minutes and had a precursor mass of m/z 384, consistent with a loss of H₂ (ketone formation) from M9. Product ions m/z 145 and m/z 255 were consistent with M3 and represent intact indazole and ketone formation on CHM, respectively (See Figure 3.S8).

A total of six metabolites that were products of ester hydrolysis, dihydroxylation and dehydrogenation were identified in the study. Three of these metabolites were hydroxylated only on the CHM ring, while the other three were monohydroxylated on both CHM and tert-butyl groups. These metabolites were separated into two groups: the former being “Type 1” and the latter, “Type 2.

Three Type 1 metabolites, M11.1 – M11.3, were identified at the following retention times: 6.54, 6.63 and 6.91 (most abundant) minutes. The precursor mass of these metabolites was m/z 402. Type 1 metabolites were dihydroxylated on CHM, as suggested by the presence of m/z 145 (intact indazole) and m/z 273, consistent with M4.
The fragment m/z 384 represents loss of H$_2$O from the precursor molecule (See Figure 3.S9).

Three Type 2 metabolites, M12.1 – M12.3, were identified at the following retention times: 7.18, 7.56 and 7.92 (most abundant) minutes. The precursor mass of these metabolites was m/z 402, as with M11. Type 2 metabolites were monohydroxylated on the CHM as well as tert-butyl groups. The presence of m/z 145 (intact indazole) and m/z 257 in the product ion spectrum indicate intact indazole and hydroxylated CHM, respectively. The fragment m/z 356 represents loss of the methyl ester group from the precursor molecule (See Figure 3.S10).
**Figure 3.6** Proposed metabolic pathways of MDMB-CHMINACA. Asterisk (*) represents that the exact mechanism for production of M5 and M6 is unknown, as no other corresponding metabolites were identified.
Discussion

Biotransformation of xenobiotics during the metabolic process occurs in many tissues, but predominantly in the liver. The liver is a rich source of a variety of enzymes that facilitate Phase I metabolism; these include, but are not limited to, cytochrome P450 (CYP450) and carboxylesterase (CES) enzymes. CYP450 enzymes are responsible for oxidation reactions and CES enzymes are involved in hydrolysis reactions of compounds containing ester and amide functionality. \cite{42,51} Both biotransformations were observed in the current study, and were the main metabolic products (M1, M2); it can therefore be presumed that these classes of enzymes contributed to the biotransformations that took place. The enzymes also work together to produce metabolic products; for example, several metabolites identified were products of both ester hydrolysis and oxidation of various forms, e.g. monohydroxylation (M7, M9), dihydroxylation (M11, M12), and ketone formation (M10).

To simulate the in-vivo Phase I metabolism of drug products, in-vitro studies using human liver microsomes (HLM) are regularly performed in research laboratories and are reported to be the most popular in-vitro model. \cite{42,51} These studies are typically facilitated by high-resolution mass spectrometry, e.g. time-of-flight (TOF) mass analyzers; this technology provides the added benefit of producing an exact mass and proposed elemental composition. \cite{51,99} HLM are subcellular fractions derived from the endoplasmic reticulum of hepatic cells; they are prepared by liver homogenization with subsequent differential centrifugation. HLM are useful for studying Phase I metabolic processes. To account for inter-individual variations in enzymatic activity, pooled HLM
are used in studies, typically ranging from 50-150 donors. Some researchers have also incubated synthetic cannabinoids with certain CYP (1A2, 2B6, 2C9, 2C19, 2D6, 2E1, 3A4) and CES enzymes (CES1 and CES2) to assess which enzymes are responsible for specific biotransformations. The authors concluded that for the compounds studied, CYP1A2, CYP2C9, CYP3A4, and CES1 were the enzymes that resulted in the greatest degree of hydroxylation, carboxylic acid formation, and hydrolytic activity, respectively. Conversely, some synthetic cannabinoids themselves act as inhibitors of drug-metabolizing CYP enzymes, potentially resulting in toxic accumulation of the drugs.

The metabolism of many synthetic cannabinoids has been determined via HLM incubations. The findings reported in the current study contributes to the body of knowledge available on indazole carboxamide synthetic cannabinoid metabolism via HLM incubations. It also complements the manuscript previously published on the metabolism of MDMB-CHMICA, a related compound which differs in structure only in that it possesses an indole core, while MDMB-CHMINACA has an indazole core (Figure 3.1).

The biotransformations of MDMB-CHMINACA that occurred in the current study are consistent with those of related synthetic cannabinoids reported in the literature. The most common biotransformations identified were hydroxylation, particularly on the CHM moiety, and ester hydrolysis with subsequent hydroxylation and ketone formation steps. This is consistent with metabolites reported for other synthetic cannabinoids bearing a CHM group, e.g. MDMB-CHMICA, MAB-CHMINACA, and AB-
CHMINACA. No biotransformations in this study occurred on the indazole ring itself. The predominant metabolites identified (based on relative abundance) were M1, M2, M8, M10, and M11. Monohydroxylated metabolites of MDMB-CHMINACA (M2) are the most appropriate candidates for inclusion in laboratory screening assays as markers for confirmation of the consumption of this synthetic cannabinoid. CHM-hydroxylated MDMB-CHMINACA metabolites are specific to this synthetic cannabinoid and allow for discrimination between metabolites of structurally-related analogs. The primary biotransformations of MDMB-CHMINACA reported in the current study are consistent with those recently presented by Franz et al. The authors reported monohydroxylation of the CHM moiety and ester hydrolysis as the major metabolic pathways. In addition to these biotransformations, several others were identified and reported in the present study.

As expected, the greatest similarities in the metabolic pathways of MDMB-CHMINACA were observed when compared to MDMB-CHMICA. Most of the predominant metabolite classes (M1, M2, M8, M11) identified for MDMB-CHMINACA were also observed for MDMB-CHMICA, except for a product of ester hydrolysis and ketone formation with dehydrogenation (M10). Other differences in the metabolism of MDMB-CHMICA when compared to MDMB-CHMINACA were: monohydroxylation of the indole ring (no hydroxylation of indazole for MDMB-CHMINACA); formation of ester hydrolysis and dihydroxylation metabolites (similar products were dehydrogenated for MDMB-CHMINACA); amide hydrolysis of carboxamide linkage (not observed for MDMB-CHMINACA).
Both MDMB-CHMINACA and MDMB-CHMICA underwent ester hydrolysis, which is an NADPH-independent biotransformation. In the current study, this metabolite was also identified in the control sample containing MDMB-CHMINACA, buffer solution and HLM, but no NADPH, which activates CYP enzymatic activity. Hydrolysis is common for ester- and amide-containing synthetic cannabinoids; this biotransformation has been attributed to CES and amidase enzymes.\textsuperscript{51–53}

Comparing the metabolic profiles of MDMB-CHMINACA, MAB-CHMINACA and AB-CHMINACA, the major similarity is the production of CHM-hydroxylated metabolites as the major metabolic products and selection of these metabolites for drug screening procedures. MAB-CHMINACA incubation resulted in no production of amide hydrolysis products; AB-CHMINACA, however produced many hydrolyzed metabolites. Differences in the profile of these analytes may be attributed to their differences in structure: MDMB-CHMINACA (ester), MAB-CHMINACA and AB-CHMINACA (amides), as well as the overall incubation process; both MDMB-CHMINACA and MAB-CHMINACA went through HLM incubation, while human hepatocytes were used for MAB-CHMINACA.\textsuperscript{46,53}

MDMB-CHMINACA possesses an array of functional groups, many of which are shared with other synthetic cannabinoids as well as non-drug substances. The metabolic profile identified in this study, therefore, can be used as a predictor for the likely metabolic fate of these compounds. Other CHM-containing synthetic cannabinoids whose metabolic profile has not been reported that may metabolize similarly to MDMB-CHMINACA include: MO-CHMINACA (difference of indazole carboxylate vs.
carboxamide) and APP-CHMINACA (also termed PX-3) (indazole carboxamide with CHM ring) (Figure 3.1). It is likely that these compounds would have hydroxylation of the CHM group as a predominant metabolite class, in addition to ester and amide hydrolysis, respectively.

Similarly, synthetic cannabinoids containing methyl 3,3-dimethylbutanoate (MDMB) and related moieties such as ethyl 3,3-dimethylbutanoate (EDMB) are likely to metabolize similarly to MDMB-CHMINACA. Recent reports from the Center for Forensic Science Research and Education’s NPS Discovery program have demonstrated that synthetic cannabinoids of the indole and indazole carboxamide chemical classes are still the most prevalent classes identified in drug seizures and toxicology casework and continue to emerge.\textsuperscript{104,105} Compounds recently identified include 4-fluoro-MDMB-BINACA (also termed 4F-MDMB-BINACA and 4F-MDMB-BUTINACA) and 5-fluoro-EDMB-PINACA (also termed 5F-EDMB-PINACA), a DEA Schedule I substance (Figure 3.1).\textsuperscript{104,105}

Many hydroxylated metabolites of synthetic cannabinoids exhibit pharmacological activity, binding to and activating the CB1 receptor to induce a greater biological effect than that of THC.\textsuperscript{11,54–56,106} These studies have demonstrated that synthetic cannabinoid metabolites potentially contribute to the overall pharmacological profile and user impairment. To the author’s knowledge, reports on the pharmacological activity of CHM-hydroxylated synthetic cannabinoids are limited. Studies to date have focused predominantly on hydroxylated metabolites of synthetic cannabinoids bearing pentyl chains. This represents a gap in the field of synthetic cannabinoid metabolism.
which needs to be filled in order to have a better understanding of the effects of metabolites of this drug class. Determining the toxicity of the metabolites identified in this study is of clinical and forensic toxicological relevance.

An ester hydrolysis metabolite of MAB-CHMINACA, shared with MDMB-CHMINACA (M1), was found to be pharmacologically active at CB₁ in a study conducted by Cannaert et al. In general, carboxylic acid metabolites of synthetic cannabinoids have not exhibited pharmacological activity; however, this compound is an exception. M1 was also identified in a powdered product seized by Korean customs officials; this represented the first reported instance of a synthetic cannabinoid metabolite identified as the predominant species in a drug product.

More research is needed in the area of synthetic cannabinoid receptor binding, activity and metabolism to fully understand the mechanisms involved. Future studies should include synthesis of metabolites identified in this study in addition to determining their pharmacological activity and effect on drug users who consume MDMB-CHMINACA.

**Conclusions**

This work represents the first extensive report on the Phase I metabolism of MDMB-CHMINACA, a potent indazole carboxamide class synthetic cannabinoid. A total of twenty-seven metabolites were identified via human liver microsome incubation and characterization by high-resolution mass spectrometry. Predominant biotransformations that occurred were hydroxylation and ester hydrolysis. Many
hydrolyzed metabolites underwent subsequent hydroxylation steps, producing an array of metabolites with and without dehydrogenation.

The metabolite types identified were consistent with those reported for other synthetic cannabinoids that share certain structural features: a cyclohexylmethyl (CHM) group; a terminal methyl ester; and a tert-butyl group adjacent to a carboxamide linkage. It is proposed that monohydroxylated metabolites of MDMB-CHMINACA (M2.1 (most abundant) – M2.5) are the most appropriate candidates for inclusion in laboratory screening assays as markers for confirmation of the consumption of this synthetic cannabinoid. CHM-hydroxylated MDMB-CHMINACA metabolites are specific to this synthetic cannabinoid and allow for discrimination between metabolites of structurally-related analogs.

The results of this research can be applied to the prediction of metabolic pathways for synthetic cannabinoids with similar structural features whose metabolism has not yet been determined. Additionally, the results provide researchers with further insight into the metabolic products of CHM-containing synthetic cannabinoids, whose pharmacological activity is currently unknown.
CHAPTER 4
METABOLISM OF APP-CHMINACA (PX-3)

Background

APP-CHMINACA (Figure 4.1), N-(1-amino-1-oxo-3-phenylpropan-2-yl)-1-(cyclohexylmethyl)-1H-indazole-3-carboxamide (common name: PX-3), is a synthetic cannabinoid of the indazole carboxamide class.\(^\text{14}\) The carboxamide group in APP-CHMINACA links the indazole ring system to a 1-amino-1-oxo-3-phenylpropan-2-yl (APP) moiety. The indazole group is substituted at the 1-position with a cyclohexylmethyl (CHM) ring, a common substituent of highly-potent synthetic cannabinoids.\(^\text{13,15}\)

![Chemical structures of synthetic cannabinoids](image)

**Figure 4.1** Chemical structures of synthetic cannabinoids: APP-CHMINACA (PX-3) (a), 5-fluoro APP-PICA (PX-1) (b), 5-fluoro APP-PINACA (PX-2) (c), APP-PICA (d), APP-BINACA (e), and 5-fluoro MPP-PICA (f)

APP-CHMINACA was originally reported by Pfizer in a 2009 patent as a cannabinoid receptor ligand being studied for potential therapeutic benefits; it is referred to as “Example 14” in the patent.\(^\text{4}\) The CB\(_1\) and CB\(_2\) binding affinity (K\(_i\)) and receptor
activation ability of APP-CHMINACA relative to $[^3\text{H}]$CP 55,940 (a full agonist at both receptors) and THC was evaluated by Schoeder et al. Receptor binding studies were performed via a radioligand binding assay, while activation was assessed through a cyclic adenosine monophosphate (cAMP) accumulation assay. $K_i$ values of 9.81 nM at CB$_1$, and 4.39 nM at CB$_2$ were determined for APP-CHMINACA; for $[^3\text{H}]$CP 55,940: 1.28 nM (CB$_1$), 1.42 nM (CB$_2$) and THC: 3.87 nM (CB$_1$), 71.6 nM (CB$_2$). In the cAMP study, APP-CHMINACA activated both receptors at a concentration of 1 µM. Activation at CB$_1$ and CB$_2$ was approximately 75% and 90% (respectively) of the values obtained by $[^3\text{H}]$CP 55,940, demonstrating its agonistic properties. Both receptors were activated by APP-CHMINACA to a greater degree than THC. 

Doi et al. conducted pharmacological evaluations of the (R)- and (S)-enantiomers of APP-CHMINACA to assess the degree to which each molecule activated the CB receptors; activation was determined via a $[^3\text{S}]$guanosine 5’-O-[gamma-thio]triphosphate (GTP$_\gamma$S) binding assay. The concentrations of the APP-CHMINACA enantiomers at which the activation response was 50% of the response of the normalization compound (EC$_{50}$), was determined through this assay; the normalization compound utilized was $[^3\text{H}]$CP 55,940. The study revealed that (S)-APP-CHMINACA functions as an agonist at CB$_1$ (EC$_{50}$: 0.251 µM), while (R)-APP-CHMINACA (EC$_{50}$: 33.7 µM) did not sufficiently activate the CB$_1$ receptor; the (S)-enantiomer was 134-times more potent than the (R)-enantiomer at CB$_1$. At CB$_2$, (S)-APP-CHMINACA (EC$_{50}$: 8.09 nM) demonstrated agonistic properties and was approximately 61-times more potent than the (R)-enantiomer (EC$_{50}$: 0.490 µM). The results showed that the (S)-enantiomer of APP-CHMINACA has
a greater ability to activate cannabinoid receptors than its \((R)\)-isomer and preferentially activates \(\text{CB}_2\) over \(\text{CB}_1\). While \((S)\)-\text{APP-CHMINACA} did activate the receptors, it did so to a lesser degree than the normalization compound \(((^3\text{H})\text{CP 55,940})\). In the study, \(^3\text{H}\text{CP 55,940}\), produced the following results: \(\text{EC}_{50}\) value of 1.28 nM at \(\text{CB}_1\); \(\text{EC}_{50}\) of 0.154 nM at \(\text{CB}_2\), with potencies approximately 196- and 53-times greater than \((S)\)-\text{APP-CHMINACA}, at \(\text{CB}_1\) and \(\text{CB}_2\), respectively.\(^{108}\) Antonides et al. also performed pharmacological evaluations of \((R)\)- and \((S)\)-isomers of indazole carboxamide synthetic cannabinoids to assess their relative potencies at \(\text{CB}_1\) and \(\text{CB}_2\).\(^{20}\) The authors tested \text{AMB-FUBINACA} (\text{FUB-AMB}), 5-fluoro ADB, \text{AB-FUBINACA} and \text{AB-CHMINACA} and found that the \((S)\)-isomers were more potent than the \((R)\)-isomers at both receptors, and preferentially activated \(\text{CB}_2\) over \(\text{CB}_1\).

\text{APP-CHMINACA (PX-3)} is a member of the “\text{PX-series}” of synthetic cannabinoids, which includes 5-fluoro \text{APP-PICA} (\text{PX-1}), and 5-fluoro \text{APP-PINACA} (\text{PX-2}) (Figure 4.1); \text{APP-PICA}, the non-fluorinated version of \text{PX-1} is also similar in structure to these compounds. The main structural difference between \text{APP-CHMINACA, PX-1 and PX-2} is the presence of the \text{CHM ring} versus a fluoropentyl side chain; additionally, \text{PX-1} and \text{APP-PICA} possess an indole core, while \text{PX-2} bears an indazole. \text{APP-CHMINACA} is the most potent of the \text{PX-series} compounds, according to the study conducted by Schoeder et al.\(^{13}\)

\text{APP-CHMINACA in powdered form} was identified in a package labeled “white pigments” seized by Belgian Customs authorities that originated from China. The material was analyzed via gas chromatography-mass spectrometry (GC-MS), high-
resolution mass spectrometry (HRMS), and nuclear magnetic resonance (NMR), Raman and Fourier transform-infrared (FT-IR) spectroscopies. Another indazole carboxamide synthetic cannabinoid, 5F-AMB, was also identified in material contained in this package.¹⁰⁹ The National Forensic Laboratory of Slovenia performed analyses of APP-CHMINACA on material submitted in September 2015, and compiled a monograph of the data for use by forensic scientists; in addition to technologies previously mentioned, this report also included analysis via ion chromatography, GC-IR, and attenuated total reflectance (ATR) FT-IR spectroscopy.¹¹⁰ APP-CHMINACA was identified in drug and paraphernalia samples collected from patients involved in an outbreak of synthetic cannabinoid-facilitated adverse events in Anchorage, Alaska (USA) between July 2015 and May 2016; the occurrence, lasting 245 days, involved a total of 1,351 emergency department visits.¹¹¹ Evaluations of the stability, recovery and detection of APP-CHMINACA in biological matrices, including serum/plasma for method development have also been performed.²³,²⁴

All three PX-series compounds were identified in seized drug casework in 2015, as reported by the United States Drug Enforcement Administration (DEA), totaling 65 identifications.¹¹² PX-1 was identified in a laboratory in Sweden and reported to the European Monitoring Center for Drugs and Drug Addiction (EMCDDA) in 2014¹¹³; it was also reported by the DEA in its 2017 annual report on findings submitted by forensic chemistry testing laboratories in the United States.¹¹⁴ PX-1 was identified in biological specimens collected from intoxicated individuals who presented to an emergency department in Turkey between 2017 and 2018.¹⁹ PX-2 (also termed PPA(N)-2201) was
extracted from seized material and characterized by Qian et al. Recent reports (2018-2019) on the identifications of PX-series synthetic cannabinoids have been compiled by the UNODC Early Warning Advisory on NPS.

Historically, PX-series synthetic cannabinoids were not as prevalent in forensic casework as other drug classes. However, structurally-related compounds are emerging and have been identified in drug seizures and biological specimens; these compounds include the indazole carboxamide APP-BINACA (also termed APP-BUTINACA), and the indole carboxamide 5-fluoro MPP-PICA (5F-MPP-PICA) (Figure 4.1e, 4.1f). APP-BINACA has a butyl side chain and shares the APP group with APP-CHMINACA, while 5F-MPP-PICA possesses a terminal methyl ester and a fluoropentyl side chain. APP-BINACA was detected in post-mortem blood samples in the state of Indiana (USA) and 5F-MPP-PICA was identified in a drug sample submitted by the United States Department of Homeland Security; both compounds were reported in early 2019 by The Center for Forensic Science Research and Education via their NPS Discovery platform.

In this study, the in vitro metabolism of APP-CHMINACA (PX-3) was elucidated. As described in Chapter 2, this was achieved by incubation of the parent molecule with human liver microsomes (HLM) and analyzing resulting incubate extracts via ultra-high performance liquid chromatography – quadrupole time of flight mass spectrometry (UHPLC-QTOF-MS). The metabolites were characterized by examining the product ion spectra and accurate masses generated by QTOF-MS, and evaluation of
the chemical formula produced. This work represents the first detailed report on the metabolic profile and proposed markers of human consumption of APP-CHMINACA.

Results

**Mass Spectral Analysis of PX-3**

A peak in the chromatogram associated with APP-CHMINACA was observed at 9.53 minutes. A precursor accurate mass of m/z 405.2286 ([M+H]+) and the elemental composition C_{24}H_{28}N_{4}O_{2} were generated via high-resolution mass spectral analysis. The product ion mass spectrum and chemical structures of APP-CHMINACA and its most prominent fragments are presented in Figure 4.2. The base peak of the mass spectrum, nominal m/z 241, represents cleavage of the amide bond with the CHM-indazole acylium moiety remaining; m/z 145 corresponds to the indazole acylium ion; m/z 360 is indicative of the loss of the terminal amide group from the precursor structure, and m/z 388 represents loss of amine. The absence of these ions from the product ion spectra of proposed metabolites, or a shift in mass (e.g. increase due to the addition of a new substituent) are indicative of biotransformation and was noted during metabolite characterization. The mass spectrum of APP-CHMINACA was consistent with those previously reported.109,110
Characterization of PX-3 Metabolites

Twelve metabolites were identified in the study, spanning seven different chemical subclasses. Two major biotransformation pathways were observed; the first was hydrolysis of the distal amide moiety; the second, hydroxylation of the CHM ring. Hydrolyzed APP-CHMINACA was subsequently oxidized, generating products of mono- and dihydroxylation, and ketone formation. Three APP-CHMINACA-specific metabolites were produced; one mono-, di-, and trihydroxylated metabolite each was identified.

A summary of the main findings of the current study are presented in Table 4.1. The characterization of each metabolite identified was conducted by evaluating the following: measured accurate precursor mass and calculated mass error (ppm); mass of
product ions and their relation to the parent molecule fragments; elemental composition of precursor and fragments; retention time. The rank (relative abundance) was calculated by dividing the peak areas of each metabolite by the peak area of the most abundant metabolite. No metabolites were identified in the control sample that only contained APP-CHMINACA and buffer solution; this indicates that no hydrolysis occurred as a result of the buffer.

The retention characteristics of the metabolites identified are presented in Figure 4.3, an extracted ion chromatogram (XIC). The product ion spectra, fragmentation pattern and chemical structures of the most-abundant metabolites and an APP-CHMINACA-specific metabolite are presented in Figures 4.4 – 4.6. The precursor molecules and their nominal masses are enclosed in a text box in the figures to differentiate them from the proposed fragment structures. The fragment structures are adjacent to their representative peaks. Similar data for all other metabolites discussed are provided in the Supplementary Materials (Figures 4.S1 – 4.S5).

The metabolic pathways proposed for APP-CHMINACA are presented in Figure 4.7. The exact site of biotransformation was not determined in the current study; potential sites are represented by Markush bonds. In order to elucidate the specific site, subsequent studies would need to be undertaken. Each metabolite and (as applicable) its isomers would need to be synthesized and analyzed chromatographically and via NMR spectroscopy to determine the absolute structure of each metabolite presented in this report.
Figure 4.3 Extracted ion chromatogram of metabolites identified.
<table>
<thead>
<tr>
<th>Metabolite ID</th>
<th>Biotransformation</th>
<th>Retention Time (min)</th>
<th>Elemental Composition</th>
<th>Measured Accurate Mass (m/z)</th>
<th>Mass Error (ppm)</th>
<th>Diagnostic Product Ions</th>
<th>Rank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parent</td>
<td>N/A</td>
<td>9.53</td>
<td>C_{24}H_{23}N_{4}O_{2}</td>
<td>405.2286</td>
<td>0.3</td>
<td>145, 241, 360</td>
<td>N/A</td>
</tr>
<tr>
<td>M1*</td>
<td>Distal Amide Hydrolysis</td>
<td>9.81</td>
<td>C_{24}H_{23}N_{3}O_{3}</td>
<td>406.2130</td>
<td>1.3</td>
<td>145, 241, 360</td>
<td>1</td>
</tr>
<tr>
<td>M2.1</td>
<td>Distal Amide Hydrolysis and Monohydroxylation (CHM)</td>
<td>7.83</td>
<td>C_{24}H_{23}N_{3}O_{4}</td>
<td>422.2074</td>
<td>0.0</td>
<td>145, 257, 376</td>
<td>2</td>
</tr>
<tr>
<td>M2.2</td>
<td>Distal Amide Hydrolysis and Monohydroxylation (CHM)</td>
<td>8.00</td>
<td>C_{24}H_{23}N_{3}O_{4}</td>
<td>422.2074</td>
<td>-0.1</td>
<td>145, 239, 404</td>
<td>4</td>
</tr>
<tr>
<td>M2.3</td>
<td>Distal Amide Hydrolysis and Monohydroxylation (CHM)</td>
<td>8.19</td>
<td>C_{24}H_{23}N_{3}O_{4}</td>
<td>422.2073</td>
<td>-0.3</td>
<td>145, 257, 376</td>
<td>5</td>
</tr>
<tr>
<td>M2.4</td>
<td>Distal Amide Hydrolysis and Monohydroxylation (CHM)</td>
<td>8.44</td>
<td>C_{24}H_{23}N_{3}O_{4}</td>
<td>422.2069</td>
<td>-1.4</td>
<td>145, 257, 376</td>
<td>7</td>
</tr>
<tr>
<td>M2.5</td>
<td>Distal Amide Hydrolysis and Monohydroxylation (CHM)</td>
<td>8.54</td>
<td>C_{24}H_{23}N_{3}O_{4}</td>
<td>422.2074</td>
<td>-0.1</td>
<td>145, 239, 404</td>
<td>3</td>
</tr>
<tr>
<td>M3.1</td>
<td>Distal Amide Hydrolysis and Ketone (CHM) Formation</td>
<td>7.96</td>
<td>C_{24}H_{23}N_{3}O_{4}</td>
<td>420.1915</td>
<td>-0.7</td>
<td>145, 255, 374</td>
<td>9</td>
</tr>
<tr>
<td>M3.2</td>
<td>Distal Amide Hydrolysis and Ketone (CHM) Formation</td>
<td>8.04</td>
<td>C_{24}H_{23}N_{3}O_{4}</td>
<td>420.1921</td>
<td>0.7</td>
<td>145, 255, 374</td>
<td>6</td>
</tr>
<tr>
<td>M4</td>
<td>Distal Amide Hydrolysis and Dihydroxylation (CHM)</td>
<td>7.36</td>
<td>C_{24}H_{23}N_{3}O_{5}</td>
<td>438.2024</td>
<td>0.1</td>
<td>145, 273, 420</td>
<td>8</td>
</tr>
<tr>
<td>M5</td>
<td>Monohydroxylation (CHM)</td>
<td>7.43</td>
<td>C_{24}H_{23}N_{3}O_{3}</td>
<td>421.2234</td>
<td>0.0</td>
<td>145, 257, 376</td>
<td>12</td>
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<td>M6</td>
<td>Dihydroxylation (CHM)</td>
<td>6.62</td>
<td>C_{24}H_{23}N_{3}O_{4}</td>
<td>437.2183</td>
<td>-0.1</td>
<td>145, 273, 392</td>
<td>10</td>
</tr>
<tr>
<td>M7</td>
<td>Trihydroxylation (CHM)</td>
<td>6.47</td>
<td>C_{24}H_{23}N_{3}O_{5}</td>
<td>453.2133</td>
<td>0.1</td>
<td>145, 289, 408</td>
<td>11</td>
</tr>
</tbody>
</table>

**Table 4.1.** Table of metabolites identified and associated biotransformations, average retention time (min), elemental composition, measured accurate mass (m/z), mass error (ppm), diagnostic product ions, and rank (based on relative peak area percent). The metabolite marked with an asterisk represents that it was also identified in the control sample to which no NADPH was added.
The most abundant metabolite identified, M1, was a distal amide hydrolysis product with no further substitution. M1 had a retention time of 9.81 minutes, with a nominal precursor mass of m/z 406. This metabolite was approximately equal in abundance to APP-CHMINACA (Figure 4.3). The only structural difference between M1 and APP-CHMINACA is the presence of a terminal carboxylic acid group in place of amide. The main product ions of M1 were shared with APP-CHMINACA (Figure 4.4). M1 was also identified in the QC sample, indicating that it is an NADPH-independent biotransformation; this is a common occurrence among synthetic cannabinoids and other compounds possessing amide and ester functional groups.51–53,95,102,103,119

![Product ion spectrum and chemical structures of M1 (top right) and fragment ions.](image)

**Figure 4.4** Product ion spectrum and chemical structures of M1 (top right) and fragment ions.
Five metabolites, M2.1-M2.5, were products of distal amide hydrolysis with monohydroxylation on the CHM moiety, each with a precursor mass of m/z 422. The metabolites eluted at 7.83, 8.00, 8.19, 8.44 and 8.54 minutes, respectively. M2.1 was present in the greatest abundance; overall, metabolites of this class were the most numerous of all metabolites identified. Hydroxylation on CHM was verified by the presence of representative ions in its product ion spectrum (Figure 4.5). Specifically, m/z 145, represented intact indazole; the base peak at m/z 257, an increase of m/z 16 (addition of O) relative to m/z 241 present in M1, represented monohydroxylation of CHM; m/z 376, also an increase of m/z 16 relative to m/z 360 in the product ion spectrum of M1 (Figure 4), indicated monohydroxylation. The product ion spectrum presented in Figure 4.5 was observed for M2.1, M2.3 and M2.4; for metabolites M2.2 and M2.5, a similar spectrum was produced – m/z 145, 257 and 376 were present – but the base peak of the product ion spectrum was 239 instead of 257, indicative of the loss of water from the molecule (Figure 4.S1).
Figure 4.5. Product ion spectrum and chemical structures of M2 (top right) and fragment ions

Two minor metabolites were generated, M3.1-M3.2, that were products of distal amide hydrolysis with ketone formation on the CHM moiety. These were the result of further oxidation of M2, from hydroxyl to ketone. The precursor mass was m/z 420, and the metabolites had retention times of 7.96 and 8.04 minutes, respectively. The precursor mass was consistent with loss of H$_2$ from M2, indicating the formation of a double bond. The presence of m/z 145 in the product ion spectrum (Figure 4.S2) was indicative of intact indazole; m/z 255 (a decrease in mass of 2 relative to m/z 257 observed in M2) demonstrated the formation of a ketone; and m/z 374 further verified this finding, as it is also a decrease in mass of 2 relative to m/z 376 in M2.
A minor metabolite, M4, that was the result of further hydroxylation of M2 eluted at 7.36 minutes; it was a distal amide hydrolysis metabolite with dihydroxylation on the CHM moiety and a mass of m/z 438. The m/z 145 ion, representing an intact indazole core group, was present in the product ion spectrum (Figure 4.3); dihydroxylation on CHM was proven by m/z 273, an increase in mass of 16 relative to m/z 257 found in the mass spectrum of M2 (Figure 4.5); m/z 420 represents the loss of water from the molecule.

A monohydroxylated minor metabolite specific to APP-CHMINACA, M5, was observed at 7.43 minutes, and had a precursor mass of m/z 421. Representative fragments present in the product ion spectrum (Figure 4.4) were used to demonstrate that hydroxylation took place on the CHM moiety. Intact indazole was indicated by m/z 145; m/z 257, represented an increase of m/z 16 (gain of O) relative to m/z 241 present in the spectrum of APP-CHMINACA (Figure 4.2), indicating monohydroxylation; m/z 376 was also the result of a gain of m/z 16 in the m/z 360 fragment in APP-CHMINACA. M5 was the least abundant of all APP-CHMINACA-specific metabolites.

The most-abundant APP-CHMINACA-specific metabolite, M6, was a dihydroxylation product, with hydroxylation occurring on the CHM group. The compound had a retention time of 6.62 minutes with a mass of m/z 437. Product ion m/z 273 in the mass spectrum (Figure 4.6) represented dihydroxylation of the CHM group; this was an increase of 16 and 32 mass units relative to m/z 257 and 241 observed in M5 and APP-CHMINACA, respectively. The ion m/z 392 was the result of further hydroxylation of the fragment associated with m/z 376 in M5, and dihydroxylation of the
A minor, trihydroxylated metabolite, M7, with mass m/z 453 was observed at 6.47 minutes. This APP-CHMINACA-specific metabolite had characteristic ions that were indicative of trihydroxylation on the CHM moiety (Figure 4.5). A fragment of m/z 145 indicated that hydroxylation did not take place on indazole; m/z 289 represented trihydroxylation on CHM due to an m/z increase of 16 and 48 mass units relative to the related fragments at m/z 273 and m/z 241 present in the spectra of M6 and APP-CHMINACA, respectively. Trihydroxylation on CHM was further substantiated by the presence of m/z 408, an increase in mass of 16 and 48 relative to m/z 392 and m/z 360 in M6 and APP-CHMINACA, respectively.
Figure 4.7 Proposed metabolic pathway of APP-CHMINACA

Discussion

The metabolism of several indazole carboxamide synthetic cannabinoids has previously been determined and reported, including molecules containing a CHM substituent such as: AB-CHMICA, ADB-CHMICA (MAB-CHMICA), AB-CHMINACA, ADB-CHMINACA (MAB-CHMINACA), AMB-CHMICA, AMB-CHMINACA, MDMB-CHMICA, MDMB-CHMINACA; the current work complements this collection of studies. It is essential that researchers continue to investigate the metabolic and toxicological processes associated with synthetic cannabinoids to develop a greater understanding of what occurs upon ingestion of these compounds and the effects they have on the body. Additionally, these studies allow
reference material vendors to synthesize the appropriate markers of consumption so that method development scientists can produce assays for clinical and forensic applications.

Two major biotransformation pathways were associated with APP-CHMINACA metabolism; namely: hydrolysis of the terminal amide group to carboxylic acid; and hydroxylation of the CHM moiety. These biotransformations are consistent with those reported for structurally-related synthetic cannabinoids. The metabolites identified in the greatest abundance were a distal amide hydrolysis product with no further biotransformation (M1), and a related metabolite featuring distal amide hydrolysis with hydroxylation on the CHM ring (M2.1). APP-CHMINACA-specific metabolites were identified, with a CHM-dihydroxylated product (M6) present in the greatest abundance. It is proposed that these three metabolites are the best markers for confirming consumption of APP-CHMINACA in human biological specimens. To the author’s knowledge, there is currently no synthetic cannabinoid that shares these metabolites, allowing for distinguishing between consumption of APP-CHMINACA and other compounds. The metabolites identified in the current study are consistent with those identified and included in the testing scope established by Staeheli et al.

In general, the major metabolic pathway of synthetic cannabinoids is hydroxylation facilitated by cytochrome P450 (CYP450) enzymes; in addition to hydroxylation, compounds possessing amide and ester groups typically undergo hydrolysis, facilitated by carboxylesterase (CES) or amidase enzymes. Both of these enzymes are present in HLM, making them useful for in vitro metabolism determinations.
APP-CHMINACA metabolized similarly to MDMB-CHMINACA, which also possesses a CHM group and was tested via the same incubation procedure. The two compounds shared the same major metabolic routes (hydrolysis and hydroxylation), though MDMB-CHMINACA possesses a terminal ester instead of amide. In the case of APP-CHMINACA, the prominent metabolites were amide hydrolysis products, the most abundant being hydrolyzed with no further biotransformation. Hydroxylations on APP-CHMINACA all occurred on the CHM ring and included mono-, di-, and trihydroxylation. For MDMB-CHMINACA, the most abundant metabolite was monohydroxylated on CHM. A CHM-dihydroxylated product was also generated for MDMB-CHMINACA, but no trihydroxylation took place. Like APP-CHMINACA, the main ester hydrolysis product also had no further biotransformation.

APP-CHMINACA is structurally-related to PX-1 (5F-APP-PICA), and shared some metabolic features; the two compounds were evaluated using identical procedures. The greatest degree of consistency observed between the two compounds was hydrolysis of the terminal amide group (APP moiety) as one of the most abundant metabolites; unlike PX-1, no hydroxylation of the benzyl group occurred for APP-CHMINACA. Since PX-1 possesses a fluoropentyl side chain, it metabolizes differently than CHM-containing synthetic cannabinoids, including defluorination/hydroxylation on the chain and N-dealkylation as major products.

Additional studies on APP-CHMINACA and other synthetic cannabinoids with limited pharmacological data are warranted. These include evaluations of their
pharmacokinetic and pharmacodynamic properties, in vitro assays to determine the pharmacological activity of the metabolites identified in metabolism studies, and assessments on their interactions with metabolic enzymes. Though carboxylic acid metabolites of synthetic cannabinoids have shown little to no activity at the CB receptors, many hydroxylated metabolites have proven to be receptor agonists, inducing greater effects than THC.\textsuperscript{11,54–56,79,100,122–124} Some synthetic cannabinoids even inhibit drug-metabolizing enzymes, potentially leading to severe toxic effects.\textsuperscript{48} These studies would serve to create a link between the initial identification of synthetic cannabinoids in forensic casework, determination of their metabolic pathway and toxicologic effects, and assessing the effects that synthetic cannabinoids and related NPS have on humans.

**Conclusions**

This work introduces, for the first time, the detailed in vitro Phase I metabolic profile of the cyclohexylmethyl indazole carboxamide synthetic cannabinoid APP-CHMINACA (PX-3). Following incubation with human liver microsomes (HLM), twelve metabolites were generated and characterized via high-resolution mass spectrometry, using a UHPLC-QTOF-MS platform. The predominant biotransformations that occurred in the study were hydrolysis of the distal amide moiety and hydroxylation of the cyclohexylmethyl (CHM) substituent. The metabolites present in the greatest abundance were a distal amide hydrolysis product with no further biotransformation (M1), and a related metabolite, distal amide hydrolysis with hydroxylation on the CHM ring (M2.1). Hydroxylated metabolites specific to APP-CHMINACA were also
identified, with CHM-dihydroxylation (M6) as the most prominent. It is therefore proposed that the most appropriate markers for confirming consumption of APP-CHMINACA are M1, M2.1 and M6; though M1 and M2.1 are more abundant that M6, it can be used to unequivocally determine APP-CHMINACA ingestion. The classes of metabolites identified in the current study are consistent with those reported for related synthetic cannabinoids, specifically those with CHM substituents and distal amide groups. Application of the HLM incubation procedure, mass spectral data collection parameters, and the metabolite characterization process described in this study demonstrates the utility of the method, as it has been used to identify the \textit{in vitro} metabolic pathways of multiple synthetic cannabinoids.
CHAPTER 5

METABOLISM OF 5F-APP-PICA (PX-1)

Background

PX-1 (Figure 5.1), N-(1-amino-1-oxo-3-phenylpropan-2-yl)-[1-(5-fluoropentyl)-1\textit{H}-indole]-3-carboxamide (also termed 5-fluoro APP-PICA; SRF-30), is a synthetic cannabinoid of the indole carboxamide class.\textsuperscript{14,113,125} It is the fluorinated analog of APP-PICA (Figure 5.1). The compound possesses an indole core structure, and a carboxamide linking group, which connects the indole ring to a 1-amino-1-oxo-3-phenylpropan-2-yl (APP) moiety. PX-1 also has a fluoropentyl side chain, which is attached at the nitrogen of the indole ring system. Indole carboxamide synthetic cannabinoids are one of the most prevalent classes of New Psychoactive Substances (NPS), many of which have been reported in the scientific literature.\textsuperscript{14}

\textbf{Figure 5.1} Chemical structures of (a) PX-1, (b) APP-PICA, (c) 5F-MPP-PICA, (d) APP-BINACA, (e) AM-2201, (f) 5F-ADB, (g) 5F-MDMB-PICA, (h) EG-2201, and (i) 5F-CUMYL-PEGACLONE
Studies to determine the CB<sub>1</sub> and CB<sub>2</sub> binding affinities (K<sub>i</sub>) and degree of receptor activation of PX-1 were performed by Schoeder et al.<sup>13</sup> Binding affinities were assessed through a competitive radioligand binding assay with [³H] CP 55,940, a cyclohexylphenol class synthetic cannabinoid and known CB receptor full agonist; receptor activation was tested via a cyclic adenosine monophosphate (cAMP) accumulation assay. The authors reported K<sub>i</sub> values of 485 nM (at CB<sub>1</sub>), and 164 nM (at CB<sub>2</sub>) for PX-1, demonstrating poor binding affinity for both receptors relative to [³H] CP 55,940: 1.28 nM (CB<sub>1</sub>), 1.42 nM (CB<sub>2</sub>) and THC: 3.87 nM (CB<sub>1</sub>), 71.6 nM (CB<sub>2</sub>). In the cAMP study, PX-1 activated both receptors slightly less than [³H] CP 55,940, signifying agonist properties despite its relatively low binding affinity; activation favored the CB<sub>1</sub> receptor. PX-1 activated the receptors to a greater degree than THC in this study.<sup>13</sup>

In November 2014, PX-1 was first reported (as 5F-APP-PICA) to the Early Warning System of the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) by a laboratory in Sweden.<sup>113</sup> In the United States, the Drug Enforcement Administration (DEA) reported multiple PX-1 findings in forensic exhibits between 2015 and 2017 in their publications highlighting emerging synthetic cannabinoids.<sup>112,114</sup>

Urine samples collected from multiple patients with suspected synthetic cannabinoid intoxication who presented to an emergency department in Turkey between 2017 and 2018 were found positive for PX-1.<sup>19</sup> One sample was also positive for synthetic cannabinoid MDMB-CHMICA and metabolites of AB-FUBINACA and ADB-FUBINACA. Studies on the identification and stability of PX-1 in additional biological specimens, including human serum and hair have also been conducted; PX-1 was
incorporated into the associated testing scope of the laboratories performing the evaluation.\textsuperscript{22–24}

PX-1 is similar in structure to synthetic cannabinoids that have more recently emerged. These include 5F-MPP-PICA and APP-BINACA (also termed APP-BUTINACA) (Figure 5.1), as reported by The Center for Forensic Science Research and Education’s NPS Discovery program.\textsuperscript{117,118}

The aim of the current study was to elucidate the \textit{in vitro} Phase I metabolic profile of the synthetic cannabinoid, PX-1, and to provide some basis for the prediction of the metabolic fate of related indole carboxamide synthetic cannabinoid agents. As described in Chapter 2, this was undertaken by incubating the molecule with human liver microsomes (HLM), followed by analysis of the incubates via ultra high performance liquid chromatography – high resolution mass spectrometry (UHPLC-HRMS). The mass analyzer utilized was a quadrupole time of flight mass spectrometer (QTOF-MS). QTOF-MS was used to provide an accurate mass and proposed molecular formula of each metabolite generated. This work summarizes these findings and provides suggested markers for use in laboratory assays to demonstrate consumption of PX-1.

**Results**

**Mass Spectral Analysis of PX-1**

PX-1 eluted at 8.24 minutes. An elemental composition of C\textsubscript{23}H\textsubscript{26}N\textsubscript{3}O\textsubscript{2}F was determined via mass spectral analysis, with a precursor accurate mass of m/z 396.2087 ([M+H]\textsuperscript{+}). The product ion spectrum and structures of the precursor and three fragment
ions are provided in Figure 5.2. The base peak, m/z 232, represents cleavage of the C-N carboxamide bond, and the remaining fluoropentyl indole acylium ion; m/z 144 represents the indole acylium ion (further loss of fluoropentyl); m/z 379 represents the loss of the distal amine.

**Figure 5.2** Product ion spectrum (MS/MS) and structure of PX-1 (top right)

**Characterization of PX-1 Metabolites**

A total of ten (10) metabolites were identified in the current study. The predominant biotransformation that occurred was oxidative defluorination of the side chain. Additional metabolites identified were products of hydroxylation of the indole ring and benzyl moieties; distal amide hydrolysis, including subsequent hydroxylation on the pentyl side chain; N-desfluoropentyl; and a carboxypentyl (pentanoic acid) metabolite.
Three monohydroxylated metabolites that are specific to PX-1 (intact fluoropentyl side chain) were identified.

The findings of the study are summarized in Table 5.1. The identity of all postulated metabolites was derived by evaluating the elemental composition; product ion spectra relative to the parent molecule, PX-1; accurate mass; associated mass error (ppm); and retention time. No metabolites were identified in the control sample containing only PX-1 and buffer solution, demonstrating that metabolism was enzyme-dependent. Four metabolites were identified in the control sample to which drug, buffer and microsomes were added (no NADPH); these metabolites are marked with an asterisk in Table 5.1, indicating that they were present in the both the test samples and the control.

The rank (relative abundance) was determined by dividing the raw peak areas of individual metabolites by the peak area of the most abundant metabolite identified.

An extracted ion chromatogram (XIC) of all metabolites identified in the study is provided in Figure 5.3. For the purposes of this chapter, data for the most prominent metabolites, and those most suitable for drug screening assays will be highlighted. Representative product ion spectra for these metabolites and the structures of their precursor and fragment ions are provided in Figures 5.4 – 5.6. Product ion spectra of the remaining metabolites are presented in Appendix A, Supplementary Figures 5.S1 – 5.S7. The proposed metabolic pathway of PX-1 is presented in Figure 5.7. The exact sites of hydroxylation were not determined in this study and are represented by Markush bonds. In order to verify the specific site, each proposed metabolite and its positional isomers
would need to be synthesized and analyzed, comparing the retention and mass spectral
profiles of the reference material to the data presented in the current study.

**Figure 5.3** Extracted ion chromatogram (XIC) of the metabolites identified
<table>
<thead>
<tr>
<th>Metabolite ID</th>
<th>Biotransformation</th>
<th>Retention Time (min)</th>
<th>Elemental Composition</th>
<th>Measured Accurate Mass (m/z)</th>
<th>Mass Error (ppm)</th>
<th>Diagnostic Product Ions</th>
<th>Rank</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1*</td>
<td>Oxidative Defluorination</td>
<td>7.26</td>
<td>C_{23}H_{27}N_{3}O_{3}</td>
<td>394.2128</td>
<td>0.6</td>
<td>144, 230, 377</td>
<td>1</td>
</tr>
<tr>
<td>M2</td>
<td>N-Desfluoropentyl</td>
<td>6.59</td>
<td>C_{18}H_{17}N_{2}O_{2}</td>
<td>308.1396</td>
<td>0.8</td>
<td>116, 144, 291</td>
<td>2</td>
</tr>
<tr>
<td>M3*</td>
<td>Distal Amide Hydrolysis</td>
<td>8.51</td>
<td>C_{24}H_{25}N_{2}O_{3}F</td>
<td>397.1923</td>
<td>0.3</td>
<td>144, 232, 397</td>
<td>3</td>
</tr>
<tr>
<td>M4*</td>
<td>Distal Amide Hydrolysis and Oxidative Defluorination</td>
<td>7.57</td>
<td>C_{24}H_{25}N_{2}O_{4}</td>
<td>395.1967</td>
<td>0.5</td>
<td>144, 230, 395</td>
<td>4</td>
</tr>
<tr>
<td>M5*</td>
<td>Carboxypentyl</td>
<td>7.21</td>
<td>C_{23}H_{25}N_{3}O_{4}</td>
<td>408.1923</td>
<td>1.3</td>
<td>144, 244, 391</td>
<td>5</td>
</tr>
<tr>
<td>M6</td>
<td>Oxidative Defluorination and Dihydroxylation (Indole and Pentyl Chain)</td>
<td>6.23</td>
<td>C_{23}H_{27}N_{3}O_{4}</td>
<td>410.2074</td>
<td>0.0</td>
<td>160, 246, 393</td>
<td>6</td>
</tr>
<tr>
<td>M7</td>
<td>Oxidative Defluorination and Dihydroxylation (Benzyl and Pentyl Chain)</td>
<td>6.69</td>
<td>C_{23}H_{27}N_{3}O_{4}</td>
<td>410.2076</td>
<td>0.4</td>
<td>144, 230, 393</td>
<td>7</td>
</tr>
<tr>
<td>M8</td>
<td>Monohydroxylation (Indole)</td>
<td>7.13</td>
<td>C_{23}H_{26}N_{3}O_{3}F</td>
<td>412.2034</td>
<td>0.7</td>
<td>160, 248, 395</td>
<td>8</td>
</tr>
<tr>
<td>M9</td>
<td>Monohydroxylation (Fluoropentyl Chain)</td>
<td>7.26</td>
<td>C_{23}H_{26}N_{3}O_{3}F</td>
<td>412.2031</td>
<td>0.1</td>
<td>144, 248, 395</td>
<td>9</td>
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<tr>
<td>M10</td>
<td>Monohydroxylation (Benzyl)</td>
<td>7.78</td>
<td>C_{23}H_{26}N_{3}O_{3}F</td>
<td>412.2034</td>
<td>0.6</td>
<td>144, 232, 395</td>
<td>10</td>
</tr>
<tr>
<td>Parent</td>
<td>N/A</td>
<td>8.24</td>
<td>C_{23}H_{26}N_{3}O_{3}F</td>
<td>396.2087</td>
<td>1.3</td>
<td>144, 232, 379</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Table 5.1 Summary of metabolites identified in test samples and associated biotransformations, average retention time (min), elemental composition, measured accurate mass (m/z), mass error (ppm), diagnostic product ions, and rank (based on relative peak area percent). Metabolites marked with an asterisk were also identified in the control sample to which no NADPH was added.

The most abundant metabolite identified in the study, M1, eluted at 7.26 minutes, with a precursor mass of m/z 394. The base peak at m/z 230 was consistent with the loss of fluorine via enzymatic defluorination and subsequent monohydroxylation of the pentyl side chain (termed oxidative defluorination). This metabolite was also identified in the control sample to which no NADPH was added (drug + buffer + HLM only). The authors
of previous reports conducted on the structurally-related synthetic cannabinoid AM-2201 (Figure 1) suggested that enzymatic defluorination and hydroxylation is facilitated by CYP1A2, CYP2C9 and CYP2E1\textsuperscript{41,101}; hydroxylation of this defluorinated metabolite class most commonly takes place on the terminal carbon (C-5) of the pentyl side chain, known as the omega site, but may also occur to a minor degree on the adjacent carbon (C-4), known as the omega-minus-1 site.\textsuperscript{101,126,127} The presence of m/z 144 represents the presence of intact indole, while m/z 377 suggests loss of the distal amine. The presence of M5 (described below) suggests that hydroxylation takes place at the omega site; additionally, the absence of a defluorinated metabolite further substantiates this claim. The product ion spectrum and structures of associated precursor and fragment ions is presented in Figure 5.4.
The loss of the fluoropentyl chain from PX-1, was the second-most abundant biotransformation observed. M2 was observed at 6.59 minutes and had a precursor mass of m/z 308. The m/z 144 and m/z 116 ions are indicative of an intact indole ring; m/z 263 represents loss of the distal amide, while m/z 291 indicates a loss of the distal amine (See Figure 5.S1).

Hydrolysis of the distal amide resulted in the production of M3 at 8.51 minutes, with a precursor mass of m/z 397. This metabolite was also found in the control sample to which no NADPH was added and is a CYP-independent biotransformation, as reported in other synthetic cannabinoid metabolism studies involving compounds with amide and
ester functionality.\textsuperscript{51-53} The product ions m/z 144 and m/z 232 are consistent with PX-1, indicating no modification of the indole and side chain (See Figure 5.S2).

A product of distal amide hydrolysis, oxidative defluorination, M4, was observed at 7.57 minutes, with a precursor mass of m/z 395. The product ions m/z 144 and m/z 230 were consistent with M1 and represent intact indole and the loss of fluorine and monohydroxylation of the pentyl side chain (See Figure 5.S3). This metabolite was also present in the control sample to which no NADPH was added.

Oxidation of the hydroxyl group in M1 led to the production of M5, a carboxypentyl metabolite. M5 eluted at 7.21 minutes and had a precursor mass of m/z 408. The base peak at m/z 244 represents the carboxypentylindole acylium ion; m/z 144 is indicative of intact indole and m/z 391 represents loss of loss of the distal amine from the precursor. The product ion spectrum and structures of associated precursor and fragment ions is presented in Figure 5.5. This metabolite was also identified in the control sample to which no NADPH was added. Pentanoic acid metabolites are common markers that are incorporated into drug screening assays for synthetic cannabinoids.\textsuperscript{25} The presence of M5 suggests that M1 is hydroxylated at the terminal carbon of the pentyl side chain.
In the defluorinated and dihydroxylated product M6, hydroxylation is present on the indole ring as well as the pentyl side chain. M6, which had a precursor mass of m/z 410, eluted at 6.23 minutes. The presence of m/z 160 and m/z 246 represents hydroxylation on the indole moiety; this was demonstrated by an increase in m/z of 16 relative to indole (m/z 144) and the loss of fluorine and monohydroxylation of the pentyl side chain (m/z 230), as observed in the product ion spectrum of M1. The ion at m/z 393 represents loss of the distal amine (See Figure 5.S4).
Another defluorinated and dihydroxylated metabolite, M7, eluted at 6.69 minutes, having a precursor mass of m/z 410. M7 is hydroxylated on the benzyl moiety and the pentyl side chain. The ion at m/z 144 represents intact indole; m/z 230 and 393 are consistent with the ions observed in M1 and M6, respectively (See Figure 5.5).

PX-1-specific metabolite, M8, which is hydroxylated on the indole ring, eluted at 7.13 minutes. The precursor mass of the metabolite is m/z 412. The m/z 160 fragment represents hydroxylation of indole; the base peak at m/z 248 represents monohydroxylation of the side of the molecule possessing the fluoropentyl side chain (m/z 230); m/z 395 represents loss of the distal amine from the precursor. The product ion spectrum and structures of associated precursor and fragment ions is presented in Figure 5.6. Metabolite M8 was the most abundant PX-1-specific metabolite identified.

![Product ion spectrum and structure of M8 (monohydroxylation (indole)), top right](image)

**Figure 5.6** Product ion spectrum and structure of M8 (monohydroxylation (indole)), top right
Another specific metabolite, M9 was identified, eluting at 7.26 minutes with a precursor mass of m/z 412. Fragment ions m/z 248 and m/z 395 were consistent with those generated by M8 with the exception of m/z 144 produced by M9; this is indicative of intact indole (See Figure 5.S6).

A third PX-1-specific metabolite, M10, was identified, eluting at 7.78 minutes and having a precursor mass of m/z 412. The product ions m/z 144 and m/z 232 are consistent with the parent PX-1, indicating no modification of the indole and side chain. Hydroxylation of M10 occurred on the benzyl moiety; m/z 289 is indicative of the loss of monohydroxybenzyl (See Figure 5.S7).

![Proposed metabolic pathway of PX-1](image)

**Figure 5.7** Proposed metabolic pathway of PX-1
Discussion

The metabolic profiles of several indole carboxamide synthetic cannabinoids possessing fluoropentyl side chains have been reported by Franz et al.\textsuperscript{103} These include 5F-AB-PICA, 5F-ADB-PICA, 5F-AMB-PICA and 5F-MDMB-PICA. The overall metabolic profiles of these analytes are consistent with those identified in the current study; this includes: simultaneous defluorination and monohydroxylation of the fluoropentyl side chain as one of the predominant metabolites; hydroxylation of the fluoropentyl chain with retention of fluorine; and N-dealkylation (N-desfluoropentyl). Carboxypentyl metabolites (e.g. M5) are often produced during the metabolism of synthetic cannabinoids possessing fluoropentyl chains; they are commonly present as a primary metabolite in human urine samples.\textsuperscript{41} This class of metabolites was not reported in the Franz et al.\textsuperscript{103} study, but has toxicological significance and should be considered.

A recent report by Fabregat-Safont et al. validates the primary findings of the current study; the authors determined the \textit{in vitro} metabolism of PX-1 via pooled human hepatocyte incubation over 180 minutes; seven Phase I metabolites were proposed.\textsuperscript{128} The major biotransformations reported by Fabregat-Safont et al. were consistent with those delineated in this report, including the enzymatic defluorination and monohydroxylation of the side chain (M1), amide hydrolysis (M3) and carboxypentyl (M5) as some of the most relevant metabolic products. In addition to these metabolite types, our findings include PX-1-specific metabolites (M8, M9, and M10) which allow laboratories to unequivocally determine PX-1 consumption; these are reported for the first time in this study. A fluorine-containing metabolite was identified by Fabregat-Safont et al.\textsuperscript{128} (M3);
However, this metabolite is potentially shared with 5F-MPP-PICA (Figure 8) as outlined below. M1-M4 reported in our report (non-specific metabolites) were also identified by Cooman and Bell; these were the only metabolites reported. Fabregat-Safont et al. identified five Phase II metabolites corresponding to the Phase I metabolites identified in their study. The primary focus of the current study was on Phase I metabolism in HLM, while the incubation process utilized by Fabregat-Safont et al. also differed. The reports are complementary and demonstrate the usefulness of both HLM and human hepatocyte incubation processes in determining the metabolic profiles of synthetic cannabinoids. HLM are a more cost-effective option for in vitro metabolism studies, and has been successfully applied to the determination of many synthetic cannabinoids.

Other synthetic cannabinoids containing fluoropentyl side chains whose in vitro and/or in vivo metabolic profile has been reported include AM-2201, 5F-ADB, 5F-MDMB-PICA, EG-2201, and 5F-CUMYL-PEGACLONE (Figure 5.1). Structural differences exist between these compounds and PX-1. AM-2201, the first halogenated synthetic cannabinoid identified in drug samples, possesses a naphthyl group linked to indole; 5F-ADB is an indazole carboxamide, while 5F-MDMB-PICA is its indole analog; EG-2201 contains a carbazole core moiety; and 5F-CUMYL-PEGACLONE possesses a γ-carbolinone core group linked to a benzyl derivative (cumene). Despite differences in certain structural components, the overall metabolic profile of these compounds is related to those produced by PX-1 and those described by Franz et al., including the major biotransformations described in the current report. Like PX-1, 5F-ADB and 5F-MDMB-PICA also metabolized to hydrolysis products.
Additionally, carboxypentyl metabolites were reported for AM-2201, 5F-ADB, 5F-MDMB-PICA and 5F-CUMYL-PEGACLONE. The benzyl group of 5F-CUMYL-PEGACLONE was a site of biotransformation, particularly, monohydroxylation, as with PX-1. AM-2201 metabolites are of particular interest, as many are shared with its non-fluorinated analog, JWH-018.\textsuperscript{134} It is proposed, therefore, that similar to AM-2201/JWH-018, the non-fluorinated analog of PX-1, APP-PICA (Figure 5.1) would metabolize similarly and have certain shared metabolites, for example: M1, M2, M4, M5, M6 and M7.

Metabolites of JWH-018 and AM-2201 often selected for inclusion in drug screening procedures include monohydroxylated metabolites (indole or pentyl/fluoropentyl chain), and the carboxypentyl metabolite, which are generally the primary metabolites identified in human urine samples.\textsuperscript{101,127} The author of the current study, therefore, propose M1, M5 and M8 as the most appropriate markers of PX-1 consumption for implementation into laboratory assays. M8 was the most abundant PX-1-specific metabolite identified in the study and was therefore selected as the primary specific metabolite. Though not specific to PX-1, M1 and M5 are useful markers, and can be reported by laboratories as “APP-PICA and PX-1 metabolite” if identifying the exact substance consumed is not imperative. These metabolites were present in greater quantities in vitro than the specific metabolite M8. It should be noted, however, that M8 would need to be reported in order to definitively verify PX-1 consumption.

To the author’s knowledge, clinical studies evaluating the pharmacodynamics and pharmacokinetics of PX-1 have not yet been performed; this information would give
further insight into the effects induced by PX-1. Additionally, determining the specific enzymes responsible for the biotransformations reported in this study by incubation with individual isozymes would complement this work, as would verification of these metabolites in authentic specimens (human urine, blood, etc.). Unfortunately, no authentic specimens were available to us during the course of this work, though multiple attempts to acquire them were made. The monohydroxylated metabolites of many synthetic cannabinoids have been shown to exhibit pharmacological activity, activating the CB1 receptor to induce pharmacological effects to a greater degree than THC. It is of interest, therefore, to gain insights into the toxicity of the metabolites of PX-1 and structurally-related synthetic cannabinoids, as they may contribute to drug user impairment.

Conclusions

This work delineates the in vitro Phase I metabolism of PX-1, a member of the prevalent class of indole carboxamide synthetic cannabinoids. A total of ten metabolites were identified via human liver microsome incubation and characterization by high-resolution mass spectrometry. The primary biotransformation that occurred was oxidative defluorination of the fluoropentyl side chain with subsequent monohydroxylation (M1). Further oxidation of this metabolite led to the formation of dihydroxylated as well as carboxylated metabolites; one such compound was a distal amide hydrolysis (to carboxylic acid) product and the other a pentanoic acid metabolite (M5). Other markers identified included an N-desfluoropentyl metabolite; an amide hydrolysis product
possessing an intact fluoropentyl side chain; and three PX-1-specific (fluorinated) monohydroxylated metabolites (M8, most abundant). Despite differences in certain structural elements between PX-1 and related compounds (those bearing a fluoropentyl side chain), the overall metabolic profile is consistent.

It is proposed that M1, M5, and M8 are the most appropriate candidates for inclusion in laboratory screening assays as markers for confirmation of the consumption of PX-1. This is consistent with markers selected by authors of previously reported metabolism studies on structurally-related compounds. Though less in abundance than M1 and M5, PX-1-specific metabolite M8 allows for discrimination between PX-1 metabolites and those of current structurally-related analogs (e.g. APP-PICA, 5F-MPP-PICA) and those that may soon appear on the illicit drug market.

The results of this research can be applied to the prediction of metabolic pathways for synthetic cannabinoids as well as non-drug substances with similar structural elements whose metabolic profile has not yet been elucidated. Additionally, the results provide reference standard manufacturers and research scientists with further insight into the metabolic products of PX-1 and related compounds for the synthesis of materials for the development of laboratory assays.
CHAPTER 6

METABOLISM OF 5F-ADB AND ANALYSIS OF BLOOD SAMPLES

Background

5F-ADB (Figure 1), methyl 2-[1-(5-fluoropentyl)-1H-indazole-3-carboxamido]-3,3-dimethylbutanoate (also termed 5F-MDMB-PINACA and 5-fluoro ADB), is a synthetic cannabinoid of the indazole carboxamide class.\textsuperscript{14}

![Chemical structures of various synthetic cannabinoids](image)

Figure 6.1 Chemical structures of (a) 5F-ADB, (b) 5F-AMB, (c) 5F-ADB-PINACA, (d) 5F-CUMYL-PICA, and (e) AM-2201

The United States Drug Enforcement Administration (DEA) has classified 5F-ADB as a Schedule I substance, a category into which drugs with no approved medicinal use and a high potential for abuse are placed.\textsuperscript{36} It has undergone international assessment by multinational authoritative agencies including a risk assessment by the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) and the United Nations Office on Drugs and Crime (UNODC). At the time of the EMCDDA risk assessment (2017), 14 Member States had controlled 5F-ADB.\textsuperscript{135} In 2018, 5F-ADB was internationally controlled by the UNODC.\textsuperscript{38}
This synthetic cannabinoid has been prevalent in the illicit drug market worldwide for several years. One of the earliest reports of 5F-ADB identified in non-biological (botanical) and biological samples (fluids and tissues) was based on the autopsy laboratory results published by Hasegawa et al. after a nationwide outbreak of multiple synthetic cannabinoids in Japan in 2014.\textsuperscript{136,137} According to annual reports by the DEA Special Testing and Research Laboratory, between 2016 and 2017, 5F-ADB was one of the most-detected synthetic cannabinoids (top 3) in the United States, and the most prevalent in 2018-2019, totaling approximately 500 cases for all years combined.\textsuperscript{114,138–140} DEA 2018 data are consistent with reports by The Center for Forensic Science Research and Education’s NPS Discovery program, highlighting 5F-ADB as one of the most prevalent compounds in circulation.\textsuperscript{141,142}

5F-ADB was identified in multiple cases of seized herbal mixtures from criminal investigations in Bulgaria between 2017 and 2018 and characterized via mass spectrometry and nuclear magnetic resonance (NMR).\textsuperscript{143} In Kuwait, an investigation of 434 botanical and powder materials containing synthetic cannabinoids provided by customs, drug control and correctional authorities between January and December 2018 was conducted.\textsuperscript{32} The study revealed that 5F-ADB was the most prevalent compound in these seizures, present in 244 cases. 5F-ADB was often found in combination with another synthetic cannabinoid, FUB-AMB, as well as other drugs of abuse including THC, methamphetamine and nicotine. Other uncommon findings reported by Poklis et al. include 5F-ADB in combination with cannabidiol (CBD) and THC in e-liquids.\textsuperscript{144}
Among other synthetic cannabinoids, 5F-ADB was identified in powder and botanical samples seized at the New Zealand border and domestically between January and December 2017 and reported\textsuperscript{145}; of all seizures (184 total), 40\% of the powder samples and 30\% of the botanical samples contained 5F-ADB. Quantitative analyses performed on the botanical samples resulted in an average concentration of 20 mg/g (range: 1-102 mg/g), indicating a lack of homogeneity between samples. In Germany, concentrations of 5F-ADB contained in herbal samples purchased by researchers online in 2018 were reported at 29-41 mg/g.\textsuperscript{130} Antonides et al. reported 10 botanical cases seized between March 2017 and January 2018 which were positive for 5F-ADB; the concentration range reported was <1.0–91.5 mg/g\textsuperscript{20}; chiral chromatographic separation was conducted on the samples and the (S)-enantiomer was the prevalent species identified, with greater than 99\% (S)-enantiomer present in 9 out of 10 cases; the 10\textsuperscript{th} case had 94\% (S)-enantiomer. In the same study the authors reported that the (S)-enantiomer of multiple synthetic cannabinoids (including 5F-ADB) were more pharmacologically active at the CB\textsubscript{1} receptor than the (R)-enantiomer. Evaluations were performed via a G-protein coupled receptor activation assay, monitoring protein-protein interactions via NanoLuc Binary Technology. The EC\textsubscript{50} and E\textsubscript{max}, the concentration of 5F-ADB enantiomer at which the activation response was 50\% of the response of the normalization compound (JWH-018, a known CB receptor agonist), and the efficacy, the maximum response achieved relative to the normalization compound, respectively, were determined. The study revealed that (S)-5F-ADB functions as an agonist at CB\textsubscript{1} (EC\textsubscript{50}: 1.78 nM; E\textsubscript{max}: 331\%), relative to JWH-018 (EC\textsubscript{50}: 45.1 nM; E\textsubscript{max}: 103\%). (R)-5F-ADB
(EC$_{50}$: 131 nM; E$_{\text{max}}$: 180%) did not activate the CB$_1$ receptor as effectively as the (S)-enantiomer; the (S)-enantiomer was approximately 74-times more potent than the (R)-enantiomer at CB$_1$.$^{20}$

Additional studies to determine the CB$_1$ binding affinity (K$_i$) and receptor activation of 5F-ADB were previously conducted by Schoeder et al. and Banister et al., respectively.$^{13,15}$ Binding affinity was determined via competitive binding assays with $[^3\text{H}]$CP 55,940, a cyclohexylphenol class synthetic cannabinoid and known receptor agonist; receptor activation was tested via a fluorometric imaging plate reader (FLIPR) assay. A K$_i$ value of 23.3 nM was reported for 5F-ADB and 1.28 nM for $[^3\text{H}]$CP 55,940, demonstrating that 5F-ADB does not bind to the receptor as strongly. THC was also evaluated in the study, and 5F-ADB bound to CB$_1$ approximately 6-times less strongly (THC Ki: 3.87 nM at CB$_1$). Banister et al. reported an EC$_{50}$ value of 0.59 nM (at CB$_1$) for 5F-ADB. Both CP 55,940 and THC were tested in that study and 5F-ADB was found to be approximately 71- and 290-times more potent that these substances, respectively (CP 55,940 EC$_{50}$: 42 nM; THC EC$_{50}$: 171 nM). These results show that though 5F-ADB did not bind to the receptor as strongly as the other test compounds, it activated the receptor significantly more, and may have greater pharmacological effects in users.

5F-ADB has been implicated in numerous acute toxicity events and fatalities throughout the world. A number of toxicology reports from Bulgaria, Germany, Japan, New Zealand, Spain, Turkey, and the United States have included overdose, drowning, driving under the influence of drugs (DUID), and prison inmate fatalities linked to the consumption of 5F-ADB.$^{3,32,61–63,89,92,97,137,146–152}$ Analysis of human biological specimens
for the presence of synthetic cannabinoids typically entails identifying the parent drug in blood specimens, and/or its metabolites in urine. \(^{25}\) However, this approach is incomplete, as many synthetic cannabinoid metabolites have been determined to be pharmacologically active and also appear in blood samples. \(^{54-56,62,150}\) It is important, therefore, to have a more complete understanding of how synthetic cannabinoid metabolites are distributed in vivo, and whether the predominant metabolites are pharmacologically active.

The aim of the current study was to determine the in vitro metabolic profile of the synthetic cannabinoid 5F-ADB as well as its kinetic properties, namely the microsomal half-life (\(T_{1/2}\)) and intrinsic clearance (\(CL_{\text{int, micr}}\)), human hepatic clearance (\(C_{H}\)) and extraction ratio (ER). Additionally, nine authentic forensic human blood samples provided by NMS Labs that screened positive for 5F-ADB during 2017 and 2018 were analyzed for the presence of the metabolites identified during the metabolic profile elucidation.

As outlined in Chapter 2, 5F-ADB was incubated for 120 minutes with human liver microsomes (HLM) and sampled at various timepoints, followed by analysis of the incubates via ultra high performance liquid chromatography – tandem mass spectrometry (UHPLC-MS/MS). The mass analyzer utilized was a quadrupole time of flight mass spectrometer (QTOF-MS). QTOF-MS was used to provide an accurate mass and proposed molecular formula of each metabolite generated. Blood samples underwent a liquid-liquid extraction prior to analysis by UHPLC-MS/MS. This work summarizes these findings and provides suggestions for biomarkers to use in laboratory assays to
substantiate consumption of 5F-ADB, especially for human blood samples in which the parent drug is not detected.

Results

**Metabolic Stability of 5F-ADB**

The half-life ($T_{1/2}$) of 5F-ADB was 3.1 min ± 0.14 min (mean ± standard deviation; n=3). The in vitro microsomal intrinsic clearance ($CL_{int, micr}$) was 0.271 mL min$^{-1}$ mg$^{-1}$ corresponding to an intrinsic clearance ($CL_{int}$) of 256.2 mL min$^{-1}$ kg$^{-1}$ after scaling to whole liver dimensions. The predicted $CL_H$ was 18.6 mL min$^{-1}$ kg$^{-1}$ with an extraction ratio (ER) of 0.93.

5F-ADB has a relatively short half-life of 3.1 minutes. The high $CL_{int}$ and predicted ER of 0.94 is indicative of a drug that can be rapidly and extensively metabolized in the body with a potential short window of detection of the parent compound in blood and urine. In comparison, the half-lives, $CL_H$, and ER of two other 5-fluoropentyl synthetic cannabinoids, 5F-AMB and 5F-CUMYL-PICA (Figure 6.1), have been reported.$^{68,69}$ 5F-AMB differs from 5F-ADB in that it possesses an isopropyl group instead of a tert-butyl group adjacent to the ester group. 5F-CUMYL-PICA is an indole carboxamide compound with a cumyl group connected at the carboxamide nitrogen. 5F-AMB data are as follows: $T_{1/2}$, 1.0 ± 0.2; $CL_H$, 19.4; and ER, 1.00. 5F-CUMYL-PICA data are as follows: $T_{1/2}$, 1.77; $CL_H$, 19.15; and ER, 0.96. Overall, these values are comparable to 5F-ADB (and within the same order of magnitude), demonstrating that some other 5-fluoropentyl synthetic cannabinoids are also rapidly metabolized.
Mass Spectral Analysis of 5F-ADB

5F-ADB eluted at 9.60 minutes. The elemental composition of $C_{20}H_{28}F_3N_3O_3$ was determined via mass spectral analysis, with a precursor accurate mass of m/z 378.2186 ([M+H]$^+$). The product ion spectrum and structures of the precursor and three fragment ions are provided in Figure 6.2. The base peak, m/z 233 (nominal mass) represents the remaining fluoropentylindazole acylium ion after cleavage of the C-N carboxamide bond; m/z 145 represents the indazole acylium ion (further loss of fluoropentyl); m/z 318 represents the loss of the ester functional group. In the time study, 5F-ADB decreased rapidly in abundance and was undetectable after 15 minutes (see Figure 6.3).

![Product ion spectrum (MS/MS) and structure of 5F-ADB (top left)](image)

Figure 6.2 Product ion spectrum (MS/MS) and structure of 5F-ADB (top left)
Figure 6.3 Plot of the formation of 5F-ADB metabolites over 120 minutes during incubation in HLM. Values represent the mean ± S.E.M. (n=3). The peak area of 5F-ADB is shown on the left y-axis, while the peak area of the metabolites is shown on the right y-axis. The insert above the plot is a zoom-in of the 0-15-minute timeframe.

Characterization of 5F-ADB Metabolites

Seven metabolites were identified in the current study. A variety of biotransformations were observed including oxidative defluorination (M1); carboxypentyl (pentanoic acid) (M2); monohydroxylation of the fluoropentyl chain (M3.1/M3.2) and of the indazole ring system (M4); ester hydrolysis (M5); and ester hydrolysis in combination with oxidative defluorination (M6). Three metabolites
maintained the integrity of the fluoropentyl chain and ester group, (M3.1, M3.2, and M4), and are specific to 5F-ADB. Reference material for M1 and M5 was commercially available and was purchased and analyzed in order verify that the retention times and mass spectra were consistent with metabolites identified during in vitro incubations and in human blood samples.

A plot of the production of metabolites in vitro over the 120-minute incubation period is presented in Figure 6.3. A description of the characteristics of each metabolite and 5F-ADB is provided in Table 6.1. The data are compiled from the 10-minute incubation replicates where all metabolites were present simultaneously. The identity of all postulated metabolites was elucidated by evaluating the elemental composition; product ion spectra relative to the parent molecule, 5F-ADB; measured accurate mass and associated mass error; and retention time. Also included in Table 6.1 are the theoretical logP (octanol/water partition coefficient) values of 5F-ADB and its metabolites calculated using ChemDraw Professional software, version 16.0.1.4 (77).

No metabolites were identified in the control sample containing only 5F-ADB and buffer solution, proving that metabolism was enzyme-dependent. Three metabolites (M1, M5 and M6) were identified in the control sample to which drug, buffer and microsomes were added (no NADPH); these metabolites are marked with an asterisk in Table 6.1, indicating that they were present in the both the test samples and the control. An extracted ion chromatogram (XIC) of a 10-minute incubation sample in which all metabolites were present is provided in Figure 6.4. For the purposes of this chapter, data for the metabolites identified in the human blood samples will be highlighted.
Representative product ion spectra for these metabolites and the structures of their precursor and fragment ions are provided in Figure 6.5 and Figure 6.6. Product ion spectra of the remaining metabolites are presented in Appendix A, Supplementary Figures 6.S1 – 6.S4. The proposed metabolic pathway of 5F-ADB is presented in Figure 6.7. The exact sites of hydroxylation were not determined in this study and are represented by Markush bonds. In order to verify the specific biotransformation site, each proposed metabolite and its positional isomers would need to be synthesized and analyzed, comparing the retention and mass spectral profiles of the reference material to the data presented in the current study.

Figure 6.4 Extracted ion chromatogram (XIC) of the metabolites identified at 10 minutes during HLM incubation
<table>
<thead>
<tr>
<th>Metabolite ID</th>
<th>Biotransformation</th>
<th>Retention Time (min)</th>
<th>Elemental Composition</th>
<th>Measured Accurate Mass (m/z)</th>
<th>Mass Error (ppm)</th>
<th>Diagnostic Product Ions</th>
<th>logP</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1*</td>
<td>Oxidative Defluorination</td>
<td>8.57</td>
<td>C_{20}H_{29}N_{3}O_{4}</td>
<td>376.2228</td>
<td>-0.9</td>
<td>145, 231, 316</td>
<td>2.89</td>
</tr>
<tr>
<td>M2</td>
<td>Carboxypentyl</td>
<td>8.50</td>
<td>C_{20}H_{29}N_{3}O_{5}</td>
<td>390.2017</td>
<td>-1.6</td>
<td>145, 245, 330</td>
<td>2.69</td>
</tr>
<tr>
<td>M3.1</td>
<td>Monohydroxylation (Fluoropentyl Chain)</td>
<td>8.42</td>
<td>C_{20}H_{28}F_{2}N_{3}O_{4}</td>
<td>394.2137</td>
<td>0.1</td>
<td>145, 249, 334</td>
<td>2.48</td>
</tr>
<tr>
<td>M3.2</td>
<td>Monohydroxylation (Fluoropentyl Chain)</td>
<td>8.49</td>
<td>C_{20}H_{28}F_{2}N_{3}O_{4}</td>
<td>394.2137</td>
<td>0.1</td>
<td>145, 249, 334</td>
<td>2.48</td>
</tr>
<tr>
<td>M4</td>
<td>Monohydroxylation (Indazole)</td>
<td>9.23</td>
<td>C_{20}H_{28}F_{2}N_{3}O_{4}</td>
<td>394.2135</td>
<td>-0.3</td>
<td>161, 249, 334</td>
<td>3.21</td>
</tr>
<tr>
<td>M5*</td>
<td>Ester Hydrolysis</td>
<td>8.92</td>
<td>C_{19}H_{27}F_{2}N_{3}O_{3}</td>
<td>364.2031</td>
<td>0.1</td>
<td>145, 233, 318</td>
<td>3.34</td>
</tr>
<tr>
<td>M6*</td>
<td>Ester Hydrolysis and Oxidative Defluorination</td>
<td>7.82</td>
<td>C_{19}H_{27}N_{3}O_{4}</td>
<td>362.2069</td>
<td>-0.3</td>
<td>145, 231, 316</td>
<td>2.63</td>
</tr>
<tr>
<td>Parent</td>
<td></td>
<td>9.60</td>
<td>C_{20}H_{28}F_{2}N_{3}O_{3}</td>
<td>378.2186</td>
<td>-0.3</td>
<td>145, 233, 318</td>
<td>3.60</td>
</tr>
</tbody>
</table>

Table 6.1 Summary of metabolites identified in test samples and associated biotransformations, average retention time (min), elemental composition, measured accurate mass (m/z), mass error (ppm), diagnostic product ions and logP (via ChemDraw). Metabolites marked with an asterisk were also identified in the control sample to which no NADPH was added. (Note: Data are taken from the 10-minute incubation replicates where all metabolites were present simultaneously.)

A product of simultaneous enzyme-mediated defluorination and monohydroxylation, M1 was identified in the study. This metabolite eluted at 8.57 minutes and had a precursor mass of m/z 376. The base peak at m/z 316 was consistent with the loss of the ester group; m/z 231 represents defluorination and simultaneous monohydroxylation of the pentyl side chain; m/z 145 was indicative of unchanged indazole; and m/z 213 represents the loss of water from m/z 231, resulting in the formation of a pentene chain on indazole (Figure 6.5). The retention time and mass spectrum of M1 was consistent with data generated by analysis of the reference standard.
In the time study, M1 was present from 0-15 minutes; its abundance decreased rapidly, and it was not detected for the remainder of the incubation. M1 is a metabolite that is shared with ADB, the non-fluorinated version of 5F-ADB, and if detected in biological specimens would not allow for differentiation between the two species. This metabolite was also identified in the control sample to which no NADPH was added (drug + buffer + HLM only).

Figure 6.5 Product ion spectrum and structure of M1 (oxidative defluorination), middle right

Oxidation of the hydroxyl group in M1 led to the production of M2, a carboxypentyl (pentanoic acid) metabolite. M2 eluted at 8.50 minutes and had a precursor mass of m/z 390. The base peak at m/z 330 represents the loss of the ester group; m/z 245 is attributed to the carboxypentylindazole acylium ion; m/z 145 is indicative of intact indazole. The product ion spectrum is presented in Figure 6.S1. The presence of M2
suggests that M1 is hydroxylated at the terminal carbon of the pentyl side chain. M2 was generated after 5 minutes in the incubation and increased in abundance slightly through 15 minutes and decreased gradually for the remainder of the study (through 120 minutes).

Two metabolites with monohydroxylation on the fluoropentyl side chain, M3.1 and M3.2, were identified at 8.42 and 8.49 minutes, respectively. These are 5F-ADB-specific metabolites and can be used to unequivocally substantiate the consumption of 5F-ADB in human biological specimens. The precursor mass of the M3 metabolites was m/z 394. The base peak of m/z 249 represents the hydroxyfluoropentylindazole acylium ion; m/z 334 represents the loss of the ester group; and m/z 145 indicates intact indazole. The product ion spectrum is presented in Figure 6.2. M3.2 coeluted with M2 (see Figure 6.4) but was differentiable via MS/MS. During the time study, both M3.1 and M3.2 were present from 0-15 minutes; both metabolites increased in abundance at 5 minutes but steadily decreased and were undetectable after 15 minutes. Their detection in the 0-minute samples may be attributed to the rapid biotransformation of 5F-ADB, as the samples were taken immediately after the addition of the NADPH cofactor.

Another 5F-ADB specific metabolite, M4, was identified in the study. This metabolite is hydroxylated on the indazole ring system and eluted at 9.23 minutes, and like M3.1 and M3.2 has a precursor mass of m/z 394. The m/z 161 fragment represents hydroxylation of the indazole; the base peak at m/z 249 represents the fluoropentylhydroxylindazole acylium ion; m/z 334 represents the loss of the ester group. The product ion spectrum is presented in Figure 6.3. In the time study, M4 was present and in low abundance at only two timepoints, 5 and 10 minutes.
Hydrolysis of the terminal ester group resulted in the production of M5 at 8.92 minutes, with a precursor mass of m/z 364. M5 shares common product ions with 5F-ADB; the base peak, m/z 233 represents the remaining fluoropentylindazole acylium ion after cleavage of the C-N carboxamide bond; m/z 145 represents unchanged indazole; m/z 318 represents the loss of the carboxylic acid group (Figure 6.6). The retention time and mass spectrum of M5 was consistent with data generated by analysis of the reference standard. M5 was the only species present throughout the entire 120-minute incubation process; it increased in abundance through 15 minutes, followed by a slight decrease at 30 minutes and leveled off through 120 minutes. This metabolite was also identified in the control sample to which no NADPH was added and is considered a CYP-independent biotransformation.51

![Figure 6.6 Product ion spectrum and structure of M5 (ester hydrolysis), top left](image-url)
Further metabolism of M1 or M5 (see Figure 6.7) led to the production of M6, which was observed at 7.82 minutes, with a precursor mass of m/z 362. The base peak at m/z 316 represents the loss of the carboxylic acid moiety; m/z 231 represents oxidative defluorination; m/z 145 is indicative of unchanged indazole (Figure 6.S4). This metabolite was also present in the control sample to which no NADPH was added. M6 was present throughout almost the entire 120-minute incubation process; it appeared at 5 minutes and increased slightly throughout 120 minutes.

FIGURE 6.7 Proposed metabolic pathway of 5F-ADB
Identification of Metabolites in Blood Samples

Authentic forensic human blood samples (n=9) were provided by NMS Labs, a private forensic toxicology laboratory in Horsham, PA, USA. The samples were submitted to NMS during 2017 and 2018 and screened positive for 5F-ADB. The goal of the current study was to screen for the presence of 5F-ADB metabolites identified in vitro in the blood samples. A summary of the findings and demographic information for the samples is outlined in Table 6.2. Neither 5F-ADB nor its metabolites were identified in the blank blood control. Additionally, all analytes were recovered in the positive control.

All nine samples tested positive for M1; this was the most-abundant substance identified in each sample. Two samples also contained M5, present at 33-35% abundance relative to M1; one sample was positive for 5F-ADB, M1 and M5 (Figure 6.8). All three substances are pharmacologically active at CB₁/CB₂ receptors and M1 and M5 may contribute to a user’s impairment profile.15,56,122 The metabolites identified in the samples were consistent with the retention time and mass spectra of reference standards purchased for the study. No other metabolites were identified in the samples.
**Figure 6.8** Extracted ion chromatogram of Sample 9, positive for 5F-ADB, M1 and M5

<table>
<thead>
<tr>
<th>Sample Number</th>
<th>Matrix Type</th>
<th>Case Type</th>
<th>Gender</th>
<th>Age</th>
<th>Findings</th>
<th>Relative Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Blood (NS)</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>M1</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>Femoral Blood</td>
<td>Postmortem</td>
<td>Male</td>
<td>24</td>
<td>M1</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>Blood (NS)</td>
<td>DUID</td>
<td>Male</td>
<td>30</td>
<td>M1</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>Central Blood</td>
<td>Postmortem</td>
<td>Female</td>
<td>NS</td>
<td>M1</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>Blood (NS)</td>
<td>DUID</td>
<td>Female</td>
<td>48</td>
<td>M1</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>Cardiac Blood</td>
<td>Postmortem</td>
<td>NS</td>
<td>NS</td>
<td>M1</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>Heart Blood</td>
<td>Postmortem</td>
<td>NS</td>
<td>42</td>
<td>M1</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>Blood (NS)</td>
<td>DUID</td>
<td>Male</td>
<td>26</td>
<td>M1 M5</td>
<td>100% 33%</td>
</tr>
<tr>
<td>9</td>
<td>Blood (NS)</td>
<td>DUID</td>
<td>Male</td>
<td>25</td>
<td>M1 M5 5F-ADB</td>
<td>100% 35% 11%</td>
</tr>
</tbody>
</table>

**Table 6.2** Summary of findings and demographic information from nine authentic blood samples tested. (Acronym key: DUID – Driving under the influence of drugs; NS – not specified)
Multiple samples contained trace amounts of 5F-ADB, but due to poor chromatographic and/or mass spectral data, it was not identified. These are important findings, as they provide examples of instances in which the parent compound is undetectable, potentially due to a short half-life or instrument limitations. However, the metabolites of the parent compound may be present in much greater quantities.

**Discussion**

Earlier in vitro studies conducted on the structurally-related synthetic cannabinoid AM-2201 (Figure 6.1) suggested that oxidative defluorination was facilitated by CYP1A2, CYP2C9 and CYP2E1.\textsuperscript{41,101} However, additional studies on AM-2201 by Holm et al.\textsuperscript{153} showed that it is possible to produce the oxidative defluorination product in HLM without the addition of NADPH as a primary metabolite. A related compound, THJ-2201 which also possesses a 5-fluoropentyl chain, was incubated with recombinant enzymes and the CYP2B6 and CYP2C19 types proved to be the most effective at producing the oxidative defluorination product. These studies demonstrate that there may be multiple routes to the production of M1, both in the presence and absence of NADPH. To the author’s knowledge, the non-CYP enzyme responsible for this biotransformation has not been reported to date. Hydroxylation of this defluorinated metabolite class most commonly takes place on the terminal carbon (C-5) of the pentyl side chain, known as the omega site, but may also occur to a minor degree on the adjacent carbon (C-4), known as the omega-minus-1 site.\textsuperscript{101,126,127}
Cannabinoid receptor CB₁ and CB₂ binding affinity (Kᵢ), potency and efficacy (EC₅₀ and Eₘₐₓ) studies were conducted on M1 along with other synthetic cannabinoids and corresponding metabolites by Gamage et al.⁵⁶ The competitive binding and receptor activity were assessed against a known CB₁ and CB₂ receptor agonist, CP 55,940. In the study, 5F-ADB was observed to possess a strong binding affinity to both CB₁ and CB₂ receptors at 0.692 nM and 0.677 nM, respectively. The potency and associated efficacy reported at CB₁ and CB₂ was 0.294 nM (111%) and 0.469 nM (103%), respectively. The values achieved for M1 at CB₁ were: Kᵢ: 37.1 nM; EC₅₀: 6.46 nM; Eₘₐₓ: 110%; at CB₂: Kᵢ: 7.49 nM; EC₅₀: 3.33 nM; Eₘₐₓ: 85%. In comparison, CP 55,940, a known CB₁ receptor agonist, at CB₁: Kᵢ: 1.25 nM; EC₅₀: 0.359 nM; Eₘₐₓ: 95%; at CB₂: Kᵢ: 1.15 nM; EC₅₀: 0.294 nM; Eₘₐₓ: 92%. These data show that M1 does not bind to nor activate the CB receptors as effectively as full agonists (5F-ADB and CP 55,940), and binds preferentially to CB₂, activating this receptor to a greater degree than CB₁. However, the efficacy of M1 was comparable to 5F-ADB and CP 55,940 at CB₁ and cannot be discounted in its ability to contribute to a user’s overall impairment profile.

Pentanoic acid metabolites are common markers that are incorporated into drug screening assays for synthetic cannabinoids, particularly for urine analyses. It was observed in previous in vitro studies using a human hepatocytes model that synthetic cannabinoid pentanoic acid metabolites undergo Phase II biotransformation, i.e. glucuronidation and are frequently identified in human urine samples.⁶⁸,⁹⁷,¹²⁷ M2 may therefore be a reliable urinary biomarker for 5F-ADB intake.
The binding affinity, potency and efficacy of M3 (omega-minus-1 site) was also determined by Gamage et al.\textsuperscript{56} The values achieved for M3 at CB\textsubscript{1} were: \(K_i\): 19.8 nM; \(EC_{50}\): 4.77 nM; \(E_{\text{max}}\): 91%; M3 binds more tightly to CB\textsubscript{1} than M1, and is also slightly more potent and less efficacious. Like M1, its ability to contribute to a user’s overall impairment profile should be considered.

Hydrolyzed metabolic products have been reported in other synthetic cannabinoid metabolism studies involving compounds with ester and amide functional groups.\textsuperscript{52,53,70–72} Thomsen et al.\textsuperscript{52} reported that synthetic cannabinoids can be hydrolyzed by carboxylesterase type-1 (CES1) and type-2 (CES2) enzymes.

A study to determine the potency and efficacy of M5 at CB\textsubscript{1} was conducted by Wouters et al.\textsuperscript{122} The values achieved for M5 were: \(EC_{50}\): 113 nM; \(E_{\text{max}}\): 246%. For JWH-018, a known CB\textsubscript{1} receptor agonist, \(EC_{50}\): 36.8 nM; \(E_{\text{max}}\): 102%. These data show that M5 is less potent than JWH-018 but has greater efficacy and may contribute to a user’s impairment profile. M5 is a metabolite that is shared with 5F-ADB-PINACA\textsuperscript{122} (Figure 6.1); this is important to note since if it is used to screen samples, the exact source would not be differentiable in the absence the parent compound or compound-specific markers.

The metabolites of 5F-ADB identified in the current study are consistent with those recently reported by Yeter and Öztürk\textsuperscript{97} and Franz et al.\textsuperscript{103} Yeter and Öztürk reported all of the metabolites identified in this study with the exception of M4, while Franz et al. reported all metabolites except M2 and M6. Metabolites containing carboxylic acid groups, namely M2, M5 and M6 were detected at later timepoints than
hydroxylated metabolites M1, M3 and M4, potentially resulting in longer detection windows in human samples.

It is common for synthetic cannabinoid-positive blood samples to contain more metabolite than parent compound. Similar to the results of the current study, this phenomenon was observed in an evaluation of 600 blood samples by Öztürk et al.\textsuperscript{57} in which the parent compound was present in only 3 samples, but the hydroxylated metabolite and pentanoic acid metabolite were present in 92 and 145 samples, respectively.

In laboratory assay panels that do not include relevant synthetic cannabinoid primary metabolites, a sample screen could miss markers that point to a causative agent. It is therefore proposed that in the instance of 5F-ADB, M1 and M5 are appropriate markers to include in laboratory blood tests which screen for 5F-ADB. This is consistent with a recent report by Krotulski et al.\textsuperscript{58} who suggested that M5 be included in test panels. It is imperative that synthetic cannabinoid assay panels include metabolites, especially known pharmacologically active metabolites; this is particularly important for drugs with short half-lives.

Reports on the identification of synthetic cannabinoid metabolites in blood are currently limited, but awareness of the necessity of this practice appears to be increasing. In the instance of 5F-ADB, multiple reports on the identification of M5 in blood have been published\textsuperscript{61,62,146–148,150}, however, the author only found one published report on the identification of M1 in blood.\textsuperscript{149}
The presence of M1 and M5 in blood samples may be attributed to CES enzymes present in the blood that rapidly metabolize 5F-ADB, an ester group-containing molecule.\textsuperscript{154} Another consideration is CYP and CES enzymes that may be present in lung tissues that metabolize 5F-ADB upon smoking.\textsuperscript{25,52,134} Additionally, the production of breakdown products and pyrolysis products (from easily-hydrolyzable synthetic cannabinoids) that have an identical chemical structure as hydrolysis metabolites has been investigated by Franz et al.\textsuperscript{155} This complicates the interpretation of toxicological analyses, as it has become increasingly difficult to determine which compounds identified in biological samples are due to breakdown, pyrolysis and true metabolism.

Synthetic cannabinoids range from slightly to highly lipophilic compounds.\textsuperscript{156} Several compounds, including 5F-ADB, have been reported to be stored in fatty tissues upon consumption, as described in multiple autopsy reports.\textsuperscript{136,157–160} The logP of the compounds evaluated in the current study are reported in Table 6.1, ranging from 2.48–3.60. Though 5F-ADB and its metabolites are theoretically not highly lipophilic relative to logP values reported for other synthetic cannabinoids, Hasegawa et al.\textsuperscript{136} reported the identification of 5F-ADB in multiple biological tissues including brain, adipose, lung, heart muscle, liver, spleen, skeletal muscle and pancreas. What is of interest is whether the metabolites of 5F-ADB can effectively cross the blood-brain barrier and induce effects like those of the parent drug.
Conclusions

This work describes the in vitro Phase I metabolism of 5F-ADB, a synthetic cannabinoid of the prevalent indazole carboxamide class. In total, seven metabolites were identified via human liver microsome incubations and characterization by high-resolution mass spectrometry. 5F-ADB is rapidly and extensively metabolized, as demonstrated by its short half-life of 3.1 minutes; hepatic clearance of 18.6 mL min\(^{-1}\) kg\(^{-1}\), and extraction ratio of 0.93, as reported for the first time in this study. The biotransformation products identified included oxidative defluorination; monohydroxylation of the fluoropentyl chain and indazole ring system; carboxypentyl (pentanoic acid) formation; ester hydrolysis; and ester hydrolysis with oxidative defluorination. It was observed that the metabolites containing carboxylic acid functional groups were detected in incubation samples longer than the hydroxylated metabolites, potentially indicative of longer detection windows in human samples. Authentic human blood samples were screened for the presence of the metabolites identified in vitro; M1 and M5 were identified in these samples, with M1 as the most abundant species, often in the absence of parent drug. It is proposed that M1 and M5 are useful markers for inclusion in laboratory blood tests that screen for 5F-ADB, as both are pharmacologically active, may appear in samples in which the parent drug is undetectable and can point to the causative agent. It is imperative that synthetic cannabinoid assay panels include metabolites, especially known pharmacologically active metabolites, especially for compounds with short half-lives.
CHAPTER 7

METABOLISM OF FUB-AMB AND ANALYSIS OF BLOOD SAMPLES

Background

FUB-AMB, methyl N-{1-[(4-fluorophenyl)methyl]-1H-indazole-3-carbonyl}-L-valinate, (also termed AMB-FUBINACA and MMB-FUBINACA) (Figure 7.1), is an indazole carboxamide class synthetic cannabinoid.\(^{14}\)

![Chemical structures of (a) FUB-AMB, (b) AB-FUBINACA, (c) FUB-AMB/AB-FUBINACA 3-methylbutanoic acid metabolite, and (d) FUB-AMB monohydroxylated metabolite](image)

**Figure 7.1** Chemical structures of (a) FUB-AMB, (b) AB-FUBINACA, (c) FUB-AMB/AB-FUBINACA 3-methylbutanoic acid metabolite, and (d) FUB-AMB monohydroxylated metabolite

It was originally reported by Pfizer in a 2009 patent as a cannabinoid receptor ligand being studied for potential therapeutic benefits.\(^{161}\) The United States Drug Enforcement Administration (DEA) has classified FUB-AMB as a Schedule I substance, the most highly-restricted chemical class, with no approved medical use and a high potential for abuse.\(^{37}\) The World Health Organization (WHO) recognized FUB-AMB as a drug that has had a negative impact on human health on a global level, and cited numerous fatalities and severe intoxications experienced by consumers.\(^{162}\)

Based on reports issued by the DEA Special Testing and Research Laboratory, FUB-AMB was the most-detected synthetic cannabinoid in the United States between 2016 and 2018, and was also reported numerous times in 2019; a total of 857 cases were
reported during these years.\textsuperscript{114,138–140} FUB-AMB has been identified in a variety of matrices including seizures of botanical materials, powders, infused papers, both from the justice system and online (dark web) in Germany, Kuwait, New Zealand, Turkey and the United Kingdom.\textsuperscript{32,35,145,163–166}

FUB-AMB has been implicated in numerous acute intoxications and fatalities, including co-ingestion with additional synthetic cannabinoids.\textsuperscript{89,147,149,151,152,167} One of the first outbreaks associated with FUB-AMB occurred in 2016 in New York where 33 people were intoxicated within a one-block radius, 18 of whom presented to the emergency room.\textsuperscript{16} The measured concentration of FUB-AMB in herbal materials collected from patients ranged from 14.2 – 25.2 mg/g. FUB-AMB was also identified in products consumed during an outbreak of coagulopathy in the state of Illinois, USA in 2018, when rodenticides brodifacoum, difenacoum and bromadilone were added to the herbal mixtures.\textsuperscript{168,169} During toxicology testing, FUB-AMB and its ester hydrolysis metabolite were identified in many of the samples collected from affected patients during this outbreak.

Studies to determine the CB$_1$ and CB$_2$ binding affinity (K$_i$) and receptor activation of FUB-AMB were conducted by Banister et al. and Schoeder et al., respectively.\textsuperscript{13,15} Binding affinity was determined via competitive binding assays with [$^3$H]CP 55,940, a cyclohexylphenol class synthetic cannabinoid and known CB$_1$ receptor agonist; receptor activation was tested via a fluorometric imaging plate reader (FLIPR) assay. K$_i$ values of 0.387 nM and 0.536 nM at CB$_1$ and CB$_2$, respectively were reported for FUB-AMB and 1.28 nM and 1.42 nM for [$^3$H]CP 55,940, demonstrating that FUB-AMB binds more
strongly to both receptors. THC was also evaluated in the study, and FUB-AMB bound to
CB₁ and CB₂ approximately 10-times and 133-time more strongly, respectively (THC Ki:
3.87 nM at CB₁ and 71.6 nM at CB₂).¹³ Banister et al. reported an EC₅₀ value of 2.0 nM
(at CB₁) and 18.0 nM (at CB₂) for FUB-AMB. Both CP 55,940 and THC were tested in
that study and FUB-AMB was found to be approximately 21- and 4-times more potent at
CB₁ and CB₂, respectively than CP 55,940; for THC, FUB-AMB was approximately 86-
times more potent at CB₁ (THC activity at CB₂ was not evaluated).¹⁵ Based on these data,
FUB-AMB is likely to induce a greater pharmacological effect in users.

To determine whether acute toxicity or impairment can be attributed to a
causative agent like synthetic cannabinoids, laboratories recommend sampling blood to
screen for the parent compound, while metabolites are often detected in urine. However,
this approach may overlook the possible prolonged toxicity from pharmacologically
active synthetic cannabinoid metabolites in blood.²⁵,⁵⁴-⁶,¹⁷⁰ It is important, therefore, to
gain a more complete understanding of how synthetic cannabinoid metabolites are
distributed in vivo, and whether the predominant metabolites are pharmacologically
active.

Other authors have conducted in vitro evaluations of FUB-AMB metabolism and
found that the ester hydrolysis metabolite was the most abundant metabolite generated in
vitro; this included studies in human hepatocytes, human liver microsomes and
zebrafish.¹⁰³,¹²⁸,¹⁷¹ However, none of these studies analyzed authentic human samples
from FUB-AMB users to verify the presence of this metabolite. The aim of the current
study was to verify these findings, to assess the stability of FUB-AMB and assess in vivo
if the major metabolites were also present in twelve authentic human blood samples provided by NMS Labs, a private forensic toxicology laboratory in Horsham, PA, USA. The samples screened positive for FUB-AMB during 2017 and 2018, and were stored under frozen conditions until evaluated in this study. Additionally, the metabolism kinetics of FUB-AMB was assessed to determine the rate of disappearance and the production of the major metabolites.

As described in Chapter 2, FUB-AMB was incubated for 120 minutes with human liver microsomes (HLM) and sampled at various timepoints, followed by analysis of the incubates via ultra high performance liquid chromatography – tandem mass spectrometry (UHPLC-MS/MS). A quadrupole time of flight mass spectrometer (QTOF-MS) was used to provide an accurate mass and proposed molecular formula for the parent compound and metabolite generated. Blood samples underwent a liquid-liquid extraction prior to analysis by UHPLC-MS/MS. Reference material for M1 was commercially available and was purchased and analyzed in order to verify that the retention time and product ion spectrum were consistent with the species identified during in vitro incubations and in human blood samples.

This work summarizes the findings and provides suggestions for modifications to the current practice of forensic and clinical toxicology laboratories as it pertains to testing for synthetic cannabinoids in human biological specimens, especially for human blood samples in which the parent drug is not detected.
**Results**

A plot of the production of the major metabolite (M1) and decrease in abundance of FUB-AMB over the 120-minute incubation period is presented in Figure 7.3. A summary of the characteristics of FUB-AMB and M1 are provided in Table 7.1, and includes the measured accurate mass and associated mass error; retention time; and diagnostic product ions. These data are compiled from the 0-minute incubation replicates where both species were present simultaneously. Also included in Table 7.1 are the theoretical logP values (log of the octanol/water partition coefficient) of FUB-AMB and M1, calculated using ChemDraw Professional software, version 16.0.1.4 (77).

No metabolites were identified in the control sample containing only FUB-AMB standard and buffer solution, demonstrating that metabolism was enzyme-dependent. M1 was identified in the control sample to which drug, buffer and microsomes were added (no NADPH); this indicates that this biotransformation is NADPH-independent. Representative extracted ion chromatograms of FUB-AMB and M1 in HLM and of M1 in the authentic blood samples are presented in Figure 7.5.

**Mass Spectral Analysis of FUB-AMB**

FUB-AMB was retained for 9.40 minutes, with a precursor accurate mass of m/z 384 ([M+H]+). The product ion spectrum and chemical structures of the precursor and three fragment ions are provided in Figure 7.2. The base peak, m/z 253, represents the remaining fluorobenzylindazole acylium ion after cleavage of the C-N carboxamide bond; m/z 109 represents the fluorobenzyl ion; m/z 324 represents the loss of the methyl
ester functional group; m/z 352 is indicative of the loss of methoxy from the molecule. In the time study, FUB-AMB was rapidly metabolized and was undetectable after 5 minutes (see Figure 7.3).

**Figure 7.2** Product ion spectrum (MS/MS) and chemical structure of FUB-AMB (top left)
Figure 7.3 Plot of the formation of M1 over 120 minutes during incubation in HLM. Values represent the mean ± S.E.M. (n=3). Also shown is the decrease in abundance of FUB-AMB over time

Characterization of FUB-AMB Metabolite (M1)

Hydrolysis of the ester group of FUB-AMB resulted in the production of M1, the 3-methylbutanoic acid metabolite, which eluted at 8.74 minutes, with a precursor mass of m/z 370. M1 shares common product ions with FUB-AMB (see Figure 7.4), the main differences being that m/z 324 and m/z 352 represent loss of carboxylic acid and water from the molecule, respectively. The retention time and mass spectrum of M1 was consistent with data generated by analysis of the reference standard.
M1 was present throughout the entire 120-minute incubation process; it increased in abundance through 10 minutes, and remained relatively consistent through 120 minutes (see Figure 7.3). This metabolite was also identified in the control sample to which no NADPH was added and is considered a NADPH-independent biotransformation. An XIC of M1 and FUB-AMB in HLM at the 0-minute time point is presented in the left panel of Figure 7.5. Additionally, in a study by Xu et al., further glucuronidation of M1 was observed, similar to what was detected in authentic urine samples.49,172
Figure 7.5 Extracted ion chromatogram (XIC) of FUB-AMB and M1 identified at 0 minutes during HLM incubation (left); extracted ion chromatogram of Sample 1 (blood), positive for M1 only (right)

<table>
<thead>
<tr>
<th>ID</th>
<th>Retention Time (min)</th>
<th>Elemental Composition</th>
<th>Measured Accurate Mass (m/z)</th>
<th>Mass Error (ppm)</th>
<th>Diagnostic Product Ions</th>
<th>logP</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1*</td>
<td>8.74</td>
<td>C₂₀H₂₀FN₃O₃</td>
<td>370.1561</td>
<td>-0.2</td>
<td>109, 253, 324</td>
<td>3.52</td>
</tr>
<tr>
<td>FUB-AMB (Parent)</td>
<td>9.40</td>
<td>C₂₁H₂₂FN₃O₃</td>
<td>384.1719</td>
<td>0.3</td>
<td>109, 253, 324</td>
<td>3.79</td>
</tr>
</tbody>
</table>

Table 7.1 Summary of data obtained for FUB-AMB and its ester hydrolysis metabolite FUB-AMB 3-methylbutanoic acid, identified in test samples. Information includes the average retention time (min), elemental composition, measured accurate mass (m/z), mass error (ppm), diagnostic product ions and logP (via ChemDraw). The asterisk represents that the metabolite was also identified in the control sample to which no NADPH was added. (Note: data are taken from the 0-minute incubation replicates)

Identification of M1 in Blood Samples

Authentic forensic human blood samples (n=12) were provided by NMS Labs for the current research project. The samples were submitted to NMS during 2017 and 2018 and screened positive for FUB-AMB (qualitative LC-MS/MS analysis; reporting limit of
0.1 ng/mL). The goal of the current study was to screen for the presence of M1 in the blood samples, and to determine if FUB-AMB was still detectable. A summary of the findings and demographic information for the samples is outlined in Table 7.2. Neither FUB-AMB nor M1 were identified in the blank blood control. Additionally, all analytes listed in Section 2.3 were recovered in the positive control.

All twelve samples tested positive for M1. Multiple samples showed trace amounts of FUB-AMB, but due to poor chromatographic and/or mass spectral data, it was not reported as positive. A representative XIC of M1 in an authentic blood specimen (Sample 1) is presented in the right panel of Figure 7.5.

<table>
<thead>
<tr>
<th>Sample Number</th>
<th>Matrix Type</th>
<th>Case Type</th>
<th>Gender</th>
<th>Age</th>
<th>Findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Peripheral Blood</td>
<td>Postmortem</td>
<td>Male</td>
<td>26</td>
<td>M1</td>
</tr>
<tr>
<td>2</td>
<td>Blood (NS)</td>
<td>DUID</td>
<td>Male</td>
<td>51</td>
<td>M1</td>
</tr>
<tr>
<td>3</td>
<td>Blood (NS)</td>
<td>DUID</td>
<td>Male</td>
<td>26</td>
<td>M1</td>
</tr>
<tr>
<td>4</td>
<td>Femoral Blood</td>
<td>Postmortem</td>
<td>NS</td>
<td>NS</td>
<td>M1</td>
</tr>
<tr>
<td>5</td>
<td>Femoral Blood</td>
<td>Postmortem</td>
<td>Male</td>
<td>21</td>
<td>M1</td>
</tr>
<tr>
<td>6</td>
<td>Heart Blood</td>
<td>Postmortem</td>
<td>Male</td>
<td>45</td>
<td>M1</td>
</tr>
<tr>
<td>7</td>
<td>Blood (NS)</td>
<td>NS</td>
<td>Male</td>
<td>35</td>
<td>M1</td>
</tr>
<tr>
<td>8</td>
<td>Blood (NS)</td>
<td>DUID</td>
<td>Male</td>
<td>30</td>
<td>M1</td>
</tr>
<tr>
<td>9</td>
<td>Femoral Blood</td>
<td>Postmortem</td>
<td>Male</td>
<td>26</td>
<td>M1</td>
</tr>
<tr>
<td>10</td>
<td>Blood (NS)</td>
<td>DUID</td>
<td>Male</td>
<td>22</td>
<td>M1</td>
</tr>
<tr>
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<td>Blood (NS)</td>
<td>NS</td>
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<td>22</td>
<td>M1</td>
</tr>
<tr>
<td>12</td>
<td>Femoral Blood</td>
<td>Postmortem</td>
<td>Male</td>
<td>27</td>
<td>M1</td>
</tr>
</tbody>
</table>

Table 7.2 Summary of findings and demographic information from nine authentic blood samples tested. (Acronym key: DUID – driving under the influence of drugs; NS – not specified)
Discussion

The metabolite identified in the current study, FUB-AMB 3-methylbutanoic acid (M1) is a shared metabolite of AB-FUBINACA. Both parent compounds are hydrolyzed at the terminal end of the molecule; FUB-AMB undergoes ester hydrolysis while AB-FUBINACA undergoes amide hydrolysis (see Figure 7.1). If detected in biological specimens, M1 alone would not allow for differentiation between FUB-AMB and AB-FUBINACA, as it is not a specific metabolite; in that instance, a specific metabolite (e.g. hydroxylated) would be a good marker for consumption. A trace amount of a hydroxylated FUB-AMB metabolite (Figure 7.1) was identified in all three 0-minute incubation samples.

In a study conducted by Franz et al. where one of the authors ingested 5 mg of AB-FUBINACA (one-time consumption), the parent compound was detected in serum for up to 72 hours; the 3-methylbutanoic acid metabolite (M1 in the current study) was detected for up to 311 hours in urine. During this self-administration study, the maximum serum concentration, 2.3 ng/mL, was reached after approximately 2 hours; the maximum urine concentration, 48 ng/mL, was achieved after approximately 7 hours. The authors also tested samples from a drug abstinence clinic patient who ceased using synthetic cannabinoid products after a period of extensive consumption. Urine samples were collected from the subject over a two-year time frame, and each sample tested positive for M1. Franz et al. therefore proposed that due to the possibility of long-term storage of synthetic cannabinoids in fatty tissues in the body and given a period of chronic extensive consumption, an elimination phase of several months to a year seems
possible. Adamowicz et al. reported a fatality case in which FUB-AMB was identified in autopsy samples including the following tissues: kidney, liver, stomach, intestine, lung and brain, supporting the possibility that parent drug may be stored in tissue and released over time. The data by Franz et al. are consistent with the HLM findings in the current study which showed prolonged stability over the course of the incubations; this may lead to long detection windows of M1 in blood and urine.

Aside from the stability of M1, it is structurally-related to the ester hydrolysis metabolite of another synthetic cannabinoid, MDMB-FUBINACA. This metabolite was studied by Wouters et al. and shown to retain activity at CB₁, though at a relatively high concentration, EC₅₀ = 450 nM, with an E_max of 177%, compared to the parent molecule: EC₅₀ = 0.36 nM, with an E_max of 241%. M1 therefore may also have activity at the CB₁ receptor and may contribute to a user’s impairment profile; this hypothesis would need to be verified via pharmacological evaluations.

In the current study, M1 was present in greater abundance that FUB-AMB at the zero time point both in the presence and absence of NADPH, demonstrating that FUB-AMB is highly unstable. This was also observed by Fabregat-Safont et al.; some possibilities for this phenomenon (as observed with other synthetic cannabinoids) are the instability of the terminal ester bond of FUB-AMB; the apparent short half-life of the compound (see Figure 4); the presence of hydrolyzing enzymes, including but not limited to carboxylesterase type-1 and type-2 (CES1 and CES2) and amidase.

The logP of M1 is 3.52, and that of FUB-AMB is 3.79 (see Table 7.1). While in blood, these compounds have the likelihood of crossing the blood-brain barrier and could
therefore induce impairment to the user even hours after intake. Parent molecule FUB-AMB as well as M1 (and in some instances other synthetic cannabinoids) have been identified in acute toxicity and post-mortem blood samples.\textsuperscript{61,73,89,147,149,151,152,167,169}

For all the blood samples tested in the present study, no parent compound was reported as positive. This demonstrates that though the potential agent of interest (parent compound) was not identified, there may still be other components that can point to its identity present in the sample (i.e. metabolites). The absence of FUB-AMB in the blood samples that had previously tested positive for the parent compound may be attributed to the relative instability of the molecule under various storage conditions, including at room temperature and under refrigerated conditions. Krotulski et al. recently reported a study in which multiple synthetic cannabinoids were evaluated and showed an overall decrease in their abundance in spiked blank blood samples over time.\textsuperscript{58} Additionally, the quantity of FUB-AMB present in the blood samples provided by NMS Labs is unknown, and some may have screened positive but at a low abundance. A similar phenomenon was observed in a 2015 study by Erol Öztürk et al. in which 600 blood samples were tested for synthetic cannabinoids and the parent compound was present in only 3 samples, but the hydroxylated metabolite and pentanoic acid metabolite were present in 92 and 145 samples, respectively.\textsuperscript{57} These types of findings cannot be overlooked, as they provide examples of instances in which the parent compound is undetectable, potentially due to a short half-life or instrument limitations. However, the metabolites of the parent compound may be present in much greater quantities.
In light of the findings of the current study and given consideration to studies described and cited above in which metabolites were found in blood samples, it is proposed that M1 is a reliable marker to include in blood and (hydrolyzed) urine assays that screen for FUB-AMB and AB-FUBINACA intake. In laboratory assay panels that do not include relevant synthetic cannabinoid primary metabolites, a sample screen could miss markers that point to a causative agent. It is imperative that synthetic cannabinoid assay panels include metabolites, especially known (or potentially) pharmacologically active metabolites; this is particularly important for drugs with short half-lives.

Conclusions

This work describes the assessment of the primary 3-methylbutanoic acid metabolite (M1) of FUB-AMB, a prevalent indazole carboxamide synthetic cannabinoid in human liver microsomes (HLM) and authentic forensic blood case samples. FUB-AMB was rapidly metabolized and generated M1 which was stable in HLM through a 120-minute incubation period, which is potentially indicative of a longer detection window in human biological samples. Twelve authentic human blood samples were screened for the presence of M1 and FUB-AMB. M1 was identified in all samples in the absence of parent drug. It is proposed that M1 is a reliable marker to include in blood and urine assays that assess FUB-AMB and AB-FUBINACA intake; this is particularly important, as M1 may be pharmacologically active like its precursor FUB-AMB. Additionally, M1 may frequently appear in samples in which the parent drug is undetectable and can point to the causative agent. It is imperative that synthetic
cannabinoid assay panels in forensic and clinical laboratories include metabolites, especially known or potentially pharmacologically active metabolites, especially for compounds with short half-lives.
CHAPTER 8
CONCLUSIONS, LIMITATIONS AND FUTURE DIRECTIONS

The aim of this research project was to evaluate the in vitro Phase I metabolism of five forensically-relevant synthetic cannabinoids of varying chemical structure, namely MDMB-CHNINACA; APP-CHMINACA (PX-3); 5F-APP-PICA (PX-1); 5F-MDMB-PINACA (5F-ADB); and FUB-AMB. Each compound was incubated with pooled human liver microsomes (HLM) for 120 minutes, generating an array of metabolites which were characterized via high-resolution mass spectrometry, using a UHPLC-QTOF-MS platform. For several of these compounds (MDMB-CHMINACA, PX-1 and PX-3), the detailed metabolic profile and unique metabolites were reported for the first time. Additionally, the metabolic stability and kinetic profiles of 5F-ADB and FUB-AMB were reported for the first time. It was observed that these compounds were metabolized rapidly, resulting in short half-lives and relatively elevated metabolic clearances.

Each of the analytes studied possessed either a terminal amide or ester functional group, which was a primary biotransformation site. Enzyme-mediated hydrolysis of this moiety resulted in the formation of a carboxylic acid group, and was often the most abundant metabolite identified in the incubation extracts. This metabolic pathway is NADPH-independent, as determined by the presence of these metabolites in all control samples to which no NADPH was added. Hydroxylation of the molecules was another prominent biotransformation and, like hydrolysis, is consistent with what has been observed in metabolism studies reported by other authors in the field for structurally-
related compounds. For compounds possessing a cyclohexylmethyl group (MDMB-CHMINACA and APP-CHMINACA), this moiety was the primary hydroxylation site. Compounds with a fluoropentyl side chain (PX-1 and 5F-ADB) generated an oxidative defluorination metabolite with simultaneous defluorination and hydroxylation of the side chain as a primary metabolite. It is proposed that hydroxylated metabolites and hydrolysis metabolites of the synthetic cannabinoids tested, particularly those which are analyte-specific (not shared with other analytes), are implemented into laboratory assay panels for unequivocal identification of the causative synthetic cannabinoid agent in human biological specimens, including urine and blood.

In the case of 5F-ADB, the oxidative defluorination metabolite was the most prevalent species identified in the authentic human blood samples tested in that study; in some samples tested, the ester hydrolysis metabolite was also identified but to a lesser degree. In most samples, no parent compound was identified. These findings were similar to that of FUB-AMB. The ester hydrolysis metabolite was the only species identified in the blood samples; no parent molecule was present in any specimens. Additionally, it was observed in the time studies of 5F-ADB and FUB-AMB that the metabolites containing carboxylic acid functional groups were detected in incubation samples longer than the hydroxylated metabolites, potentially indicative of longer detection windows in human samples. These findings have significant toxicological implications. The hydroxylated metabolite of 5F-ADB has been proven pharmacologically-active, and the FUB-AMB hydrolysis metabolite is similar in structure to a known pharmacologically-active metabolite of a related synthetic cannabinoid. It is proposed that these metabolites are
reliable markers to include in blood and urine assays that assess 5F-ADB and FUB-AMB intake, especially when the parent drug is undetectable. These species can point to the causative agent. It is imperative that synthetic cannabinoid assay panels in forensic and clinical laboratories include metabolites, especially known or potentially pharmacologically active metabolites, especially for compounds with short half-lives.

The results of this research can be applied to the prediction of metabolic pathways for synthetic cannabinoids as well as non-drug substances with similar structural elements whose metabolic profile has not yet been elucidated, and whose pharmacological activity is currently unknown. Additionally, the results provide reference standard manufacturers and research scientists with further insight into the metabolic products of synthetic cannabinoids and related compounds for the synthesis of materials facilitating the development of laboratory assays.

One of the limitations of the research is that no biological specimens that were positive for MDMB-CHMINACA, PX-1 and PX-3 were available to verify the presence of metabolites in vivo. It is generally ideal to possess drug-positive specimens to verify metabolites and determine the degree of correlation between in vitro and in vivo data. Another limitation is that the study only assessed Phase I metabolism in pooled human liver microsomes. Pooled human hepatocytes are a better model for elucidating metabolic pathways in vitro, as they contain the necessary cofactors for both Phase I and Phase II metabolic pathways. However, hepatocytes are more costly than and require special handling, which is more complex than HLM. In general, there is a good degree of
correlation between hepatocytes and HLM with respect to the generation of major metabolites.

Future studies include synthesis of the major metabolites identified in this study that are not commercially-available; and acquisition of drug-positive samples from toxicology laboratories in order to verify the presence of metabolites in vivo. Additionally, in vitro and in vivo pharmacological evaluations of the metabolites should be conducted to assess their activity at the cannabinoid receptors and to determine their effect on mice models, respectively. This will give insight into the potential ability for synthetic cannabinoid metabolites to contribute to the drug user impairment profile.
REFERENCES CITED


(40) Cheng, Y.; Prusoff, W. H. Relationship between the Inhibition Constant (K1) and the Concentration of Inhibitor Which Causes 50 per Cent Inhibition (I50) of an Enzymatic Reaction. *Biochem. Pharmacol.* 1973, 22 (23), 3099–3108.


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(88) International Narcotics Control Board. Green List: List of Psychotropic Substances under International Control


APPENDIX A

SUPPLEMENTAL PRODUCT ION SPECTRA

This Appendix contains product ion spectra of metabolites not included in the main text.

Figure 3.S1 Product ion spectrum of M3 (ketone formation)

Figure 3.S2 Product ion spectrum of M4 (dihydroxylation)
Figure 3.S3 Product ion spectrum of M5 (dihydroxylation and dehydrogenation)

Figure 3.S4 Product ion spectrum of M6 (trihydroxylation and dehydrogenation)
Figure 3.S5 Representative product ion spectrum of M7 (ester hydrolysis and monohydroxylation)

Figure 3.S6 Product ion spectrum and structure of M8 (ester hydrolysis and dehydrogenation), top right
**Figure 3. S7** Representative product ion spectrum of M9 (ester hydrolysis and monohydroxylation and dehydrogenation)

**Figure 3. S8** Representative product ion spectrum and structure of M10 (ester hydrolysis and ketone formation and dehydrogenation), top right.
Figure 3.S9 Representative product ion spectrum and structure of M11 (ester hydrolysis and dihydroxylation (CHM) and dehydrogenation), top left

Figure 3.S10 Representative product ion spectrum of M12 (ester hydrolysis and dihydroxylation and dehydrogenation (CHM hydroxy/tert-butyl hydroxy))
Figure 4.S1 Representative product ion spectrum of M2 (distal amide hydrolysis and monohydroxylation (CHM)). This spectrum was observed for M2.2 and M2.5; m/z 239 was more prominent than m/z 257, relative to that observed for M2.1, M2.3 and M2.4.

Figure 4.S2 Representative product ion spectrum of M3 (distal amide hydrolysis and ketone formation (CHM))
**Figure 4.S3** Product ion spectrum of M4 (distal amide hydrolysis and dihydroxylation (CHM)).

**Figure 4.S4.** Product ion spectrum of M5 (monohydroxylation (CHM)).
Figure 4.S5. Product ion spectrum of M7 (trihydroxylation (CHM))

Figure 5.S1 Product ion spectrum of M2 (desfluoropentyl)
Figure 5.S2 Product ion spectrum of M3 (distal amide hydrolysis)

Figure 5.S3 Product ion spectrum of M4 (distal amide hydrolysis and oxidative defluorination)
**Figure 5.S4** Product ion spectrum of M6 (oxidative defluorination and dihydroxylation (indole and pentyl chain))

**Fig. 5.S5** Product ion spectrum of M7 (oxidative defluorination and dihydroxylation (benzyl and pentyl chain))
**Figure 5.6** Product ion spectrum of M9 (monohydroxylation (fluoropentyl chain))

**Figure 5.7** Product ion spectrum of M10 (monohydroxylation (benzyl) metabolite)
Figure 6.S1 Product ion spectrum of M2 (carboxypentyl)

Figure 6.S2 Product ion spectrum of M3 (monohydroxylation (fluoropentyl chain))
Figure 6.S3 Product ion spectrum of M4 (monohydroxylation (indazole))

Figure 6.S4 Product ion spectrum of M6 (ester hydrolysis and oxidative defluorination)