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An insight into the metabolism of New Psychoactive Substances

Structural elucidation of urinary metabolites of synthetic
cannabinoids and fentanyl analogues using synthesized
reference standards

Jakob Wallgren

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Jakob Wallgren

Division of Organic Chemistry
Department of Physics, Chemistry and Biology,
Linköping University, Sweden
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Cover: A depiction of (a) Khat, illustrating the complexity and mind-altering effects of New Psychoactive Substances.

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What is now proved was once only imagined

- *William Blake*

Till Morfar från din lilla stora Jakob

ABSTRACT

New Psychoactive Substances (NPS) is an umbrella term covering hundreds of substances across different drug groups. Many of these substances were originally developed for therapeutic use but have later appeared on the recreational drug market. The use of NPS has been associated with many outbreaks leading to hospitalizations and has been implicated in numerous fatalities worldwide. To be able to analytically detect drugs in a forensic setting is vital in the fight against the abuse of NPS. One of the most notable challenges in detection of NPS is the identification of major urinary metabolites for use as biomarkers. Furthermore, given the lack of reference standards in most metabolism studies, the major urinary metabolites can often only be tentatively determined.

This thesis describes the synthesis and analysis of potential metabolites used to identify the exact structures of major metabolites of the synthetic cannabinoid AKB-48, fentanyl and five fentanyl analogues in authentic human urine samples and/or hepatocyte incubations. Synthetic targets were chosen based on previous metabolism studies by our research group. Subsequently, synthetic routes were developed to produce numerous potential metabolites across the studied NPS. The synthesized reference standards were analyzed by LC-QTOF-MS alongside hepatocyte incubations and authentic human urine samples. Comparison of the resulting analytical data was used to determine the exact structures of many metabolites. This included urinary metabolites of AKB-48 with a single hydroxyl group situated on a secondary carbon of the adamantane moiety, or position 3 or 5 of the pentyl side chain. For the studied fentanyls, the β -OH and the 4'-OH metabolites were abundant metabolites identified in hepatocyte incubations while the 4'-OH, 4'-OH-3'-OMe and 3',4'-diOH were the favored metabolic motifs among the metabolites identified in urine.

Additionally, a concise synthetic route to produce synthetic cannabinoid metabolites with the 4-OH-5F pentyl side chain motif was developed and demonstrated for four synthetic cannabinoids.

These findings and the developed synthetic routes can be used to provide forensic toxicology laboratories with urinary biomarkers for drug detection. Moreover, the synthesized reference standards of major metabolites can be studied to better understand the toxicity of their parent drugs.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Nya psykoaktiva substanser (NPS) är det officiella namnet för den grupp droger som tidigare har kallats för designerdroger, nättdroger eller internetdroger. NPS definieras som droger som utgör ett likvärdigt hot mot folkhälsan som droger som återfinns på Förenta Nationernas narkotikakonventioner men som själva inte återfinns under dessa konventioner.

Det finns hundratals olika rapporterade NPS spridda över olika droggrupper, såsom syntetiska cannabinoider och syntetiska opioider. Vissa av dessa droger syntetiserades ursprungligen i forskningssyfte, men tog sig senare in på den illegala drogmarknaden. De mer nyligen framtagna NPS är ofta designade att efterlikna effekterna av etablerade droger, såsom morfin eller Δ^9 -THC, vilket är den huvudsakliga psykoaktiva substansen i cannabis. Användandet av NPS har associerats till många kluster av intoxikationer som har lett till hospitaliseringar. Många har även dött till följd av användandet av NPS. Inte minst i USA där en grupp av NPS kallad för fentanylanaloger är högst delaktig i den pågående opioidkrisen.

Den ständiga inströmningen av nya NPS leder till att detektion av och diskriminering mellan dem utgör en svår utmaning för forensiska toxikologilaboratorier. Tillgången av lämpliga referenssubstanser möter inte deras efterfrågan, delvis på grund av att vilka biomarkörer som är optimala för drogdetektion inte alltid är uppenbart. Till exempel så är metabolismen av syntetiska cannabinoider i regel både snabb och omfattande. Av den anledningen kan användandet av modersubstansen som biomarkör vid analys av urin leda till falskt negativa resultat. Urin som biologisk matris har många fördelar jämfört med blod. Till exempel så har urin ett längre detektionsfönster och högre drogkoncentrationer. För att kunna identifiera optimala biomarkörer för droganalys av urinprover så måste drogernas metabolism utredas.

De flesta metabolismstudier använder sig av humana levermikrosomer eller hepatocyter som inkuberas tillsammans med droger för att generera metaboliter *in vitro*. Urinprover från individer i vilkas blod det har återfunnits droger används också men tillgången är tyvärr begränsad. Dessa metaboliter separeras sedan med hjälp av kromatografiska tekniker och deras kemiska strukturer

bestäms med hjälp av masspektrometri. Dock så är utvärderingen av masspektrometridata komplicerad och det är heller inte möjligt att skilja på vissa positionsisomerer genom att enbart analysera masspektrometridata. För att kunna möjliggöra exakt strukturutredning så krävs referensstandarder. Därför var målet med denna avhandling att addera syntes och analys av referensstandarder till de etablerade tillvägagångssätten att studera metabolismen av NPS.

Ett stort antal potentiella metaboliter av AKB-48 och andra syntetiska cannabinoider samt av fentanyl och fentanylanaloger syntetiserades. Genom att använda dessa referensstandarder i metabolismstudier så kunde de exakta kemiska strukturerna för många metaboliter bestämmas. Dessutom så identifierades mönster i de metaboliska profilerna bland fentanyl och fentanylanaloger. Dessa mönster kan användas för att förbättra predikteringen av metaboliter för andra nuvarande och kommande fentanylanaloger.

Dessa resultat samt de utvecklade syntesvägarna kan nyttjas i framställningen av referenssubstanter i syfte att användas som biomarkörer för att i urin kunna detektera drogmissbruk. Referenssubstanserna kan även användas för att studera metaboliternas farmakologiska egenskaper vilket kan leda till en djupare förståelse kring toxiciteten hos modersubstanserna.

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PAPERS INCLUDED IN THE THESIS

- I. **Synthesis and Identification of an Important Metabolite of AKB-48 with a Secondary Hydroxyl Group on the Adamantyl Ring**
Jakob Wallgren, Svante Vikingsson, Anders Johansson, Martin Josefsson, Henrik Gréen, Johan Dahlén, Xiongyu Wu and Peter Konradsson. *Tetrahedron Lett.* **2017**, 58 (15), 1456–1458.
- II. **Synthesis and Identifications of Potential Metabolites as Biomarkers of the Synthetic Cannabinoid AKB-48**
Jakob Wallgren, Svante Vikingsson, Anna Åstrand, Martin Josefsson, Henrik Gréen, Johan Dahlén, Xiongyu Wu and Peter Konradsson. *Tetrahedron* **2018**, 74 (24), 2905–2913.
- III. **Concise Synthesis of Potential 4-Hydroxy-5-Fluoropentyl Side-Chain Metabolites of Four Synthetic Cannabinoids**
Jakob Wallgren, Anders Rexander, Erik Vestling, Huiling Liu, Johan Dahlén, Peter Konradsson and Xiongyu Wu. *Synlett* **2020**, 31 (05), 517–520.
- IV. **LC-QTOF-MS Identification of Major Urinary Cyclopropylfentanyl Metabolites Using Synthesized Standards**
Svante Vikingsson, Tobias Rautio, *Jakob Wallgren,* Anna Åstrand, Shimpei Watanabe, Johan Dahlén, Ariane Wohlfarth, Peter Konradsson, Xiongyu Wu, Robert Kronstrand and Henrik Gréen. *J. Anal. Toxicol.* **2019**, 43 (8), 607–614.
- V. **Structure Elucidation of Urinary Metabolites of Fentanyl and Five Fentanyl Analogs Using LC-QTOF-MS, Hepatocyte Incubations and Synthesized Reference Standards**
Jakob Wallgren, Svante Vikingsson, Tobias Rautio, Enas Nasr, Anna Åstrand, Shimpei Watanabe, Robert Kronstrand, Henrik Gréen, Johan Dahlén, Xiongyu Wu and Peter Konradsson. *J. Anal. Toxicol.* **2020**. (Online ahead of print)

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CONTRIBUTION TO INCLUDED PAPERS

- I. Planned the synthesis. Performed all the synthetic work and characterization of the synthesized compounds. Contributed to the writing of the paper.
- II. Planned the synthesis. Performed all the synthetic work and characterization of the synthesized compounds. Contributed to the writing of the paper.
- III. Contributed to the planning of the synthesis. Performed the synthesis and characterization of some of the compounds. Wrote the paper.
- IV. Planned most of the synthesis. Prepared the synthesized reference standards for analysis. Wrote parts of the paper.
- V. Planned most of the synthesis. Performed the synthesis and characterization of many of the compounds. Contributed to the hepatocyte experiments. Prepared the synthesized reference standards for analysis. Performed the data analysis of the LC-QTOF-MS measurements. Wrote most of the paper.

PAPERS NOT INCLUDED IN THE THESIS

Synthesis and identification of metabolite biomarkers of 25C-NBOMe and 25I-NBOMe

Xiongyu Wu, Caroline Eriksson, Ariane Wohlfarth, Jakob Wallgren, Robert Kronstrand, Martin Josefsson, Johan Dahlén and Peter Konradsson.

Tetrahedron **2017**, 73 (45), 6393–6400.

Synthesis of Nine Potential Synthetic Cannabinoid Metabolites with a 5F-4OH Pentyl Side Chain from a Key Scalable Intermediate

Xiongyu Wu, Daniel Bopp, Jakob Wallgren, Johan Dahlén and Peter Konradsson. (in manuscript)

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Syntes och karaktärisering av metaboliter och fentanylanaloger

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ABBREVIATIONS

4-ANPP	4-Anilino- <i>N</i> -phenethylpiperidine
APINACA	<i>N</i> -(1-Adamantyl)-1-pentyl-1 <i>H</i> -indazole-3-carboxamide
Boc ₂ O	Di- <i>tert</i> -butyl decarbonate
BOC	<i>tert</i> -Butyloxycarbonyl
CB ₁	Cannabinoid receptor 1
CB ₂	Cannabinoid receptor 2
CNS	Central nervous system
COMT	Catechol- <i>O</i> -methyl transferase
COSY	Correlation spectroscopy
CYP	Cytochrome P450
DCE	1,2-Dichloroethane
DCM	Dichloromethane
DEA	United States Drug Enforcement Administration
DEPT	Distortionless enhancement by polarization transfer
diOH	Dihydroxy
DIPEA	<i>N,N</i> -Diisopropylethylamine
DMF	Dimethylformamide
ED ₅₀	Effective dose in 50% of the population that takes it
EDC	3-(Ethyliminomethyleneamino)- <i>N,N</i> -dimethylpropan-1-amine
EMCDDA	European Monitoring Centre for Drugs and Drug Addiction
Et ₃ N	Triethylamine
EtOH	Ethanol
EWA	United Nations Office on Drugs and Crime Early Warning Advisory
EWS	European Union Early Warning System
HLM	Human liver microsome
HMBC	Heteronuclear multiple-bond correlation

HOBt	Hydroxybenzotriazole
HR-MS	High-resolution mass spectrometry
HSQC	Heteronuclear single quantum correlation
<i>i</i> -PrOH	Isopropanol
KHB	Krebs-Henseleit buffer
LC	Liquid chromatography
LC-MS	Liquid chromatography mass spectrometry
LC-QTOF-MS	Liquid chromatography quadrupole time of flight mass spectrometry
LC-UV	Liquid chromatography ultraviolet light
<i>m</i> CPBA	<i>meta</i> -Chloroperoxybenzoic acid
MeCN	Acetonitrile
MeOH	Methanol
MS/MS	Tandem mass spectrometry
MW	Microwave-assisted heating
NA	Not available
NADP ⁺	Nicotinamide adenine dinucleotide phosphate
NEPTUNE	The Novel Psychoactive Treatment UK Network
NMO	<i>N</i> -Methylmorpholine <i>N</i> -oxide
NMR	Nuclear magnetic resonance
NOESY	Nuclear Overhauser effect spectroscopy
NPP	<i>N</i> -Phenethyl-4-piperidone
NPS	New psychoactive substances
OH	Hydroxy
OMe	Methoxy
pK _a	Acid dissociation constant in logarithmic scale
PPh ₃	Triphenylphosphine
rt	Room temperature
SCRAs	Synthetic cannabinoid receptor agonists

STAB	Sodium triacetoxyborohydride
TBAF	Tetra- <i>n</i> -butylammonium fluoride
TBTU	<i>O</i> -(Benzotriazol-1-yl)- <i>N,N,N',N'</i> -tetramethyluronium tetrafluoroborate
<i>t</i> -BuOK	Potassium <i>tert</i> -butoxide
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
UGT	Uridine 5'-diphospho-glucuronosyltransferase
UHPLC	Ultra-High-Performance Liquid Chromatography
UNODC	United Nations Office on Drugs and Crime
WHO	World Health Organization
Δ^9 -THC	(-)- Δ^9 -trans-Tetrahydrocannabinol

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1. INTRODUCTION

1.1. DEFINITION

New psychoactive substances (NPS) is the accepted name for the group of drugs that has been previously known as internet drugs, designer drugs, legal highs and research chemicals among other names.¹⁻⁴ The formal definition of NPS by the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) is the following: 'a new narcotic or psychotropic drug, in pure form or in preparation, that is not controlled by the United Nations drug conventions, but which may pose a public health threat comparable to that posed by substances listed in these conventions (Council Decision 2005/387/JHA)'.⁵ The definition by the United Nations Office on Drugs and Crime (UNODC) is similar: New psychoactive substances are substances of abuse, either in a pure form or a preparation, that are not controlled by the 1961 Single Convention on Narcotic Drugs or the 1971 Convention on Psychotropic Substances, but which may pose a public health threat.³ However, the definitions become problematic as numerous NPS, mostly synthetic cannabinoids and fentanyl analogues, have been listed under the 1971 Convention on Psychotropic Substances and the 1961 Single Convention on Narcotic Drugs since 2015.³ By definition, the added substances are no longer NPS. However, for the purpose of this thesis the term NPS will be used for any substance that has been classified as an NPS at one time or another.

The umbrella term NPS constitutes a vast number of diverse substances. As of 2018, 731 different NPS had been reported across several different subgroups to the EU Early Warning System (EWS) and 892 to the UNODC Early Warning Advisory (EWA).^{6,7} These subgroups include synthetic cannabinoids, cathinones, benzodiazepines, phenethylamines, opioids and tryptamines. A significant number of them were designed to mimic the effects of controlled drugs to allow for a legal alternative.⁶ Previously, they were produced by organized crime groups in clandestine laboratories.⁴ Presently, the majority of the production of NPS is done by chemical and pharmaceutical companies in China, and these produced substances are then distributed worldwide.⁶ 55 novel NPS were reported to the EWS in the year of 2018, which is a testament to the

rapid rate at which NPS are introduced to the market (Figure 1).⁶ Although the number of reported novel NPS has stabilized in recent years, NPS as a phenomenon is likely here to stay.⁶⁻⁸ The ensuing situation results in a health problem that needs to be dealt with by authorities and provides an analytical challenge for forensic toxicology laboratories.^{3,6,9,10}

Figure 1. The number of novel NPS reported yearly to the EWS from 2007-2018.⁶

1.2. EFFECTS ON HUMAN HEALTH

The large number of existing NPS are diverse. They belong to different subgroups of psychoactive compounds and their chemical structures vary widely both within and between these groups.⁶ This diversity can be exemplified by the four different new psychoactive substances JWH-018, one of the first identified synthetic cannabinoids,¹¹ the hallucinogenic phenethylamine 25I-NBOMe,¹² the potent synthetic opioid acrylfentanyl and the anxiolytic benzodiazepine, clonazepam (Figure 2).^{13,14} Consequently, the pharmacological properties of NPS also vary widely.¹⁵ It is therefore difficult to discuss the specific effects on human health from NPS abuse in terms of toxicity. However, there are various risks and effects on human health associated with NPS as a phenomenon.

Figure 2. Chemical structures of JWH-018, 25I-NBOMe, acrylfentanyl and clonazepam.

Firstly, the time between the introduction of an NPS to the recreational drug market and the time of scheduling of the NPS can be substantial.¹⁶ During this period the NPS can be sold legally. The Internet is a significant source of supply for NPS, which brings an increased accessibility.^{6,17} The ease of purchasing NPS online has been mentioned to be contributing to the abuse of NPS by young people.¹⁸ Furthermore, marketing strategies using terms as “legal” or “safe” have successfully been employed in the selling of NPS to entice users.^{19,20} Young people especially, run the risk of not realizing the dangers of NPS in instances of them being legal, mistaking the legality of the drugs with safe of use to their own detriment.²¹

The big diversity in potency and adverse effects of NPS results in a difficult and complex situation for the drug users.^{15,22} Reports suggest that some people are unaware of the concept of NPS, this might lead to situations where NPS are being thought of as interchangeable or as one specific drug.^{23,24} Furthermore, potential differences in concentration between batches, inhomogeneous samples, erroneous labeling and the presence of adulterants increase the complexity of NPS abuse.^{3,25-28} Therefore, the knowledge and precision required to be able to accurately replicate a dose is seemingly unattainable. In conclusion, these difficulties are prone to cause unwanted overdoses, especially among inexperienced users.

Data on pharmacology, toxicity and health risks associated with the abuse of NPS is generally unavailable or sparse.^{3,15,29} NPS have not undergone clinical trials that are required of pharmaceutical drugs. Studies on NPS have been limited to animal models, such as in the studies by Fantegrossi *et al.* and Wiley *et al.*³⁰⁻³² The lack of information regarding the effects of specific NPS and the difficulties in identifying which NPS an individual has ingested, especially in acute situations, make choosing the correct treatment by medical personnel

difficult.³³ The Novel Psychoactive Treatment UK Network (NEPTUNE) suggests diagnosis and treatment based on the drug that the ingested NPS was designed to mimic, with the exception of synthetic cannabinoids.³⁴⁻³⁶

To be able to analytically detect NPS, forensic laboratories need certified reference standards. With the high turnover rate of new psychoactive substances, it can be difficult for companies synthesizing certified reference standards to keep up.^{37,38} Further difficulties in detection can arise as a result of the low concentrations of NPS in biological samples due to their high potencies.^{8,39} Additionally, lack of pharmacokinetic data complicates the use of metabolites as biomarkers.^{38,40} These difficulties have been exploited by inmates in order to avoid detection during routine drug tests.^{18,41,42} As a consequence of the difficulties in detection of NPS, it is expected that the abuse of NPS is underreported. Different measures have been employed to try to assess the frequency of abuse,¹⁵ such as the monitoring of wastewater.^{43,44} However, the extent of the abuse of NPS is ultimately difficult to measure.

1.3. CONTROL MEASURES

As previously mentioned, one of the reasons that NPS are problematic is the time it takes for a substance to be scheduled after its emergence on the recreational drug market.¹⁶ Once a drug has been scheduled, it can no longer be sold or used legally, which limits its accessibility.¹⁶ Control of substances can be achieved on international, regional and national levels.^{15,45} An NPS that is under international control is listed under the international drug conventions: the 1961 Single Convention on Narcotic Drugs or the 1971 Convention on Psychotropic Substances.^{46,47} For a substance to become under international control, the World Health Organization (WHO) needs to recommend it after reviewing its abuse potential and risks associated with its use.⁴⁸ The gathered information of the reviewed NPS are compiled into reports.⁴⁹ As of March 2019, 48 NPS had been placed under international control.³ Similarly, the EWS collects information from its Member States' experts, performs risk assessments and provides them to the Council of the European Union.^{15,50,51} 32 risk assessments had been carried out as of August 2020.⁵⁰

At national level, a series of different approaches have been tried. Thus, what is required for a substance to be scheduled and what it means for a substance to be scheduled differs between countries.^{15,16,45,52} The different approaches can broadly speaking be divided into two control measures, *i.e.* individual and generic control. The most common approach is individual control of substances.^{15,45} This works comparably to the way of international and regional control; each substance is assessed based on its own potential to cause harm.^{15,45,52} However, this system can easily be exploited by the producers of NPS. After an NPS has been scheduled and can no longer be sold legally, the producers will synthesize a novel NPS.¹⁶ This novel NPS will have had its chemical structure altered slightly as to retain its pharmacological effects, while being a new uncontrolled substance that effectively replaces its predecessor.⁴ For example, one of the first synthetic cannabinoids to be identified in the recreationally abused drug called Spice was JWH-018.¹¹ Following the identification and scheduling of JWH-018 new structurally similar synthetic cannabinoids emerged on the market (Figure 3).⁴ This tactic adopted by the NPS producers created a cat and mouse game where it is impossible for the control measures to keep up with the pace at which novel NPS enter the market.

Figure 3. An example of the evolution of novel synthetic cannabinoids by making small alterations to their chemical structures (highlighted in red).⁴

To speed up the process of scheduling, various strategies have been employed.^{15,16,52} In Sweden, a law regarding substances hazardous to health was established.⁵³ The requirements for a substance to be scheduled as a substance hazardous to health are less extensive compared to being scheduled as a narcotic.⁵³ A drug that has been scheduled as a substance hazardous to health can no longer be sold legally, which limits the accessibility of the drug.⁵³ Furthermore, the law of destruction of hazardous substances of abuse was put into place to reduce the amount of NPS on the market during the scheduling

process.⁵⁴ This law allows the police and the customs to destroy seized material that is under investigation of being scheduled.⁵⁴ Additionally, temporary scheduling of NPS that are expected to cause harm have been employed to limit their abuse during the time in which the NPS are properly assessed.^{15,16,52}

The unprecedented proliferation of NPS has evoked a response to the time-consuming processes of individual scheduling.^{15,16,52} Thus, several countries have adopted generic frameworks of control with different sets of rules, to try a more proactive approach.^{15,16,52} Substances can be included in these generic frameworks for eliciting similar effects as previously scheduled substances or for containing structurally similar building blocks.^{15,16,55} Regardless of approach, the intention is to control all harmful NPS currently existing on the market and to stifle future analogues from ever entering the market.^{15,56} However, using such approaches risk restricting research on therapeutic benefits associated with NPS.⁵⁷ Furthermore, such frameworks can be difficult to construct and enforce due to ambiguity in what is included under the frameworks.^{29,56} Hopefully, the different strategies adopted by different countries will pave the way for a unified and optimal approach to deal with the NPS phenomenon.

1.4. COUNTERMEASURES

There are many different actions that could potentially reduce the harm caused by NPS, and it is likely that a multipronged approach will be optimal.^{6,58} A key action is the sharing of information regarding NPS. Such a system of information sharing, referred to as the EWS is in place in the European Union.⁵⁹ The United Nation of Drugs and Crime's counterpart is called the EWA and is operating worldwide.³ The purposes of these systems are to gather, evaluate and distribute information regarding NPS among its Member States to increase awareness and to aid in the development of improved responses.^{3,6,59} Sharing information of specific NPS with countries in which the NPS in question have not yet been encountered can result in an increased level of preparedness.

Efforts can be focused with different targets in mind such as the production, distribution or the abuse itself.⁶ The production can be targeted by disrupting the NPS producers' businesses through an improved scheduling framework with quicker response times or proactivity.^{6,52,60} It has been suggested that the

decrease in the number of novel NPS reported could be a result of such actions undertaken in countries producing NPS, for example China.⁶ Additionally, the production of NPS can be limited by the control of their precursors.⁶⁰ In October 2017, 4-anilino-*N*-phenethylpiperidine (4-ANPP) and *N*-phenethyl-4-piperidone (NPP), two precursors of fentanyl analogues, were put under international control.^{60,61}

The distribution of NPS can be hampered by supporting law enforcement agencies, *e.g.* the customs, to develop their ability to seize shipments. In 2017, 64160 seizures were made in Europe, with the seized material weighing close to five metric tons. Furthermore, disruption and monitoring of online marketplaces can be employed to try to prevent NPS from reaching users.⁶

Finally, focus can be put on current and future drug users by educating the population about the dangers of NPS.³³ This should be most beneficial in the case of children and young adults who, as previously stated, are more likely to believe that a substance can be safely used because it is legal to do so.²¹ A different approach, which is also focused on the user is what is called harm reduction. Instead of limiting the drug abuse, the focus is instead set upon reducing the harm caused by the drug abuse. To illustrate, four different nasal spray formulations of Naloxone, an antidote that can effectively reverse opioid overdoses, have been approved for laymen use.⁶² These nasal spray formulations are part of the take-home Naloxone programs and can be distributed to and administered by non-medical personnel who are likely to encounter opioid abuse privately or professionally.^{9,62}

Regardless of the legal status of NPS and the different countermeasures taken against their abuse, being able to identify specific NPS in preparative form, but also in biological matrices, such as blood and urine, is important.^{6,37,40} Without this ability, forensic laboratories cannot prove intake of specific NPS. To be able to prove a drug intake through chemical analysis, reference standards of the specific drug, or its key metabolites, which need to be identified through pharmacological studies, must be available.^{6,37,40} The reference standards need to be synthesized, certified and made available to forensic laboratories and incorporated in their routine analyses of tablets, powders, solutions, blotters, blood and/or urine to avoid false negative results. Many forensic laboratories have limited access to such reference standards, making it difficult for them to measure and monitor the current abuse of NPS.^{9,40,63} This concerns both how

widespread and frequent their abuse is but also their effects on human health. Additionally, research concerning the pharmacology and toxicity of NPS is necessary for being able to make appropriate decisions regarding how to best reduce the harm cause by them and should be supported.⁶

While there are many ways to combat the NPS phenomenon, the ability to identify ingested NPS in biological matrices as well as studying their pharmacology stand out as vital measures. To accomplish this, reference standards of NPS will need to be synthesized and studied.

1.5. DEMARCATIONS

There is a vast number of different NPS across several different groups of compounds.⁶ Thus, to focus the scope of the thesis, restrictions on what NPS to cover had to be made. Therefore, the thesis has an emphasis on synthetic cannabinoids and a specific group of synthetic opioids called fentanyl analogues.

When the phrase NPS was coined, the synthetic cannabinoids were very much at the heart of it. They got plenty of attention in the media given the curiosity and the naivete surrounding the synthetic cannabinoids, especially among younger people.¹⁸ Furthermore, synthetic cannabinoids constitute one of the largest groups within the world of NPS and new synthetic cannabinoids arrive at the drug scene on a yearly basis.⁶ While it is difficult to measure the extent to which the synthetic cannabinoids are abused, the number of case reports suggests that synthetic cannabinoid abuse is prominent when comparing NPS.¹⁵ Moreover, the amount of seizures and the large number of different synthetic cannabinoids suggest that synthetic cannabinoids will remain on the drug market for the foreseeable future.^{6,64}

Many fentanyl analogues have recently emerged on the recreational drug market and the mortality caused by these substances make them a necessary target for research.^{15,28,29} While fatalities have occurred in Europe, the number pales in comparison to how many people that have perished in the US, where the fentanyl analogues play a substantial role in the ongoing opioid crisis.^{6,65,66}

1.6. BACKGROUND

1.6.1. SYNTHETIC CANNABINOIDS

Synthetic cannabinoids or synthetic cannabinoid receptor agonists (SCRAs) are, as the names entail, manmade cannabinoids that act on the cannabinoid receptors.⁶⁷ While there are many cannabinoids, such as the endogenous anandamide, the most infamous one is (-)- Δ^9 -trans-tetrahydrocannabinol (Δ^9 -THC), which is the main psychoactive substance in cannabis.^{68,69} Furthermore, it is the substance that many recreationally used synthetic cannabinoids have been designed to mimic.⁸ The structural differences between Δ^9 -THC and synthetic cannabinoids are quite profound and yet they both act on cannabinoid receptor 1 (CB₁) (Figure 4).⁷⁰

Figure 4. Chemical structures of JWH-018 and Δ^9 -THC.

Cannabis is by far the most common illicit drug in the world.¹⁰ While its psychoactive effect may be the biggest draw for the recreational use of cannabis, there are also therapeutic effects associated with cannabinoids.⁷¹ Therefore, synthetic cannabinoids were developed and studied as means to examine the endogenous cannabinoid system and to potentially find therapeutic applications.^{72,73} However, these studies were largely unsuccessful. To date, the only synthetic cannabinoid used in medicine is nabilone.⁷⁴

These synthetic cannabinoids, such as JWH-018,⁷⁵ were later identified in herbal smoking blends marketed as “legal highs” under names such as Spice in 2008 in Germany and Austria.^{11,76} These herbal smoking blends were sold as legal substitutes for cannabis alleged to contain different herbs and spices that would produce a similar high as cannabis.^{77,78} However, what actually produced the cannabimimetic effects were the synthetic cannabinoids.⁷⁸ The synthetic cannabinoids had been dissolved in a suitable solvent, such as acetone, and sprayed onto the plant material and left to dry before the herbal blend was ready to be made into a joint and smoked.⁷⁹ Since then, plenty of synthetic

cannabinoids have been found in similar herbal smoking blends in varying concentrations and mixtures.⁷⁸

During the period 1997-2018, 190 different synthetic cannabinoids were reported to the EMCDDA, which makes synthetic cannabinoids one of the largest groups of NPS.⁶ However, it is important to state that all of them are not circulating simultaneously.⁶⁰ In 2017, law enforcement officers in the EU, Norway and Turkey seized synthetic cannabinoids comprising 159 kg of plant material and 84 kg of powders.⁶ The seized powders indicate that some of the herbal smoking mixtures are prepared in Europe.

The use of synthetic cannabinoids has been associated with serious harm.⁸⁰ While fatalities from intoxications of synthetic cannabinoids are rare, there are reported cases.^{15,29} Interestingly, there have been several reports of outbreaks involving hundreds of people, which might be an indication of especially toxic or potent batches.¹⁵ One such outbreak transpired in Mississippi in 2015 where seventeen people perished and many more were hospitalized after ingestion of the synthetic cannabinoid MAB-CHMINACA.⁸¹

The reasons for the popularity of synthetic cannabinoids when comparing NPS is most likely multifaceted. Given the marketing of synthetic cannabinoids as “legal highs” and their resemblance to the most widely used drug globally, cannabis, especially young people underestimate the dangers of synthetic cannabinoids.^{73,82} Synthetic cannabinoids are also abused among prisoners and forensic psychiatric inpatients as they are more likely to test negative on rudimentary drug control kits than if they would have used established drugs.^{9,42,82,83}

1.6.2. FENTANYL ANALOGUES

Fentanyl analogues are as the name implies, variations of the drug fentanyl.^{28,84} Fentanyl was first synthesized by Paul Janssen in 1960 with the aim of developing an alternative to morphine, with fewer side effects.⁸⁴⁻⁸⁶ However, there is little structural resemblance between fentanyl and morphine, which is due to their different origins (Figure 5). Fentanyl is a synthetic opioid, while morphine is a naturally occurring opiate, derived from the opium poppy *Papaver*. This is advantageous for fentanyl given the cheaper costs associated with its production.^{87,88}

Figure 5. Chemical structures of fentanyl and morphine.

Fentanyl was first used clinically in 1963 in Europe and in 1968 in USA.⁸⁹ Following the success of fentanyl, several fentanyl analogues were developed.⁹⁰ Fentanyl was placed under international control in 1964 due to its liability of abuse and dependence.⁹¹ Starting in the 1980s, reports of illicit use of fentanyl emerged.⁹² Furthermore, fentanyl and fentanyl analogues, such as α -methylfentanyl, appeared on the illicit drug market during the 1970s and 1980s in packages dubbed “China White” or “Synthetic Heroin”.^{91,93} As a result, many people died, and the use of these products became synonymous with accidental overdose.^{91,93} While the fentanyl analogues receded in prevalence after their initial wave, they exploded back onto the drug scene in the 2010s in an unprecedented fashion, both in terms of clandestine production and fatalities associated with their abuse.^{90,91} Furthermore, the trend of mixing fentanyls with other drugs, such as heroin or cocaine, has resurfaced.^{90,94-96} Additionally, there are reports of an increasing number of counterfeit pills containing fentanyls, likely increasing the risk of accidental overdose.⁹⁷

Between 2009 and 2017, 48 fentanyl analogues have emerged on the drug scene, many of which are scheduled under the 1961 Single Convention on Narcotic Drugs.^{66,90} Included in the group of fentanyl analogues, are compounds that have been approved for human or veterinary use (alfentanil, carfentanil, remifentanil and sufentanil) and compounds that have not (*e.g.* acetylfentanyl, acrylfentanyl, furanylfentanyl and 4-fluoroisobutyrylfentanyl).^{90,91} Moreover, while acetylfentanyl and furanylfentanyl have previously been described in the scientific literature in the pursuit of developing pharmaceuticals, acrylfentanyl and 4-fluoroisobutyrylfentanyl have not (Figure 6).⁹¹ Thus, some fentanyl analogues have been synthesized solely for recreational use, emphasizing the threat of new fentanyl analogues.^{90,91}

Figure 6. Chemical structures of various fentanyl analogues.

Numerous deaths have been reported globally following intoxications from different fentanyl analogues.^{28,29,63,66,88,98-100} However, the situation is especially troublesome in North America with the ongoing opioid epidemic.^{6,66} The opioid epidemic in North America is a result of a complex combination of the accessible and dangerous fentanyl analogues, other illicitly used opioids as well as the liberal prescription of opioids for medical ailments.^{65,66,101} People who are treated with opioids for pain relief run the risk of becoming addicted and might start looking for replacement opioids (*e.g.* fentanyl analogues) on the illicit drug market once their prescription ends. This public health threat has become so considerable that the United States Drug Enforcement Administration (DEA) issued an emergency scheduling of all fentanyl-related substances in 2018.¹⁰² Following that, China, the country where most of the fentanyl analogues have been synthesized,⁶ placed all substances that are structurally related to fentanyl by a series of different modifications under national control in May 2019.¹⁰³ Consequently, the prevalence of fentanyl analogues is seemingly diminishing.⁶ However, there is a looming threat of increased involvement by organized crime groups, possibly because of the ease at which these high potency substances can be manufactured, concealed and transported.^{18,66}

1.7. CHEMICAL STRUCTURE

NPS are in general easy to synthesize with non-complicated organic chemistry and with few synthetic steps.^{84,91} Often, the same types of chemical reactions can be used to create an analogue of an already established NPS by exchanging a reagent or altering a synthetic step.⁹¹ With the myriad of alterations that can be made, there are seemingly infinite potential analogues.

1.7.1. SYNTHETIC CANNABINOIDS

Synthetic cannabinoids are small non-polar compounds.⁷⁰ While there are a considerable number of synthetic cannabinoids with different structures, there are recurring structural elements that they have in common.^{31,40} The chemical structures of synthetic cannabinoids can typically be deconstructed into four different parts, namely (i) the core, (ii) the tail, (iii) the linker and (iv) the linked group (Figure 7). The most common core structures are the indole and the indazole moieties with a tail structure that is often a pentyl side chain with or without a terminal fluorine atom. Another tail structure is the cyclohexylmethyl moiety. The core is connected to the linked group via a linker such as a keto, ester or amide linker.^{40,64,79} Examples of linked groups are naphthalene, adamantane and valine derivatives.

Figure 7. Illustration of some of the different substructures of synthetic cannabinoids, showcasing the numerous potential variations of synthetic cannabinoids.

The nomenclature of synthetic cannabinoids has become increasingly complicated with the increasing number of new compounds; the names of these substances have different origins and are based on different naming systems. Many synthetic cannabinoids are named after their inventor. For instance, the JWH-series was first synthesized by John W. Huffman and the AM-series was first synthesized by Alexandros Makriyannis.⁶⁷ The number following the initials is a serial number, as in JWH-018. However, it does not necessarily say anything about the structural features of the synthetic cannabinoid. Some synthetic cannabinoids have non-scientific names such as AKB-48 or XLR-11, likely designed to appeal to customers.⁶⁴ Furthermore, a naming convention was introduced by EMCDDA in 2011, which uses a system of abbreviations based on the chemical structures of the synthetic cannabinoids.⁶⁴ For example, another name for AKB-48 is APINACA (*N*-(1-adamantyl)-1-pentyl-1*H*-indazole-3-carboxamide).⁶⁷

1.7.2. FENTANYL ANALOGUES

Fentanyl is a small and highly lipophilic compound, whose structure can be divided into four different building blocks.¹⁰⁴ The nitrogen of a propanamide is bound to a phenyl group and a piperidine ring, forming a tertiary amide. Lastly, a phenethyl moiety is bound to the nitrogen of the piperidine ring. In general, the fentanyl analogues greatly resemble fentanyl structurally. Many of the recreationally used fentanyl analogues share the structural element called 4-ANPP with each other and with fentanyl.¹⁰⁴ Thus, the only structural difference that sets them apart is the replacement of the propanamide with a different amide. The amide can differ in various ways. To give some examples, it can differ in its length as is the case in acetylfentanyl, it can be cyclic as in the case of cyclopropylfentanyl, it can be branched as in isobutyrylfentanyl or it can include a heterocyclic structure such as in the case of furanylfentanyl (Figure 8).

Figure 8. Four fentanyl analogues sharing the structural element 4-ANPP (black trace).

However, the fentanyl analogues do not all adhere to the previously stated structural similarity. There are plenty of examples of fentanyl analogues that contain a modified structure of 4-ANPP as its core.¹⁰⁴ Perhaps most effectively highlighted in the structures of the therapeutically used fentanyl analogues alfentanil, carfentanil, remifentanyl and sufentanil. Alfentanil has had the phenethyl moiety replaced with a tetrazole derivative and contains a dimethyl ether moiety attached to the tertiary carbon of the piperidine ring. Sufentanil contains the same dimethyl ether moiety as alfentanil, but also contains a thienylethyl moiety instead of the phenethyl moiety. Carfentanil, also called methoxycarbonyl-fentanyl, has a methoxycarbonyl group attached to the tertiary carbon of the piperidine ring, while remifentanyl contains the same methoxycarbonyl group as carfentanil in addition to having the phenyl part of the phenethyl moiety replaced by a methoxycarbonyl group. Lastly, there are several fentanyl analogues in which one hydrogen atom has been replaced with a fluorine atom, as in the case of 4F-isobutyrylfentanyl (Figure 9).

Figure 9. Chemical structures of five fentanyl analogues with modifications of the 4-ANPP core (red trace).

1.8. PHARMACODYNAMICS

1.8.1. SYNTHETIC CANNABINOIDS

Synthetic cannabinoids act on the cannabinoid receptors, which are G-protein coupled receptors. There are two types of cannabinoid receptors, the CB₁ and CB₂ receptors.^{73,105} CB₁ receptors are expressed both in the central and the peripheral nervous systems as well as in the bones, heart, liver, lung, vascular endothelium and reproductive system.⁶⁸ Upon activation of CB₁ receptors the cannabimimetic effects, including psychoactive effects such as euphoria, are elicited.^{106,107} Included in the list of cannabimimetic effects are analgesia, catalepsy, hypothermia and suppression of locomotion, commonly known as the “cannabinoid tetrad”.^{108,109} CB₂ receptors are predominantly expressed in the immune system and are associated with anti-cancer, anti-inflammatory, anti-oxidative, cardio-protective and immunosuppressive properties.^{73,110} Synthetic cannabinoids generally have greater affinities for both CB₁ and CB₂ receptors when compared to Δ^9 -THC (Table I).^{15,32,73}

Table I. Summary of CB₁ and CB₂ receptor affinities of various cannabinoids.

Compound	CB ₁ K _i (nm)	CB ₂ K _i (nm)	Reference
Δ^9 -THC	41 ± 2	36 ± 10	111
JWH-018	9.0 ± 5.0	2.9 ± 2.7	112
AM-2201	1.0	2.6	113
XLR-11	24.0 ± 4.6	2.1 ± 0.6	114
UR-144	29.0 ± 0.9	4.5 ± 1.7	114

Moreover, most synthetic cannabinoids are full agonists enabling them to produce a higher response compared to Δ^9 -THC, which is a partial agonist.^{30,32,73} Despite producing some similar effects, synthetic cannabinoids are inherently more dangerous than Δ^9 -THC and have been correlated with swifter onsets, stronger visual hallucinations, shorter duration of action and more severe hangover effects.³⁴ Adverse effects associated with acute intoxications of synthetic cannabinoids include, heart toxicity, psychosis and acute kidney injury.^{34,73} While death as a result of synthetic cannabinoid intoxication is rare, there are reported cases.^{15,29} It is important to note the fact that synthetic cannabinoids constitute a group of substances, not a single substance. Thus, perceived adverse effects might not be shared among different synthetic

cannabinoids or might be a result of synergy effects from concomitant drug abuse.

As no antidote exists for the toxicity of synthetic cannabinoids, the treatment of synthetic cannabinoid intoxication is symptomatic. Typical treatment includes fluids, benzodiazepines, oxygen and antiemetics.^{34,73}

1.8.2. FENTANYL ANALOGUES

While the pharmacology of non-therapeutically used fentanyl analogues is generally not well established, some animal studies have been carried out.^{88,90,104} However, since the mechanism of action of fentanyl analogues is similar to that of fentanyl, some pharmacological features are seemingly identical.^{88,90} Fentanyls are extremely potent full opioid agonists, likely due to their high lipophilicity enabling easy permeation of the blood-brain barrier in conjunction with their high selectivity and specificity towards the μ -opioid receptor.^{90,115} The μ -opioid receptor is a G-protein coupled receptor, predominantly located in the brain and the gastrointestinal tract.^{104,116,117} Upon binding to the μ -opioid receptor, fentanyls produce effects such as relaxation, anxiolysis and analgesia for medical purposes and euphoria coveted by recreational users.⁸⁸

Regarding the potency of fentanyl, it has been stated that it is 50-100 times more potent than morphine, while carfentanil, the most potent fentanyl analogue, is said to be 10 000 times more potent than morphine.^{88,118} However, according to Armenian *et al.*, these numbers lack robust data to support them and should therefore be used with caution.⁹⁰ Acetylfentanyl, butyrfentanyl and isobutyrylfentanyl have been found to be 15.7, 1.5-7.0 and 1.3-6.9 times more potent than morphine, respectively.^{119,120} The potency of furanylfentanyl has been shown to be 7 times higher when compared to morphine.¹²¹ Cyclopropylfentanyl has been reported to be 3 times as potent as fentanyl.¹⁰⁴ Acrylfentanyl's potency have been described as 75% of fentanyls potency.¹²² Carfentanil out of all known fentanyl analogues, exhibits the lowest ED₅₀.¹²³ While it is important to consider that comparisons of potencies derived from different studies should be done with caution it is noteworthy that all the fentanyls mentioned here have higher potencies than morphine.

Many fentanyl analogues share structural elements, one of which is a modified N-alkyl side chain, which if removed leads to the corresponding nor-metabolite of the parent drug (Figure 10).

Figure 10. Fentanyl undergoes N-dealkylation forming nor-fentanyl as a metabolite.

The nor-metabolite of fentanyl is inactive, which suggests that the N-alkyl side chain plays a crucial role in the binding of fentanyls to the μ -opioid receptor.^{112,124}

Having described both the desired therapeutic and recreational effects, there are also serious adverse effects following the abuse of fentanyls. Effects such as, decreased consciousness and respiratory depression, which can lead to apnea and ultimately death.^{125,126} Maximum respiratory depression is reached at 2-5 minutes following intravenous administration of fentanyl, showcasing the rapid onset.^{127,128} Additionally, chest wall rigidity has been associated with the use of fentanyl.¹²⁹ Fentanyls are especially dangerous when used concomitantly with drugs that induce sedation, such as alcohol or benzodiazepines, due to the resulting synergistic effects.^{84,88}

Given the high potencies and narrow therapeutic windows of fentanyl and the medically used fentanyl analogues, (alfentanil, sufentanil and remifentanyl) great care must be taken in deciding the correct dose based on the patient's individual characteristics.⁸⁴ This holds true for the recreational market as well, where the situation becomes even more dire given the presence of additional fentanyls whose production is not controlled. These fentanyls constitute a larger pool of different fentanyl analogues with internal variations and there are also risks of them being adulterated or used as adulterants in established drugs.^{94,95,130} Consequently, the task of making sure the dose is effective, but not hazardous, becomes a nightmare for the recreational user.^{88,94,95,131}

The treatment of a fentanyl or fentanyl analogue intoxication involves the use of the competitive μ -opioid receptor antagonist called naloxone (Figure 11).⁸⁸

Figure 11. Chemical structure of the μ -opioid receptor antagonist naloxone.

Naloxone effectively reverses the effects of an opioid intoxication including respiratory depression and can be administered via various routes.¹³² However, the intranasal route has become increasingly preferable, which has led to the development of nasal sprays.⁶² These nasal sprays are especially useful as administration can be done by laypersons, which increases the accessibility of naloxone. Given the rapid onset of fentanyls, a more readily available and easy to use antidote is more likely to reach intoxicated persons in time, which should result in fewer lives lost.⁶² After administration of naloxone and the ensuing reversal of respiratory depression, careful monitoring of the intoxicated person is required. This is because there is a risk of the duration of action of the antidote being insufficient, which could result in the return of the respiratory depression that follows fentanyl intoxication. Therefore, repeated infusions of naloxone might be required.³⁶

1.9. PHARMACOKINETICS

The pharmacokinetics of a substance can be described by the way that the body acts on the substance. This entails the absorption, distribution, metabolism and elimination of a substance. Substances can be administered in various ways, such as through insufflation, intravenous injection or sublingual administration. After administration, the substance is absorbed from the site of administration to the systematic circulatory system. Subsequently, the substance is carried by the blood and distributed into various tissues to reach its site of action. The metabolism is the way of the body to deconstruct, break down or modify substances so that they can be more easily eliminated from the body. The metabolism mainly occurs in the liver where different enzymes aid in the

process of metabolizing substances. The metabolism can be categorized into two phases. Phase I metabolism often results in the addition of polar functional groups such as alcohols, aldehydes and carboxylic acids via oxidation. Reduction and hydrolysis reactions are also examples of phase I metabolism. Phase II metabolism involves conjugation of alcohols, carboxylic acids or other polar groups via reactions such as glucuronidation and acetylation. The final step is the elimination of unwanted substances and their metabolites from the body. This occurs predominately via the urine.¹³³

It is important to study the metabolism of NPS and to identify the formed metabolites. In a forensic setting, metabolites can be used as biomarkers to facilitate detection of drug abuse by routine urine analysis. Urine as a matrix has the advantages of non-invasive sampling, greater drug concentrations and a longer detection window when compared to blood.¹³⁴ Additionally, the identified metabolites can be studied to reveal information regarding their contribution to the pharmacological effects of their parent drugs.

1.9.1. SYNTHETIC CANNABINOIDS

The most common way of administering synthetic cannabinoids is through inhalation via smoking of herbal material laced with one or more synthetic cannabinoids.⁸⁴ However, there have been reports of other modes of administration such as through drinking of tea or vaping.^{64,84}

Synthetic cannabinoids being small non-polar molecules are effectively and rapidly distributed in the body after inhalation. Their lipophilicity should enable most synthetic cannabinoids to penetrate the blood-brain barrier according to *in silico* predictions.⁷⁰ The onset has been described to be within minutes after smoking and faster than that of cannabis, while the duration of intoxication has been reported to be 2-5 hours.⁷³

While pharmacological data on synthetic cannabinoids is generally sparse, it has been suggested that cytochrome P450 (CYP) enzymes take part in the metabolism of synthetic cannabinoids.³⁰ A study on the metabolism of the synthetic cannabinoids JWH-018 and AM-2201, identified CYP2C9 and CYP1A2 as the primary CYP enzymes involved in their oxidative metabolism.¹³⁵ Holm *et al.* found the oxidative metabolism of AKB-48 to be

primarily mediated by CYP3A4.¹³⁶ CYP2C9, together with CYP3A4, have also been found to oxidize Δ^9 -THC.¹³⁷

Many studies have been conducted to investigate the metabolism of different synthetic cannabinoids using different models, including hepatocyte and human liver microsome (HLM) incubations.⁴⁰ Many of the studies also included analysis of urine or blood samples.^{134,138-163}

Exclusive use of human liver microsomes has been used to study the metabolism of, MMB022, 3,5-AB-CHMFUPPYCA, ADB-FUBINACA, 5F-ADB, CUMYL-PINACA, CUMYL-4CN-B7AICA, CUMYL-4CN-BINACA, 5F-CUMYL-PINACA, AKB-48, STS-135, MAM-2201 and XLR-11 among others.^{136,164-171}

The following synthetic cannabinoids have been studied using only hepatocytes, BB-22, 5C-AKB48, EG-018, PB-22, 5F-PB-22 SDB-006, AKB-48 and XLR-11 among others.¹⁷²⁻¹⁷⁸

Several metabolism studies of synthetic cannabinoids in urine have been carried out, including of ADB-FUBINACA, AM-694, AM-2201, JWH-007, JWH-019, JWH-203, JWH-307, MAM-2201, UR-144, XLR-11, APINAC, BB-22, EG-018, EG-2201, MDMB-CHMCZCA, MDMB-FUBINACA and 5CI-THJ-018.^{134,156-162}

Many metabolism studies of synthetic cannabinoids have been carried out using combinations of hepatocytes, human liver microsomes, urine and/or blood samples. Included in this list are, 5F-MDMB-PICA, CUMYL-4CN-BINACA, MDMB-4en-PINACA, AB-FUBINACA, AKB-48, 5F-AKB-48, 4'-N-5F-ADB, AMB-CHMICA, APINAC, CUMYL-PICA, CUMYL-PINACA, 5F-CUMYL-PINACA, 5F-CUMYL-P7AICA, CUMYL-PEGACLONE, 5F-CUMYL-PEGACLONE, 5F-CUMYL-PICA, CUMYL-4CN-BINACA, MAM-2201, MDMB-CHMICA, MN-18, NM-2201, NNEI, 5F-PY-PICA, STS-135, XLR-11, AM-2201 and UR-144.^{138-155,163,164,172,179-181}

Given the recurring chemical substructures among synthetic cannabinoids, there are similarities in their metabolic pathways. For example, synthetic cannabinoids containing an ester or amide functionality often undergo hydrolysis to its carboxylic acid counterpart. Furthermore, the terminal carbon of the pentyl side chain is often hydroxylated and further oxidized to a

carboxylic acid. Additionally, if the parent compound contains a fluorinated pentyl side chain, the fluorine normally undergoes oxidative defluorination resulting in a terminal alcohol functionality, which can be further oxidized to a carboxylic acid. Lastly, a common metabolic pathway among synthetic cannabinoids is N-dealkylation that leads to the nor-metabolite of the parent compound.⁴⁰

The metabolism of synthetic cannabinoids is often both rapid and extensive, making detection of their intake a challenge for forensic toxicology laboratories. The rapid metabolism results in a narrow time window during which these cannabinoids can be detected in blood. The extensive metabolism makes the parent a poor biomarker for detection of drug abuse by urine analysis, as its concentration in this matrix is often below detection level.⁴⁰ If the parent was to be used as a urinary biomarker, the likelihood of getting false negative results would be considerable, which calls for other biomarkers to be used to prove abuse (Figure 12).

Figure 12. The parent (AKB-48) can be detected in blood while its metabolite (nor-AKB-48) can be detected in urine following intake of AKB-48.¹⁴²

1.9.2. FENTANYL ANALOGUES

Fentanyl as a pharmaceutical preparation, is available in various formulations. Such formulations include transdermal patches, nasal sprays, sublingual tablets and injectable formulations.¹⁸² Recreationally used fentanyl is normally administered via intravenous injection. However, intranasal administration using nasal sprays has become increasingly common.^{28,183}

Upon administration, fentanyl is distributed in the body and swiftly taken up in tissues from which it is redistributed into plasma, prolonging its effects.¹²⁸ The elimination half-life of fentanyl is 219 minutes.¹²⁸ Compared to morphine, fentanyl has a more rapid onset.¹⁸⁴ Intranasal administration provides a bioavailability of 89% with an onset and duration similar to that of intravenous injection.^{185,186} Transdermal patches of fentanyl are comparatively slower in onset but longer in duration.⁸⁹

Several metabolism studies of fentanyls have been carried out using case samples, hepatocytes and/or human liver microsomes.^{29,90,104} Fentanyl is rapidly and extensively metabolized by the liver and 90-92% of fentanyl is eliminated via the urine and feces in the form of metabolites.¹²⁸ Nor-fentanyl has been identified as the major metabolite in two HLM studies and the nor-metabolite together with a hydroxylated metabolite were identified in urine.¹⁸⁷⁻¹⁹⁰ A study by Kanamori *et al.* identified the nor-, 4'-OH, 4'-OH-3'-OMe, β -OH, ω -OH and the (ω -1)-OH metabolites of fentanyl using hepatocytes and synthesized reference standards.¹⁹¹

The therapeutically used fentanyl analogues sufentanil and alfentanil were found to produce the same nor-metabolite as their major metabolite.¹⁸⁷⁻¹⁸⁸ Carfentanil produced the nor-metabolite and a metabolite hydroxylated at the piperidine moiety in incubations with HLMs and hepatocytes,¹⁹² whereas remifentanil has been shown to be 95% metabolized via ester hydrolysis.¹⁹³

Acetylfentanyl was found to produce the nor-, 4'-OH, 4'-OH-3'-OMe, β -OH and the ω -OH metabolites in hepatocyte incubations, which was confirmed by comparison with synthesized reference standards.¹⁹¹ Watanabe *et al.* found acetylfentanyl to be primarily metabolized by N-dealkylation, monohydroxylation of the piperidine ring and the ethyl linker, as well as hydroxylation/methoxylation of the phenyl ring in a study using hepatocytes and urine samples.¹⁹⁴ Another study by Melent'ev *et al.* identified a metabolite

hydroxylated at the phenethyl moiety and a hydroxy/methoxy metabolite as major metabolites of acetylfentanyl in urine samples.¹⁹⁵

Watanabe *et al.* studied the metabolism of acrylfentanyl and 4F-isobutyrylfentanyl using urine samples and hepatocytes and found both fentanyl analogues to be mainly metabolized by N-dealkylation, monohydroxylation of the piperidine ring and ethyl linker and through hydroxylation/methoxylation of the phenethyl moiety.¹⁹⁴

Amide hydrolysis and dihydrodiol formation generated the major metabolites of furanylfentanyl in a study by Watanabe *et al.* using hepatocyte incubations and urine samples.¹⁹⁴ These results were further corroborated by Goggin *et al.*¹⁹⁶

The metabolism of methoxyacetylfentanyl using hepatocyte incubations, blood, urine and brain tissue samples was investigated and the major metabolic pathways were found to be amide hydrolysis, O-demethylation and N-dealkylation.¹⁹⁷

Krotulski *et al.* identified the nor-metabolite and metabolites monohydroxylated on the tetrahydrofuran (THF) and phenethyl moieties to be major metabolites of THF-fentanyl using human liver microsomes.¹⁹⁸ The metabolism was further studied by Kanamori *et al.* using hepatocytes and synthesized reference standards, which resulted in the identification of the nor-, 4'-OH, 4'OH-3'OMe, β -OH, a ring-opened alcohol metabolite and a ring-opened carboxylic acid metabolite as significant metabolites.¹⁹⁹

The nor-metabolite and a monohydroxylated metabolite of α -methylfentanyl were identified in rat urine.²⁰⁰

A metabolism study of *ortho*-, *meta*- and *para*-fluorofentanyl utilizing urine samples and hepatocytes was carried out by Gundersen *et al.* Significant metabolites were found to be the nor-metabolite, metabolites with a single hydroxyl group on the phenethyl moiety, an N-oxide and a hydroxy/methoxy metabolite.²⁰¹

Steuer *et al.* and Staeheli *et al.* investigated the metabolism of butyrylfentanyl and identified the nor-metabolite to be a major metabolite using human liver microsomes, while hydroxylation followed by further oxidation to the corresponding carboxylic acid of the butanamide chain was found to be major

in blood and urine samples.^{120,202} Another study using hepatocytes and synthesized reference standards found the nor-, ω -OH and the (ω -1)-OH metabolites to be the major metabolites of butyrylfentanyl.²⁰³

The metabolism of the alicyclic fentanyls, cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl and 2,2,3,3-tetramethyl-cyclopropyl fentanyl was investigated by Åstrand *et al.* using hepatocytes. Important metabolic pathways were found to be N-dealkylation, oxidation of the alicyclic rings and hydroxylation of the piperidineethyl and phenethyl moieties.²⁰⁴ Cutler *et al.* identified the nor-metabolite and mono- and dihydroxylated metabolites in human liver microsome incubations and in urine samples when investigating cyclopropylfentanyl.²⁰⁵ The nor-metabolite of cyclopropylfentanyl was also identified in urine by Palaty *et al.*²⁰⁶

Identified metabolites of ocfentanil were found to be formed through O-demethylation (major) and monohydroxylation in human liver microsome incubations and post-mortem samples.²⁰⁷

Although there are significant structural differences between fentanyl analogues, similarities in their metabolic pathways have been identified. Such as, N-dealkylation of the phenethyl moiety producing the nor-metabolite, or hydroxylation with or without further oxidation or methylation at different moieties of the structures. CYP3A4 and to an extent CYP2D6 are seemingly important for various metabolic pathways of fentanyls.^{187,188,203,208}

Out of all the metabolism studies found, only three had access to reference standards.^{191,199,203} It is true that all studies are important in the pursuit of understanding the metabolism. However, without access to reference standards, the exact structure of many metabolites cannot be elucidated.⁴⁰ This is because it is impossible to differentiate between some positional isomers by interpretation of mass spectrometry data alone. For example, 3'-OH-fentanyl and 4'-OH-fentanyl produce similar fragmentation patterns despite being different molecules (Figure 13).

Figure 13. Mass spectra of 4'-OH-acetylfentanyl and 3'-OH-acetylfentanyl.

Therefore, results from metabolism studies without access to reference standards, are often depicted using Markush structures,²⁰⁹ where the metabolically added chemical group is bound to a moiety of the compound, not to a specific atom (Figure 14).¹⁹⁴

Figure 14. Acrylfentanyl and some of its metabolites depicted using Markush structures.

There is not much information available regarding the potency or toxicity of metabolites of fentanyls. However, metabolites can conceivably contribute to the pharmacological effects of their parent drug.^{210,211} An example of a parent drug with an active metabolite is oxycodone, another μ -opioid receptor agonist,

which can be metabolized to oxymorphone mediated by the CYP2D6 enzyme (Figure 15).²¹² Oxymorphone is itself active at the μ -opioid receptor and listed under the 1961 Single Convention on Narcotic Drugs.^{46,213}

Figure 15. Oxycodone can undergo metabolism to form oxymorphone.²¹²

While not much is known about the potency of β -hydroxyfentanyl, it has been reported to cause significant central nervous system (CNS) and respiratory depression.²¹⁴ The same substance has been identified as a major metabolite following incubation of hepatocytes with fentanyl (Figure 16).¹⁹¹ Consequently, β -hydroxyfentanyl might contribute to the toxicity of fentanyl.

Figure 16. β -Hydroxyfentanyl is both a metabolite of fentanyl and a parent drug.

2. AIM

Abuse of synthetic cannabinoids and fentanyl analogues are significant health problems. Intoxications of synthetic cannabinoids have been associated with severe adverse effects and multiple outbreaks involving hundreds of people have taken place.^{15,29,73} Abuse of potent fentanyl analogues has led to numerous deaths worldwide, in particular in the United States with the ongoing opioid crisis.^{28,29,63,65,66,88,98-100} However, it can be complicated to attribute adverse effects to the use of a particular substance, especially with the high likelihood of polydrug abuse and the difficulties in drug detection. As a result, the number of intoxications and deaths following synthetic cannabinoid and fentanyl analogue abuse is most likely underreported.^{15,29,183}

The detection of and discrimination between the large number of existing and upcoming synthetic cannabinoids and fentanyl analogues pose a tough challenge for forensic laboratories.⁶ The availability of appropriate reference standards is generally lacking, in part because the optimal biomarker for drug detection is not always evident.^{40,183} To illustrate, synthetic cannabinoids are rapidly and extensively metabolized. Therefore, detection of the parent drug in urine can be unfeasible. Urine as a biological matrix has substantial advantages over blood in drug detection. Most notably, urine provides a wider detection window.^{134,149} Thus, the ability to better detect abuse of these drugs in urine should provide less false negative results and therefore lead to less underreporting. In turn, this would enable a more accurate assessment of how frequent the abuse of specific drugs is. To be able to identify optimal biomarkers for drug detection in urine, metabolism studies need to be carried out.

While the metabolism of synthetic cannabinoids and fentanyl analogues is in general not well established, with many substances still to be explored or which need further exploration, there have been many different studies carried out to investigate their metabolism. Predominantly by *in vitro* studies, using hepatocytes or human liver microsomes to mimic the effects of the human body in producing metabolites. The metabolite mixtures have commonly been separated using liquid chromatography (LC) and their structures have been identified using high-resolution mass spectrometry (HR-MS).^{29,40} However, given the difficulties in the interpretation of HR-MS data and the limitations in differentiating between some positional isomers using mass spectrometry, the

exact structures of most metabolites cannot be elucidated without the use of reference standards.^{40,215}

Therefore, the aim of this research was to add synthesis of reference substances to the already established procedures of metabolism studies.^{139-142,194,204} The use of reference standards would allow for identification of the exact structures of the metabolites, which can potentially be used as biomarkers to detect drug intake by routine urine analysis. Additionally, it is possible that some metabolites may contribute to the pharmacological effects of synthetic cannabinoids and fentanyl analogues.^{30,135,191,214,216} Thus, having access to synthesized reference standards of such metabolites can enable further studies to improve the understanding of the toxicity of synthetic cannabinoids and fentanyl analogues.

The aims of this thesis were to synthesize reference standards of potential metabolites of synthetic cannabinoids and fentanyls:

- to identify the exact structures of metabolites tentatively identified in urine samples or in incubations with HLMs or hepatocytes
- to be used as urinary biomarkers to prove intake of synthetic cannabinoids and fentanyl analogues by routine urine analysis
- to be used in pharmacological studies to improve the understanding of the toxicity of synthetic cannabinoids and fentanyl analogues

3. METHODOLOGY

3.1. WORKFLOW

This research encompassed several different tasks and they were addressed from different scientific disciplines. Thus, a thorough plan and workflow were established to create a platform for metabolism studies of both present and future NPS (Figure 17).

Figure 17. Schematic workflow of the established workflow.

The first step was to choose the NPS to be included in the study. This choice was made based upon (i) how prevalent the drug was on the market, (ii) if there were particular dangers associated with it, (iii) the novelty of the drug or (iv) if case samples were available. Having decided upon which NPS to target, the reference standards of the targeted NPS were acquired and used in *in vitro* studies. The *in vitro* studies made use of either human liver microsomes or hepatocytes, which were incubated together with the parent drug to generate metabolites. If urine samples of people having ingested the NPS of interest were available, they were also included in the study to provide a more accurate representation of the *in vivo* metabolism. The generated metabolite mixtures were subsequently analyzed by liquid chromatography quadrupole time of flight mass spectrometry (LC-QTOF-MS) to separate the formed metabolites. By analyzing the tandem mass spectrometry (MS/MS) data of the metabolites, they could be tentatively identified and Markush structures could be constructed. Following the structure elucidation, synthetic targets were chosen among the formed metabolites. Thereafter, a plan for the synthesis was developed. This plan made use of scaffolds from which many potential metabolites could be synthesized. These synthesized reference standards of the potential metabolites

were then analyzed by LC-QTOF-MS, together with metabolite mixtures generated in the *in vitro* studies and metabolites present in authentic urine samples. Finally, MS/MS data and retention times of the compounds in these different sample types were compared to determine the exact structures of targeted metabolites found in the *in vitro* studies and/or authentic urine samples.

3.2. *IN VITRO* STUDIES

While human *in vivo* studies naturally would give the most accurate representation of human metabolism, they are heavily constrained due to ethical restrictions associated with the administration of substances which lack proper toxicity data.⁴⁰ With new NPS being consistently introduced to the drug scene, studies of their toxicity are lagging behind.²¹⁷⁻²²⁰ However, there are several options to predict urinary metabolites, including incubations using hepatocytes, human liver microsomes or fungus *Cunninghamella elegans*, *in silico* prediction using different metabolism prediction software, and rat or zebrafish animal models. The most common approach for metabolism studies of NPS is *in vitro* studies using hepatocyte or HLM incubations.^{29,40}

Human liver microsomes are vesicles of the endoplasmic reticulum extracted from hepatocytes. HLMs contain different liver enzymes, primarily CYP, uridine 5'-diphospho-glucuronosyltransferase (UGT) and esterase enzymes.^{40,142} The key advantages of using HLMs are their low cost and simplicity of use.^{40,142} Furthermore, by using specific inhibitors of different enzymes, the enzyme responsible for a specific biotransformation can be identified.^{203,208,221} The main disadvantage of the HLM model is that it does not reflect the *in vivo* metabolism as accurately as the hepatocyte model.^{40,142} The reason for this is that the UGT and CYP enzymes are enriched in HLMs but other enzymes that are present in hepatocytes are absent in HLMs.⁴⁰ Most of the clearance of many drugs can be attributed to the enzymes present in HLMs. However, there are additional enzymes that if involved, can lead to discrepancies in what is formed *in vivo* compared to in HLMs.^{148,222} For example, in studies of the synthetic cannabinoids 5F-AKB-48 and AM-2201, it was shown that metabolites formed through oxidative defluorination were present in urine samples but not in HLM incubations.²²³ Furthermore, in *in vitro* studies using the HLM model, the drug is directly exposed to the enzymes.

However, in the hepatocyte model, it needs to enter through the cell membranes of the hepatocytes to be exposed to their enzymes. If the uptake of the drug to hepatocytes is rate-limiting, biotransformation will be faster in HLMs compared to hepatocytes.^{192,224}

Living cryopreserved hepatocytes are typically used in the hepatocyte incubation model. The hepatocytes contain all of the phase I and phase II metabolic enzymes that enable an accurate model for the *in vivo* metabolism.^{40,223} Furthermore, it has been found in several studies that major metabolites in hepatocyte incubations and authentic urine samples correlate well.^{177,194,225} The disadvantages of the hepatocyte incubation model are its high costs, that the cryopreserved hepatocytes require storage under liquid nitrogen and that their viability needs to be carefully monitored after thawing.⁴⁰ Issues with cryopreservation methods leading to inadequate metabolic activity and survivability of cryopreserved hepatocytes have been reported.²²⁶ However, more recently developed methods have resulted in cryopreserved hepatocytes retaining their activity of most phase I and II enzymes.²²⁶ Finally, hepatocytes used in hepatocyte incubations are normally pooled from 3-10 individuals, while 150 individuals are typically used for the HLMs.¹⁴² Thus, the usage of hepatocyte incubations is more susceptible to encountering problems due to differences in enzymatic activity among individuals.

For the purposes of the studies in this thesis, results from previously carried out HLM incubations were used as a basis for the choice of synthetic targets in papers I and II, while results from previously carried out human hepatocyte incubations were utilized for papers IV and V.^{142,194,204}

Incubations of AKB-48 with HLMs were carried out by the addition of co-factors, buffers and reagents including Tris-HCl (pH 7.5), nicotinamide adenine dinucleotide phosphate (NADP⁺), glucose-6-phosphate, glucose-6-dehydrogenase, MgCl, alamethicin and uridine 5'-diphospho-glucuronic acid to the parent drug, AKB-48, in order to activate CYP and UGT enzymes. The samples were incubated for <2, 15, 30 and 60 minutes and terminated by addition of ammonium acetate and acetonitrile.¹⁴²

Acetylfentanyl, acrylfentanyl, cyclopropylfentanyl and 4F-isobutyrylfentanyl, described in papers IV and V, were incubated with human hepatocytes and used as a basis for selection of synthetic targets.^{194,204}

First, the cryopreserved hepatocytes were thawed at 37 °C and subsequently added into a thawing medium (invitro Gro HT). Following centrifugation, the supernatant was aspirated, and the pellet was re-suspended in Krebs-Henseleit buffer (KHB). After centrifugation of the suspension, the supernatant was aspirated, and the pellet was re-suspended in KHB. Using the Trypan blue exclusion method, the cell viability and concentration of the suspension were determined. Aliquots of the cell suspension were transferred to an injection plate and mixed with drug solutions in medium. The samples were incubated for 1, 3 and 5 hours. Termination of the reactions were achieved by the addition of ice-cold acetonitrile. Three controls were included in the studies. A positive control with diclofenac, degradation controls with no hepatocytes and a negative control without drugs.^{194,204}

3.3. AUTHENTIC HUMAN URINE SAMPLES

The authentic human urine samples used were from individuals in whose blood samples the parent drug of interest had been detected. Consequently, there were traces of the drug intake in the urine samples even if the parent drug was absent.^{142,194}

The reason why authentic human urine samples are desirable is because it is ultimately the understanding of the *in vivo* metabolism that is the target. While human hepatocytes and human liver microsomes are useful tools in studying the metabolism, there are limitations to the extent which *in vitro* studies can replicate what occurs *in vivo*.^{177,194,225} However, a problem with solely analyzing authentic human urine samples is that they do not exclusively contain metabolites of the drug of interest, but a mixture of byproducts of a myriad of substances ingested by the individual. Thus, there can be definite uncertainties in claiming that a particular metabolite originates from a particular parent drug. The situation can be further exacerbated if the individual is a polydrug user of structurally similar NPS. Additionally, there can be genetic variations among the enzymes involved in the metabolism.^{199,208,227} Such enzymatic differences could have an influence on to what extent different metabolites are formed. Therefore, analysis of urine samples could result in an inaccurate representation of the metabolism of the general population. Thus, it is advisable to use urine samples from as many individuals as possible.

Given the advantages and disadvantages of both authentic human urine samples and the aforementioned *in vitro* studies, the most robust approach is to make use of both when possible. Incubations of an NPS with hepatocytes ensures the connection between the produced metabolites and the parent drug, while the authentic human urine samples provide a more accurate representation of the *in vivo* metabolism.

Based on the scarcity of studies incorporating authentic human urine samples in metabolism studies, the acquisition of such samples is seemingly difficult.⁴⁰ The studies presented herein had access to some authentic human urine samples thanks to the close collaboration with the National Board of Forensic Medicine in Sweden (Papers I, II, IV and V).^{142,194,204} Authentic human urine samples used in this research were analyzed in duplicates. One set was hydrolyzed by treatment with β -glucuronidase and sulfatase prior to analysis to identify to what extent phase II metabolism had occurred.^{142,194,204}

Generally, phase I metabolites are better biomarkers as they produce greater mass spectrometry signals and are more stable over time when compared to phase II metabolites.⁴⁰ Furthermore, the peak area of phase I metabolites can be significantly increased by hydrolysis, further increasing sensitivity.¹⁹⁴ Moreover, in routine analysis a hydrolysis step is usually incorporated.^{40,194}

3.4. ANALYSIS

The metabolite mixtures from hepatocyte or human liver microsome incubations as well as authentic human urine samples were analyzed using an LC-QTOF-MS system. The system comprised an Agilent 1290 Infinity ultra-high-performance liquid chromatography (UHPLC) system fitted to an Agilent 6550 iFunnel Quadrupole Time-of-Flight mass spectrometer. Similar analytical setups have been suggested for these purposes by various groups.^{40,84,215,228,229}

Separation of the metabolites was achieved by using reversed phase liquid chromatography utilizing an Acquity HSS T3 column (150 mm x 2.1 mm, 1.8 μ m). As the column is achiral, enantiomers could not be separated and thus metabolites expected to exist as enantiomeric pairs were treated as racemic mixtures.

Gradients were chosen with the aim of having the parent drug elute at the end of the run (12 min), thereby increasing the time for separation of the metabolites. This choice was made under the assumption that the metabolites would have shorter retention times than the parent given their expected higher polarity.

Mass spectrometry data was collected in positive electrospray ionization mode. High resolution mass spectrometry enabled by the QTOF system was used to ascertain the molecular formulae of the resulting ions. Thus, the type of functional group that had been added to or removed from the parent molecule as a result of metabolism could be determined. For example, an increased m/z of +15.9949 can be explained by the incorporation of an oxygen atom, which typically means the introduction of an alcohol functionality to the molecule.¹⁹⁴ By further analysis of the fragmentation patterns of the metabolites, the positions of the modifications could be deciphered (Figure 18).

Figure 18. The resulting Markush structure of an AKB-48 metabolite following interpretation of its mass spectrometry data.

However, as described previously, the accuracy in the determination of the position of the modification is often limited to the moiety level. The resulting proposed structures are therefore depicted as Markush structures. Following structural elucidation of all the formed metabolites a table of information could be compiled as to create an overview of the metabolic pathways of the particular NPS (Table II).^{142,194,204} These tables were utilized to support the choice of synthetic targets for exact structure elucidation .

Table II. Excerpt from compiled data of a metabolism study of AKB-48. *Adapted from Vikingsson et al. J. Anal. Toxicol. 2015, 39 (6), 426–435, with permission.¹⁴²*

Type	Retention time (min)	A ^a	P ^b	F ^c	AKB-48 (min)		
				<2	15	30	60
Area of mass chromatogram							
diOH	4.09	1	1	-	14478	23723	25716
	4.33	1	1	-	509	903	1071
	4.55	1	1	-	2359	4181	4554
	4.84		NA		60	128	132
	5.05	1	1	-	270	431	443
	6.19	2	0	-	530	949	1339
	8.12	2	0	-	323	549	707
	8.34	2	0	-	11775	17871	16838

a, number of OH on adamantyl; b, number of OH on pentyl; c, position of keto group or glucuronide.

3.5. IDENTIFICATION OF SYNTHETIC TARGETS

A set of synthetic targets were chosen based upon the compiled information obtained from the *in vitro* studies. If authentic human urine samples were available, then the results from their analysis were compared with those of the *in vitro* studies and metabolites identified in both sets of samples were targeted.

The selection criteria varied depending on the aim of the specific study. If the aim was to find biomarkers suitable for routine urine analysis, then identifying a set of key metabolites, rather than a whole range of metabolites, was considered sufficient (Papers I, III, IV and V). The inclusion of a few biomarkers in these routine methods is suggested to safeguard against genetic differences between individuals that could affect their metabolism.^{199,208,227} The synthetic targets were chosen based on criteria such as abundance (detector response), uniqueness and feasibility of synthesis. It is beneficial to choose abundant metabolites as this facilitates detection at trace levels, which is often required to detect abuse of potent drugs (*e.g.* fentanyls). Moreover, a biomarker should ideally be unique, meaning that it should be exclusively formed from a specific parent drug. On the contrary, if several NPS have a shared biomarker, then that

biomarker cannot be used to distinguish between intakes of those NPS (Figure 19).^{29,40,188} This can be highly significant if there are differences in the legal status between the NPS.

Figure 19. Amide hydrolysis of methoxyacetylfentanyl and furanylfentanyl produces the same metabolite.

Lastly, the rate at which the biomarkers are identified is critical. The drug market is changing rapidly and existing NPS do continuously disappear and new substances emerge.⁶⁰ Hence, biomarkers must be identified, synthesized and made available to forensic laboratories while the NPS is still present on the recreational market. Thus, synthetic targets were chosen with their ease and speed of synthesis in mind. Studying the Markush structures of two AKB-48 metabolites, the hydroxyl group can be situated on any of the five carbons of the pentyl side chain of the leftmost structure (Figure 20). Having two hydroxyl groups, as in the case of the rightmost structure, there are 25 different possible configurations. Thus, there can be a significant difference in the number of potential metabolites that need to be synthesized to guarantee identification of the exact structure hiding behind the Markush structure.

Figure 20. The leftmost Markush structure covers 5 different exact structures, while the rightmost one covers 25 different exact structures.

If the objective of the specific study was instead broader, *e.g.* when aiming at investigating the metabolism and the role the metabolites play in the toxicity of the drug, a large number of metabolites should be identified rather than just a set of key metabolites that are useful for routine urine analysis (Paper II). By synthesizing and analyzing many potential metabolites, the resulting information can be compiled to create a metabolite library for individual compounds that can provide information regarding which metabolites are formed and which are not. Such information could be used to predict preferred metabolic motifs among structurally similar analogues. For example, there are many recurring structural building blocks among both synthetic cannabinoids and fentanyl analogues, which could mean that there are patterns in their metabolic profiles.^{28,40,84} Thus, this approach may reduce the amount of work needed to ascertain the identity of key metabolites of future NPS.

3.6. SYNTHESIS

Based upon the collated list of synthetic targets, *i.e.* the potential metabolites, retrosynthetic analysis of the parent drugs was made to get a better understanding of the chemistry involved in their synthesis. The parent drugs were deconstructed into synthons, which were then translated to actual chemical reagents as in the example of the synthetic cannabinoid AKB-48 (Figure 21). Thereafter, common chemical reactions could be identified, and subsequently a synthetic route to produce the parent drug could be established.

Figure 21. Retrosynthetic analysis of the synthetic cannabinoid AKB-48, which generated three starting materials.

While the synthesis of NPS is generally straightforward and can be conducted in few steps, the synthesis of their metabolites is usually more difficult. With the incorporation of reactive and polar functional groups to the parent drug, such as hydroxyl groups, careful planning, more synthetic steps and changes in purification procedures were commonly required. However, given the close

structural similarities between many metabolites and their parent drug, some of the synthetic steps in creating the parent drug could be modified and used in the synthesis of its metabolites. Thus, it can be a good start to perform a retrosynthetic analysis of the parent drug for planning the synthesis of its potential metabolites.

A series of potential metabolites must be synthesized to reveal the exact compound(s) behind a Markush structure. This was a challenge that required careful planning of the synthetic route for the synthesis to be efficiently carried out. Thus, the target was to identify scaffolds from which many potential metabolites could be synthesized (Figure 22). Efficient synthetic routes were thereafter developed to afford the selected scaffolds. Such systematic planning paved the way for efficient synthesis of many potential metabolites, which is crucial for identifying biomarkers while the abuse of the NPS is still ongoing.

Figure 22. Nor-4F-isobutyrylfentanyl was identified as a scaffold from which the four depicted potential metabolites could be synthesized.

If a key metabolic motif of an NPS would have already been identified, the synthetic strategy would change. Previously, the target was to create scaffolds from which many potential metabolites of specific NPS could be synthesized (Papers II, IV and V). Now, the objective would instead be to create a scaffold

with the metabolic motif already incorporated. From this new scaffold, metabolites of structurally similar analogues containing the same metabolic motif could be synthesized. This strategy would be used under the assumption that structurally similar analogues have similar metabolic profiles (Figure 23).

Figure 23. A scaffold containing the 4'-OH motif from which metabolites of different fentanyl analogues containing the 4'-OH motif could be synthesized.

Regardless of the type of scaffold to be synthesized, the goal was always to conduct their synthesis in sufficient scale to enable their subsequent use to produce a range of potential metabolites. However, given the low amount of each reference standard required for analysis (one mg) and the high turnover rate of NPS on the drug market, a timely synthesis took precedence over a high yielding one. Moreover, with the toxicity of the potential metabolites being to a large extent unknown, a small-scale synthesis was also prudent for safety reasons. In other words, it was considered more efficient to spend time on synthesizing additional potential metabolites, rather than optimizing reaction conditions to improve yields for a limited number of compounds.

Characterization of the synthesized products was performed using liquid chromatography with ultraviolet light detection (LC-UV) and with mass spectrometry detection (LC-MS) as well as the nuclear magnetic resonance experiments $^1\text{H-NMR}$ and $^{13}\text{C-NMR}$. This was considered as minimum

requirements for identification and purity analysis. Other NMR experiments such as heteronuclear single quantum correlation (HSQC), heteronuclear multiple-bond correlation (HMBC), correlation spectroscopy (COSY) and nuclear Overhauser effect spectroscopy (NOESY) were utilized if necessary.

3.7. REANALYSIS AND EVALUATION

Following the completion of the synthesized reference standards, they were analyzed using the LC-QTOF-MS system with the same settings as were used for the analysis of the hepatocyte incubations and urine samples. However, as wear and tear of the column often results in retention time drift over time, it is advisable to carry out analysis of samples that are to be compared within a reasonable time frame. In this work, the metabolite mixtures from hepatocyte or human liver microsome incubations were generated at an early stage, while the synthesis of the corresponding metabolite standards was accomplished at a later stage. As the metabolites present in these different types of samples were to be compared, *in vitro* studies were replicated in order for the metabolite mixtures from the incubations to be analyzed at the same time as the synthesized reference standards (Paper V). This precaution was considered necessary as even small differences in retention time can prove significant when trying to match data, especially since positional isomers are likely to possess similar retention times.

The exact chemical structures of relevant metabolites could be confirmed by comparing chromatograms and mass spectra of (i) metabolites generated through hepatocyte or HLM incubations and metabolites present in authentic urine samples with (ii) those of the synthesized reference standards. The specific requirements used for positive identification were matching retention times, accurate masses and fragmentation patterns. Furthermore, the metabolites should be absent in the negative controls. Following positive identification, Markush structures could be translated to exact structures. Thus, the exact structures of metabolites previously tentatively identified in *in vitro* studies and/or authentic human urine samples could be elucidated (Figure 24).

Figure 24. Translation of the ambiguous Markush structure to the exact structure.

Finally, the results were evaluated, and depending on if the targeted metabolites were identified or not, it was decided whether the substance in question should be studied further or if the approach should be applied to identify key metabolites of other NPS.

4. RESULTS AND DISCUSSION

4.1. PAPER I – SYNTHESIS AND IDENTIFICATION OF AN IMPORTANT METABOLITE OF AKB-48 WITH A SECONDARY HYDROXYL GROUP ON THE ADAMANTYL RING

4.1.1. BACKGROUND

AKB-48 is a synthetic cannabinoid, which was first identified as a constituent of an herbal smoking blend in Japan 2012.²³⁰ The drug was later in the same year, controlled under the Pharmaceutical Affairs Law in Japan.²³¹ AKB-48 possesses as stronger binding affinity for the CB₁ receptor than Δ^9 -THC does.²³² Furthermore, pharmacological studies of AKB-48 in mice and rats revealed that the drug, like many other synthetic cannabinoids, induces analgesia, catalepsy, hypothermia and suppression of locomotion.^{232,233}

Partly due to the availability of case samples, a metabolism study of AKB-48 was carried out. The study utilized human liver microsome incubations and authentic human urine samples. Among the findings was a major metabolite with a single hydroxyl group on the adamantane moiety (Figure 25).¹⁴²

Figure 25. The structure of the synthetic cannabinoid AKB-48 and the ambiguous Markush structure of one of its major metabolites. Adapted from Wallgren et al. *Tetrahedron Lett.* 2017, 58 (15), 1456–1458, with permission.²³⁴

This finding correlates well with the findings of Holm *et al.* and Gandhi *et al.* who used human liver microsomes and cryopreserved hepatocytes in their studies, respectively.^{136,176}

Furthermore, there are additional synthetic cannabinoids which contain the same adamantane moiety as AKB-48 does (Figure 26). It is therefore likely that hydroxylation would occur in the same position of the adamantane moiety for other synthetic cannabinoids containing this motif.

Figure 26. Chemical structures of four synthetic cannabinoids containing the adamantane moiety.

Altogether, identification of the exact structure of this metabolite could result in a urinary biomarker for identification of AKB-48 abuse. To accomplish this, a set of potential metabolites were synthesized, analyzed and compared with metabolites tentatively identified in an authentic human urine sample.

4.1.2. RESULTS AND DISCUSSION

Adamantine **1** was oxidized using a concentrated mixture of H_2SO_4 and HNO_3 at 10°C for two hours to selectively afford the tertiary alcohol, 3-hydroxyadamantine **2**, in a yield of 74%.²³⁵ The oxidation of the adamantane moiety was performed prior to amide coupling to avoid side reactions, such as nitration of the indazole.²³⁶ Compound **2** was coupled with 1*H*-indazole-3-carboxylic acid in an amide coupling reaction to form compound **3** in 81% yield. No trace of the potential ester was found. Compound **3** was alkylated using 1-bromopentane under basic conditions in an $\text{S}_{\text{N}}2$ reaction to selectively achieve the final product **4** in an overall yield of 54% (Scheme 1).

Scheme 1. i) conc. H_2SO_4 , conc. HNO_3 , 10 °C, 2 h, 74%; ii) TBTU, 1*H*-indazole-3-carboxylic acid, Et_3N , THF, rt, overnight, 81%; iii) 1-bromopentane, *t*-BuOK, THF/DMF (5:1), rt overnight, 90%. Adapted from Wallgren *et al. Tetrahedron Lett.* 2017, 58 (15), 1456–1458, with permission.²³⁴

The alcohol functionality of compound **5** was transformed into an amine functionality as described by Averina *et al* (Scheme 2).²³⁷ The transformation was achieved over two steps. First, compound **5** was subjected to a Ritter reaction.²³⁸ Secondly, the formed amide was hydrolyzed using concentrated HCl (aq). Without extensive purification, the crude product was coupled with 1*H*-3-indazole-carboxylic acid to afford compound **6** in a yield of 30% over three steps.

Scheme 2. i) CH_3CN , $\text{BF}_3 \cdot \text{Et}_2\text{O}$, TFA; ii) conc. HCl (aq), 150 °C MW, 1 h; iii) TBTU, 1*H*-indazole-3-carboxylic acid, Et_3N , THF, rt, overnight, 30% over three steps; iv) 1-bromopentane, *t*-BuOK, THF/DMF (5:1), rt, overnight, 72%; v) NaBH_4 , MeOH, rt, 1h, **8**: 36%; **9**: 20%. Adapted from Wallgren *et al. Tetrahedron Lett.* 2017, 58 (15), 1456–1458, with permission.²³⁴

Compound **6** was alkylated using 1-bromopentane to afford compound **7** in 72% yield. The ketone of compound **7** was selectively reduced by NaBH₄ to give two diastereoisomers, compounds **8** and **9**, in overall yields of 8% and 4% over five steps, respectively (Scheme 2). The separation of the two diastereoisomers using preparative LC was difficult, which ultimately led to the low yield of the final step.

Compounds **8** and **9** were characterized using 2D NMR experiments to ascertain their identity. Firstly, the chemical shifts of the atoms of interest were determined by HSQC, COSY and HMBC experiments. Then, NOESY experiments were carried out to confirm the identities of compounds **8** and **9** (Figures 27 and 28). NOESY experiments are capable of correlating protons that are close to each other in space, unlike through bond, which is the case for most NMR experiments. NOESY has been used to elucidate the structures of biological macromolecules.²³⁹

Figure 27. NOESY spectrum of compound **8**. The axial hydrogen at 4.02 ppm correlates with the protons at 2.06 and 2.24 ppm.

Figure 28. NOESY spectrum of compound **9**. The equatorial hydrogen at 3.83 ppm correlates with the protons at 1.65 and 2.13 ppm.

The three synthesized positional isomers (compounds **4**, **8** and **9**) were analyzed using an LC-QTOF-MS system and their mass spectra and retention times were compared to those of tentatively identified metabolites in an authentic urine sample (Figure 29).

Figure 29. Chromatogram of compounds **4**, **8** and **9** (blue trace) as well as of the authentic urine sample (red trace). Adapted from Wallgren et al. *Tetrahedron Lett.* 2017, 58 (15), 1456–1458, with permission.²³⁴

By looking at accurate mass, retention time and fragmentation pattern, a match was found with compound **8**. It is interesting that a secondary carbon of the adamantane moiety was found to be exclusively oxidized *in vivo*, whereas the procedure used in the synthesis of compound **2** resulted in exclusive oxidation of a tertiary carbon.

A customized LC-method had to be established since all three synthesized compounds co-eluted using an LC-method used in routine analysis at the Swedish National Board of Forensic Medicine. This illustrates the difficulties with separating positional isomers.

4.1.3. CONCLUSION

To conclude, a synthetic route to afford a major metabolite of AKB-48 with a single hydroxyl group situated on a secondary carbon of the adamantane moiety (compound **8**) was established. This major metabolite was identified in an authentic human urine sample. Moreover, it is also uniquely formed from AKB-48. Hence, it can potentially be used as a urinary biomarker to prove intake of the synthetic cannabinoid AKB-48.

Furthermore, it is conceivable that other synthetic cannabinoids that contain the adamantane moiety, share this metabolic pathway. Therefore, their respective potential metabolites with the same metabolic motif as compound **8** should be among the first potential metabolites to be synthesized for their respective metabolism studies.

4.2. PAPER II – SYNTHESIS AND IDENTIFICATIONS OF POTENTIAL METABOLITES AS BIOMARKERS OF THE SYNTHETIC CANNABINOID AKB-48

4.2.1. BACKGROUND

In paper I, an important metabolite of AKB-48, compound **8**, was synthesized and characterized. While this metabolite can likely be used as a urinary biomarker for AKB-48 intake, more potential metabolites were synthesized to further study the metabolism of AKB-48.

Based on previous work done by Vikingsson *et al.*, Holm *et al.* and Gandhi *et al.*, it has been established that many of the metabolites of AKB-48 are oxidized on the pentyl side chain.^{136,142,176} This can either be exclusive or in addition to oxidation of the adamantane moiety, which was studied in paper I.

To understand which positions of the pentyl side chain that are prone to oxidation can be highly valuable, considering the frequency of the pentyl side chain structure among synthetic cannabinoids. Ideally, the information gained from a thorough metabolism study of one synthetic cannabinoid, in this case AKB-48, can be carried over to other structurally similar synthetic cannabinoids.

For these reasons, many potential metabolites with ketones, hydroxyl and/or carboxyl groups on (i) the pentyl side chain, (ii) the adamantane moiety or (iii) the two in combination, were synthesized. The resulting potential metabolites were then analyzed by LC-QTOF-MS and obtained retention times and mass spectra were compared to those of tentatively identified metabolites found in an authentic human urine sample.

4.2.2. RESULTS AND DISCUSSION

Compared to paper I, the objective of this study was to synthesize a much larger number of potential metabolites. Therefore, three scaffolds, compounds **3**, **6** and **11**, were identified and synthesized in larger scale (Figure 30). These scaffolds were used as starting materials for all the potential metabolites synthesized in this study.

Figure 30. Chemical structures of the scaffolds utilized in the synthesis of all potential metabolites in this study.

Compound **10** was coupled with 1-adamantanamine using 3-(ethyliminomethyleneamino)-*N,N*-dimethylpropan-1-amine (EDC) as the coupling reagent to produce compound **11** in 48% yield (Scheme 3). The yield was low due to incomplete conversion and difficulties with purification.

Scheme 3. i) 1-adamantanamine, EDC, HOBt, Et₃N, DMF, rt, overnight, 48%; ii) 5-bromopent-1-ene, *t*-BuOK, DMF/THF (1:5), rt, overnight, 87%; iii) OsO₄ (aq), NMO, THF/H₂O (3:1), rt, overnight, 66%. Compounds **13** and **14** are commercially available. Adapted from Wallgren et al. *Tetrahedron* 2018, 74 (24), 2905–2913, with permission.²⁴⁰

Compounds **13** and **14** were purchased. However, they can also be synthesized using hydroboration-oxidation. This procedure is explained in the synthesis of compounds **18** and **19** (Scheme 4). Compound **11** was alkylated with 5-bromopent-1-ene to afford compound **12** in a yield of 87% (Scheme 3). Compounds **16**, **22** and **26** were synthesized using similar conditions (Schemes 4 and 5). Alkylation reactions were performed prior to oxidation of the pentyl side chain to avoid intramolecular cyclization of the formed alcohols.²⁴¹

Subsequently, compound **12** was treated with OsO₄ and *N*-methylmorpholine *N*-oxide (NMO) to yield compound **15** (Scheme 3). Only a catalytic amount of the toxic OsO₄ was needed as NMO was used to regenerate the osmium.²⁴² Comparable methods were applied in the synthesis of other vicinal diols, to turn compound **16** into **17**, compound **22** into **23** and compound **26** into **27** (Schemes 4 and 5).

Scheme 4. i) 1-bromopent-2-ene, *t*-BuOK, DMF/THF (1:5), rt, overnight, 98%; ii) OsO₄ (aq), NMO, THF/H₂O (3:1), rt, overnight, 80%; iii) a) BH₃·THF, N₂ (g), 0 °C, 2 h. b) NaOH (aq), H₂O₂, rt, overnight, **18**: 69%; **19**: 8%. Adapted from Wallgren *et al. Tetrahedron* 2018, 74 (24), 2905–2913, with permission.²⁴⁰

Hydroboration-oxidation of compound **16** was achieved by treatment with BH₃·THF followed by NaOH (aq) and H₂O₂ (Scheme 4).²⁴³ The reaction gave a mixture of compounds **18** and **19** in a ratio of 9:1. This selectivity is interesting given that the two possible positions for the added alcohol functionality are both secondary carbons. Compounds **24** and **25** were synthesized from compound **22** in a similar manner and with similar results (Scheme 5).

Scheme 5. i) Conc. H_2SO_4 , conc. HNO_3 , 10°C , 2 h, 74%; ii) TBTU, 1*H*-indazole-3-carboxylic acid, Et_3N , THF, rt, overnight, 81%; iii) 1-bromopentane, *t*-BuOK, THF/DMF (5:1), rt, overnight, 90%; iv) 1-bromo-2-pentene, *t*-BuOK, THF/DMF (5:1), rt, overnight, 82%; v) OsO_4 (aq), NMO, THF/ H_2O (3:1), rt, overnight, 82%; vi) a) $\text{BH}_3\cdot\text{THF}$, N_2 , 0°C , 2 h; b) NaOH (aq), H_2O_2 , rt, overnight, **24**: 64%; **25**: 7%; vii) 5-bromo-1-pentene, *t*-BuOK, THF/DMF (5:1), rt, overnight, 79%; viii) OsO_4 (aq), NMO, THF/ H_2O (3:1), rt, overnight, 82%; ix) a) $\text{BH}_3\cdot\text{THF}$, N_2 , 0°C , 2 h; b) NaOH (aq), H_2O_2 , rt, overnight, **28**: 32%; **29**: 3%; x) a) *m*CPBA, 66%; b) NaBH_4 , *i*-PrOH, 60°C , 3 h, 75%. Adapted from Wallgren et al. *Tetrahedron* 2018, 74 (24), 2905–2913, with permission.²⁴⁰

Given the low yields of compounds **19** and **25**, a better synthetic route should be to reduce the ketones of compounds **20** and **21** using NaBH_4 (Scheme 6). Furthermore, hydroboration-oxidation was also used to add water over the alkene of compound **26** to achieve compounds **28** and **29** (Scheme 5). The yield of compound **29** was very low (3%), this is because the hydroboration-oxidation reaction is an anti-Markovnikov reaction. Therefore, the selectivity is heavily skewed towards the formation of the less substituted alcohol, which in this case is the terminal one (compound **28**). To achieve compound **29** in better yield,

another synthetic route was developed. Compound **26** was first epoxidized by the addition of *meta*-chloroperoxybenzoic acid (*m*CPBA). Secondly, the epoxide was reduced by NaBH₄ in isopropanol (*i*-PrOH) at 60 °C to afford compound **29** in a yield of 49% over two steps (Scheme 5).

The synthesis of compounds **2**, **3** and **4** from compound **1** has already been described in paper I (Scheme 5). However, it is noteworthy that the yield of compound **3** (81%) was much better than that of compound **11** (48%). Conceivably because *O*-(benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium tetrafluoroborate (TBTU) was used as the coupling agent instead of EDC.²⁴⁴

Compounds **20** and **21** were synthesized from compounds **11** and **3** by the addition of 1-chloro-3-pentanone and potassium *tert*-butoxide (*t*-BuOK) in THF using microwave-assisted heating (MW) at 120 °C for one hour (Scheme 6). The resulting yields, 24% and 28% respectively, were low due to incomplete conversion. Enough product for our purposes was achieved, hence optimization was not attempted.

Scheme 6. i) 1-chloro-3-pentanone, *t*-BuOK, THF, 120 °C MW, 1 h, **20**: 24%; **21**: 28%. Adapted from Wallgren et al. *Tetrahedron* 2018, 74 (24), 2905–2913, with permission.²⁴⁰

The synthesis of compounds **6**, **7**, **8** and **9** was covered in paper I (Scheme 2). Compounds **30** and **31** were achieved through reduction of compound **6** using NaBH₄ in methanol (MeOH) for one hour (Scheme 7).

Scheme 7. i) CH_3CN , $\text{BF}_3 \cdot \text{Et}_2\text{O}$, TFA, 70°C , 3 h; ii) Conc. HCl , 150°C MW, 1 h; iii) TBTU, 1*H*-indazole-3-carboxylic acid, Et_3N , THF, rt, overnight, 30% over 3 steps; iv) NaBH_4 , MeOH, rt, 1 h, **30**: 31%; **31**: 21%; v) 1-Bromopentane, *t*-BuOK, DMF/THF (1:5), rt, overnight; vi) NaBH_4 , MeOH, rt, 1 h, **8**: 36%; **9**: 20%. Adapted from Wallgren et al. *Tetrahedron* 2018, 74 (24), 2905–2913, with permission.²⁴⁰

A mixture of CrO_3 in H_2O and concentrated H_2SO_4 (Jones reagent) was added to compound **28** in acetone at room temperature (rt) to afford compound **32** in 59% yield (Scheme 8). Excessive CrO_3 was neutralized using *i*-PrOH.

Scheme 8. Jones reagent, acetone, rt, 59%. Adapted from Wallgren et al. *Tetrahedron* 2018, 74 (24), 2905–2913, with permission.²⁴⁰

All 19 synthesized potential metabolites and the two purchased metabolites (compounds **13** and **14**) were analyzed by LC-QTOF-MS together with an authentic urine sample. The results were compared and based on retention time, accurate mass and fragmentation patterns, compounds **8**, **9**, **11**, **14**, **18** and **19** were identified in the urine sample (Figures 31 and 32). However, compound

18 was only present in trace amounts. Furthermore, the presence of small amounts of compound **13** could not be ruled out.

From these six metabolites, compounds **8** and **19** are the preferred urinary biomarkers of AKB-48. While compound **14** was found to be a major metabolite, it can also be formed from 5F-AKB-48. Therefore, it would be a poor biomarker since its presence cannot be used to distinguish intake of AKB-48 from 5F-AKB-48.^{142,163} The same reasoning applies for compound **11**. Out of the four remaining candidates, compounds **8** and **19** were found to be much more abundant than compounds **9** and **18** and were therefore considered to be better choices.

Ideally, more than one urinary biomarker should be used given potential enzymatic differences between individuals. Holm *et al.* identified that the CYP3A4 enzyme mediates the oxidative metabolism of AKB-48.¹³⁶ The variability of expression and function of CYP3A4 has been found to be significant between individuals.²⁴⁵ Furthermore, coadministration of drugs and foods, which inhibits the CYP3A4 enzyme may influence the metabolism of AKB-48.^{245,246} Therefore, both compounds **8** and **19** should be considered for use as urinary biomarkers to prove intake of AKB-48 (Figure 31).

Figure 31. Chemical structures of the metabolites identified in the urine sample. Adapted from Wallgren *et al. Tetrahedron* 2018, 74 (24), 2905–2913, with permission.²⁴⁰

Figure 32. A) Chromatogram of compounds **13**, **14**, **18** and **19** (blue trace) as well as of the authentic urine sample (red trace). B) Chromatogram of compounds **4**, **8** and **9** (blue trace) as well as of the authentic urine sample (red trace). Adapted from Wallgren et al. *Tetrahedron* 2018, 74 (24), 2905–2913, with permission.²⁴⁰

In prior research, compound **4** was analyzed and compared with the metabolites in urine samples. The preliminary findings suggested compound **4** to be a major metabolite in the urine samples. However, the results of these preliminary findings were inaccurate. The reason for this inaccuracy was an inadequate LC-method, which could not separate compounds **4**, **8** and **9**. Therefore, many metabolites with a hydroxyl group on a tertiary carbon were synthesized. However, as was established in paper I, the correct position for the hydroxyl group on the adamantane moiety is the one found in compound **8**.

Regarding the pentyl side chain, position 5 (compound **14**) was found to be the position most prone to oxidation *in vivo*, followed by position 3 (compound **19**).

Thus, future studies of the metabolism of AKB-48 should aim for synthesizing potential metabolites with a hydroxyl group in the same position as in compound **8** and with a hydroxyl group in position 3 or 5 of the pentyl side chain. Moreover, dihydroxylation of the adamantane moiety should also be targeted. The attempts in this study were regrettably unsuccessful. Despite using longer reaction times and higher temperatures in the synthesis of compound **2** using the procedure of Lavrova *et al.*, no dihydroxylation occurred.²³⁵

4.2.3. CONCLUSION

In summary, 19 potential metabolites of AKB-48 were synthesized. These metabolites together with two purchased potential metabolites and an authentic urine sample were analyzed by LC-QTOF-MS. Upon comparison, six of the synthesized or purchased metabolites were identified in the urine sample. Given that the requirements for a biomarker are to be both abundant and unique to its parent drug, compounds **8** and **19** are the best suited candidates for use as urinary biomarkers of AKB-48.

Moreover, synthetic routes have been established to oxidize different positions of both the pentyl side chain and the adamantane moiety. These synthetic routes and the findings of this study may be useful for structural elucidation of metabolites of novel synthetic cannabinoids.

4.3. PAPER III – CONCISE SYNTHESIS OF POTENTIAL 4-HYDROXY-5-FLUOROPENTYL SIDE-CHAIN METABOLITES OF FOUR SYNTHETIC CANNABINOIDS

4.3.1. BACKGROUND

The pentyl side chain motif is a common inclusion among synthetic cannabinoids.⁴⁰ Furthermore, a common modification of the pentyl side chain is fluorination at its terminal carbon.²⁴⁷ For example, the only difference between the synthetic cannabinoid JWH-018 and its analogue, AM-2201, is the replacement of a hydrogen atom with a fluorine atom (Figure 33).

Figure 33. Chemical structures of JWH-018 and its fluorinated analogue, AM-2201. Adapted from Wallgren et al. *Synlett* 2020, 31 (05), 517–520, with permission.²⁴⁸

This is a simple modification that can be done by the producers of synthetic cannabinoids to circumvent control measures. Additionally, the fluorinated synthetic cannabinoids typically have higher potencies compared to their non-fluorinated counterparts.^{247,249}

A major metabolic pathway of fluorinated synthetic cannabinoids is oxidative defluorination.⁴⁰ This has been reported to be the case for AM-2201, MAM-2201, NM-2201, XLR-11, 5F-PB-22, THJ-2201 and 5F-AKB-48.^{40,134,153,163,170,177,178} However, as was established in paper II, one of the major metabolites of AKB-48 is 5-OH-AKB-48. The same metabolite is also produced via oxidative defluorination of 5F-AKB-48 (Figure 34).

Figure 34. Chemical structures of AKB-48, 5F-AKB-48 and their shared metabolite 5-OH-AKB-48.

For this reason, the 5-OH pentyl side chain metabolite is a poor urinary biomarker as it cannot be used, in a forensic setting, to distinguish between an intake of a synthetic cannabinoid from its fluorinated counterpart. The consequences of using such a urinary biomarker can be highly significant if the illicit status of the two drugs in question is different. For example, 5F-PB-22 and 5F-AKB-48 are internationally controlled under the 1971 Convention on Psychotropic Substances, while PB-22 and AKB-48 are not.⁴⁷

Therefore, another biomarker, namely a metabolite with the 4-OH-5F pentyl side chain motif is a better alternative. This motif has been reported for AM-2201 by Hutter *et al.*¹⁵⁷ Consequently, it is expected that other synthetic cannabinoids containing a fluorinated pentyl side chain will also produce their corresponding 4-OH-5F pentyl side chain metabolite.

The synthesis of synthetic cannabinoids with this motif has already been reported in the literature. McKinnie *et al.* have described a synthetic route to produce various synthetic cannabinoids with this motif. However, while the yields were remarkable, the route required many steps.²⁵⁰ Moreover, the synthesis of the different enantiomers of 4-OH-AM-2201 has been reported by Patton *et al.*²⁵¹

To provide forensic laboratories with reference standards of urinary biomarkers of synthetic cannabinoids with the 5F pentyl side chain motif, a concise synthetic route was developed to synthesize 4-OH-5F pentyl side chain metabolites. The route was applied for the four synthetic cannabinoids, STS-135, MAM-2201, AM-2201 and XLR-11 (Figure 35).

Figure 35. Chemical structures of the fluorinated synthetic cannabinoids, STS-135, MAM-2201, AM-2201 and XLR-11. *Adapted from Wallgren et al. Synlett 2020, 31 (05), 517–520, with permission.*²⁴⁸

4.3.2. RESULTS AND DISCUSSION

Compound **33** was used in a coupling reaction with adamantan-1-amine hydrochloride, TBTU and triethylamine (Et_3N) in THF at 70 °C to achieve compound **34** in a yield of 68% (Scheme 9). Compound **34** was treated with 2-(3-bromopropyl)oxirane and *t*-BuOK in THF at 0 °C to give compound **35** in an $\text{S}_{\text{N}}2$ reaction. The 2-(3-bromopropyl)oxirane was synthesized by the addition of *m*CPBA to 5-bromo-pent-1-ene in dichloromethane (DCM). Tetra-*n*-butylammonium fluoride trihydrate ($\text{TBAF} \cdot 3\text{H}_2\text{O}$) in THF was used to open the epoxide of compound **35** using microwave-assisted heating at 165 °C to afford the final product **36** in a yield of 49%.

Scheme 9. (i) Adamantan-1-amine hydrochloride; ii) 2-(3-bromopropyl)oxirane. *Adapted from Wallgren et al. Synlett 2020, 31 (05), 517–520, with permission.*²⁴⁸

The TBAF·3H₂O was co-evaporated using methanol and toluene before use to remove the water as it could otherwise react with the epoxide of compound **35** to form the vicinal diol. During the ring opening of compound **35**, a minor byproduct was formed through ring opening at position 4 of the pentyl side chain. To verify the identity of compound **36**, a distortionless enhancement by polarization transfer (DEPT-135) experiment was carried out (Figure 36). The result showed that the split fluorinated carbon at 86.8 ppm was indeed a CH₂ carbon. Furthermore, the result of the DEPT-135 was corroborated by the double triplet splitting pattern of the ¹⁹F-NMR spectrum of compound **36**. Thus, the ring opening of compound **35** proceeded with high selectivity.

Figure 36. DEPT-135 spectrum of compound **36**, confirming the fluorine's position at the terminal carbon of the pentyl side chain.

By acylation of compound **37** using 4-methyl-1-naphthoyl chloride, 1-naphthoyl chloride and 2,2,3,3-tetramethylcyclopropanecarbonyl chloride, respectively, compounds **38a-c** were afforded in yields of 33-85% (Scheme 10). The acylation reactions were carried out under nitrogen gas at 0 or 30 °C using either DCM or 1,2-dichloroethane (DCE) as the solvent. Furthermore, the mild Lewis acid, ZrCl₄, was used in the acylation reactions, which proved to be effective as decomposition of the relatively unstable 2,2,3,3-tetramethylcyclopropanecarbonyl moiety was kept to a minimum.²⁵² The low yield of compound **38a** was a result of its poor solubility in DCM.

Therefore, DCE was used as the solvent in the synthesis of compound **38b**, which is also a naphthoyl derivative, to almost double the yield. Compounds **38a-c** were alkylated in similar fashion as in the synthesis of compound **35** to achieve compounds **39a-c** in yields of 75-95% (Scheme 10).

Scheme 10. (i) **38a**: 4-Methyl-1-naphthoyl chloride, DCM, 0 °C, 8 h, 33%; **38b**: 1-naphthoyl chloride, DCE, 30 °C, 20 h, 64%; **38c**: 2,2,3,3-tetramethylcyclopropanecarbonyl chloride, DCM, 0 °C, 4 h, 85%; (ii) 2-(3-bromopropyl)oxirane; **39a**: t-BuOK, DMF–THF, overnight, 81%; **39b**: NaH, DMF, overnight, 95%; **39c**: t-BuOK, DMF–THF, 17 h, 75%; (iii) **40a**: THF, 70 °C, overnight, 40%; **40b**: Method A: toluene, 165 °C MW, 45 min, 43%; Method B: THF, 80 °C, overnight, 86%; **40c**: THF, 80 °C, 4 h, 40%. Adapted from Wallgren et al. *Synlett* 2020, 31 (05), 517–520, with permission.²⁴⁸

Ring opening was performed using two different methods (methods A and B) to successfully form compounds **40a-c** in yields of 40-86% (Scheme 10). In method A, microwave-assisted heating at 165 °C for 45-60 minutes was used, while method B made use of conventional heating at 70-80 °C for 4-16 hours. Both methods produced similar results in terms of yield. Reaction times were minimized using microwave-assisted heating (method A),²⁵³ whereas conventional heating led to fewer byproducts. However, method A was ineffective in the synthesis of compound **40c** due to the instability of the 2,2,3,3-tetramethylcyclopropanecarbonyl moiety. Therefore, the milder conditions of method B were utilized instead.

As an alternative source of TBAF to the azeotropically dried TBAF·3H₂O, TBAF in THF was tried. Both sources of TBAF produced similar yields.

However, the use of TBAF in THF was significantly simpler and should therefore be used if available.

In order to attain >98% purity of the final products (**36** and **40a-c**), purification by preparative LC was applied after the last reaction step. However, the yields suffered as a result of that (40-49%), possibly due to solubility issues (Schemes 9 and 10). This can be demonstrated by the yield of 80% in the synthesis of compound **40b** using method B, where only silica gel chromatography was used for purification (Scheme 10).

This concise synthetic route has been shown to be capable of producing potential 4-OH-5F pentyl side chain metabolites of four different synthetic cannabinoids with ketone or amide functionality. While various reaction conditions and reagents have been tried, more optimization can be carried out to improve yields.

Patton *et al.* made use of similar conditions as were used in this study apart from the usage of KHF_2 and $\text{Bu}_4\text{NH}_2\text{F}_2$ as fluorinating agents.²⁵¹ Through the use of TBAF in this study, improvements in both reaction time and yield were achieved.

McKinnie *et al.* also provided a synthetic route for synthetic cannabinoids with the 4-OH-5F pentyl side chain motif with better yields than were achieved in this study.²⁵⁰ However, the number of steps far exceed the three steps required in the route presented herein.²⁵⁰ Considering the small amount of reference material required by forensic toxicology laboratories and the number of novel synthetic cannabinoids reported each year, time of synthesis should take precedence over yield. Therefore, a more concise method is more appropriate for the task.

4.3.3. CONCLUSION

In conclusion, a straightforward and concise synthetic route for the synthesis of potential 4-OH-5F pentyl side chain metabolites of synthetic cannabinoids was developed. The synthetic route has been demonstrated in the synthesis of 4-OH-STS-135 (**36**), 4-OH-MAM-2201 (**40a**), 4-OH-AM-2201 (**40b**) and 4-OH-XLR-11 (**40c**). This route should be applicable in the synthesis of the 4-OH pentyl side chain metabolite of other synthetic cannabinoids containing the fluorinated pentyl side chain moiety.

Biomarkers with the 4-OH-5F pentyl side chain motif can, unlike biomarkers with the 5-OH pentyl side chain motif, unambiguously prove drug intake of synthetic cannabinoids with a terminally fluorinated pentyl side chain. Thus, their reference standards can likely be used as urinary biomarkers to assist in drug detection conducted at forensic toxicology laboratories.

4.4. PAPER IV – LC-QTOF-MS IDENTIFICATION OF MAJOR URINARY CYCLOPROPYLFENTANYL METABOLITES USING SYNTHESIZED STANDARDS

4.4.1. BACKGROUND

Cyclopropylfentanyl is a fentanyl analogue, which has been associated with numerous deaths worldwide.^{219,254,255} For these reasons it was put under international control in 2019.²⁵⁶

The metabolism of cyclopropylfentanyl has previously been studied using hepatocytes, human liver microsomes and blood samples.^{204,205} Åstrand *et al.* identified the nor-metabolite as the major metabolite using hepatocyte incubations. Further findings included two monohydroxylated metabolites, a dihydroxylated metabolite and a hydroxy/methoxy metabolite, with all metabolic modifications occurring on the phenethyl moiety.²⁰⁴ Cutler *et al.* reported metabolites, which had undergone N-dealkylation, hydroxylation or N-oxidation using human liver microsome incubations, while the N-dealkylated metabolite together with two monohydroxylated metabolites and one dihydroxylated metabolite were identified in blood samples.²⁰⁵ However, these studies did not have access to reference standards. Therefore, the exact position of the hydroxyl and methoxy groups could not be ascertained. Based upon the results of the studies by Åstrand *et al.* and Cutler *et al.*, oxidation of the phenethyl moiety was identified as a common metabolic pathway (Figure 37).^{204,205}

Figure 37. The chemical structure of cyclopropylfentanyl with the phenethyl moiety highlighted.

To identify the exact structures of these metabolites, synthetic routes to synthesize seven potential metabolites were developed. The potential metabolites were the following: four monohydroxylated metabolites (2', 3', 4' and β -OH), the 3',4'-dihydroxylated metabolite as well as the two different hydroxy/methoxy metabolites that can be generated from the aforementioned catechol (3',4'-diOH). The specific isomers of the dihydroxylated metabolite and its two methylated metabolites were chosen based on the belief that the methylation of the hydroxyl group is mediated by catechol-*O*-methyl transferase (COMT). COMT has been described to have a preference for the 3'-position.²⁵⁷

To aid in the identification of the metabolites that are formed *in vivo*, the synthesized reference standards were analyzed by LC-QTOF-MS and cross-checked against metabolites present in thirteen authentic human urine samples.

4.4.2. RESULTS AND DISCUSSION

The developed synthetic route described in this paper was loosely based on the Siegfried method.²⁵⁸ However, the structural differences between the targeted potential metabolites were solely positioned on the phenethyl moiety. Therefore, the nor-metabolite (**A1**) was identified as a scaffold from which all the targeted potential metabolites could be achieved (Scheme 11).

Scheme 11. (i) Boc_2O , NaOH, H_2O –THF, rt, 72 h, 84%; (ii) aniline, acetic acid, STAB, DCM, rt, 16 h, 63%; (iii) cyclopropanecarbonyl chloride, DIPEA, DCM, rt, 16 h, 81%; (iv) DCM:TFA (5:1), rt, 1 h, quant.

First, the nitrogen of compound **41** had to be protected to prevent reductive amination between two 4-piperidone molecules in the following step (Scheme 11). This was achieved by the addition of di-*tert*-butyl dicarbonate

(Boc₂O) and NaOH to compound **41** in H₂O–THF to afford compound **42** in a yield of 84%. Aniline, acetic acid and Na(CH₃COO)₃BH (STAB) was added to compound **42** in DCM to afford compound **43** through reductive amination in 63% yield. The mild reductive agent, Na(CH₃COO)₃BH, was not capable of reducing the ketone of compound **42**, which enabled a one-pot reaction.²⁵⁹ Subsequently, compound **43** was acylated by the addition of *N,N*-diisopropylethylamine (DIPEA) and cyclopropanecarbonyl chloride in DCM to give compound **44** in 81% yield. The (*tert*-butyloxycarbonyl) BOC group of compound **44** was removed by treatment with trifluoroacetic acid (TFA) in DCM to yield the scaffold, compound **A1**, in quantitative yield (Scheme 11).

Compounds **A2-A8** were synthesized from compound **A1** through N-alkylation using different bromides in the presence of Cs₂CO₃ in acetonitrile (MeCN) (Scheme 12). For fear of further alkylation of the final products the use of Cs₂CO₃ was sometimes omitted. The yields of the N-alkylation reactions varied considerably, 23-83%. The reason for this was mainly attributed to the use of preparative LC for purification, which led to considerable loss of product. Purification by silica gel flash chromatography was tried but deemed to be unfeasible given the similar pK_a values of the phenol groups and the amines of the metabolites.

The alkylating agents used in the synthesis of compounds **A2-A8** were procured in different ways. For example, 4-(2-bromoethyl)phenol was purchased, while several other bromides had to be prepared (Schemes 13 and 14). Furthermore, some phenethyl side chains had to be modified after N-alkylation to achieve the targeted final products (Scheme 13).

Scheme 12. (i) 4-(2-bromoethyl)phenol, Cs_2CO_3 , MeCN, 60 °C, 1 h, 35%; (ii) 3-(2-bromoethyl)phenol, MeCN, 60 °C, overnight, 23%; (iii) 2-methoxyphenethyl bromide, Cs_2CO_3 , MeCN, 60 °C, 72 h; BBr_3 , DCM, rt, overnight, 36% over two steps; (iv) phenacyl bromide, Cs_2CO_3 , MeCN, 60 °C, 2 h; NaBH_4 , EtOH, rt, 2 h, 71% over two steps; (v) 3,4-dimethoxyphenethyl bromide, Cs_2CO_3 , MeCN, 60 °C, overnight; BBr_3 , DCM, rt, overnight, 41% over two steps; (vi) 4-(2-bromoethyl)-6-methoxyphenol, MeCN, rt, 72 h, 50%; (vii) 3-(2-bromoethyl)-6-methoxyphenol, MeCN, 60 °C, overnight, 83%.

Scheme 13. (i) PPh_3 , CBr_4 , DCM, rt, 2 h, 85%; (ii) BBr_3 , DCM, rt, overnight.

Compound **46**, which was used in the synthesis of compound **A3**, was synthesized from compound **45** in an Appel reaction,²⁶⁰ using triphenylphosphine (PPh₃) and CBr₄ in DCM for one hour at room temperature in 85% yield (Scheme 13).

In the synthesis of compound **A4**, compound **A1** was first alkylated using 2-methoxyphenethyl bromide to form compound **47** (Schemes 12 and 13). Compound **47** was subsequently treated with BBr₃ in DCM to obtain the alcohol, compound **A4**. This synthetic route was chosen to avoid intramolecular cyclization of 2-hydroxyphenethyl bromide, which occurred when demethylation was attempted before N-alkylation. In the synthesis of compound **A6**, the two methyl groups of the intermediate were removed using similar conditions (Scheme 12).

Compound **A5** was synthesized by N-alkylation of **A1** using phenacyl bromide followed by reduction of the ketone to the corresponding alcohol using NaBH₄ in ethanol (EtOH) (Scheme 12).

Lastly, the bromides used in the synthesis of compounds **A7** and **A8** were prepared prior to N-alkylation (Schemes 12 and 14). Compounds **48** and **51** were reduced using LiAlH₄ in THF to form their respective alcohols, compounds **49** and **52**. Following Appel reactions of compounds **49** and **52** using PPh₃ and CBr₄ in DCM, compounds **50** and **53** were achieved (Scheme 14).

Scheme 14. (i) LiAlH₄, THF, rt, overnight; (ii) PPh₃, CBr₄, DCM, rt, 72 h, 37% over two steps; (iii) LiAlH₄, THF, rt, overnight, 91%; (iv) PPh₃, CBr₄, DCM, rt, 1 h, 85%.

The thirteen authentic human urine samples were analyzed by LC-QTOF-MS and the structures of the detected metabolites were tentatively elucidated by interpretation of their MS/MS spectra.

The urine samples were analyzed in duplicates. One set was hydrolyzed in order to measure to what extent phase II metabolism had occurred. Conjugation was found to be extensive, with the exception of the nor-metabolite the metabolites were found to be between 40 and 91% conjugated. This highlights the importance of hydrolyzing the urine samples before analysis.

In total, eleven metabolites were tentatively identified in the urine samples. The major metabolites in the hydrolyzed urine samples were the nor-metabolite (**M1**), one dihydrodiol (**M2**) one dihydroxylated metabolite (**M5**), one monohydroxylated metabolite (**M8**) and one hydroxy/methoxy metabolite (**M9**) (Figure 38).

Upon comparison with the seven synthesized reference standards (**A2-A8**), five matches were identified (Scheme 12). The major monohydroxylated metabolite (**M8**) and the minor monohydroxylated metabolite (**M11**) were matched with the 4'-OH metabolite (**A2**) and the β -OH metabolite (**A5**), respectively (Figure 38). Compounds **A3** and **A4** were not identified in the urine samples, indicating a clear preference for position 4' in the *in vivo* oxidation of cyclopropylfentanyl. The dihydroxylated metabolite (**M5**) was positively matched with compound **A6**.

Furthermore, the synthesized reference standards **A7** and **A8** were matched with **M9** and **M10**, respectively. While both isomers were detected, **M9** was much more abundant. This selectivity for methylation of the phenol group at position 3' indicates that the formation of **M9** from **M5** is mediated by COMT.

Figure 38. Suggested metabolic pathways and extracted ion chromatograms of the metabolites that were identified in case #1. All peaks are from the hydrolyzed sample of case #1 apart from the glucuronidated metabolites **M3** and **M4**. They were found in the non-hydrolyzed sample. The signals of metabolites **M6**, **M7**, **M10** and **M11** in the panels were magnified tenfold for visibility. Metabolites that were verified by reference materials are emphasized in bold text. *Other potential structures are possible. Adapted from Vikingsson *et al. J. Anal. Toxicol.* 2019, 43 (8), 607–614, with permission.²⁶¹

Generally, the metabolites identified in the urine samples matched those identified in hepatocyte incubations by Åstrand *et al.*²⁰⁴ Additionally, the findings of the 4'-OH, β -OH and the 4'-OH-3'-OMe metabolic motifs are in line with the findings of Kanamori *et al.* from studying the metabolism of fentanyl and acetylfentanyl using hepatocytes.¹⁹¹

4.4.3. CONCLUSION

Eleven metabolites of cyclopropylfentanyl were identified across thirteen authentic human urine samples by LC-QTOF-MS analysis. Five of these metabolites were major metabolites, including the nor-metabolite (**M1**). The exact structures of three of the major metabolites and two minor metabolites were verified using synthesized reference standards. The 4'-OH (**M8**), 3',4'-diOH (**M5**) and the 4'-OH-3'-OMe (**M9**) metabolites were major metabolites while the β -OH (**M11**) and the 3'-OH-4'-OMe (**M10**) metabolites were minor metabolites.

To conclude, **M1**, **M5**, **M8** and **M9** could serve as urinary biomarkers to prove abuse of cyclopropylfentanyl. Moreover, these findings and the provided synthetic routes can hopefully be used to facilitate the production of reference standards to be used in urinary drug detection at forensic toxicology laboratories as well as in pharmacological studies.

4.5. PAPER V – STRUCTURE ELUCIDATION OF URINARY METABOLITES OF FENTANYL AND FIVE FENTANYL ANALOGS USING LC-QTOF-MS, HEPATOCYTE INCUBATIONS AND SYNTHESIZED REFERENCE STANDARDS

4.5.1. BACKGROUND

In paper IV, synthesized reference standards were used to determine the exact structures of major metabolites of cyclopropylfentanyl identified in authentic human urine samples. Building on that research, four other fentanyl analogues were chosen to be further studied using synthesized reference standards, hoping to identify patterns in their metabolism. Such patterns could be valuable in predicting the metabolism of novel fentanyl analogues.

The metabolism of three out of four analogues (*i.e.* acetylfentanyl, acrylfentanyl and 4F-isobutyrylfentanyl) has previously been studied by our research group using hepatocyte incubations and authentic human urine samples.¹⁹⁴ The metabolism of the fourth fentanyl analogue, isobutyrylfentanyl, has not yet been studied. The four analogues were in part chosen based upon their prevalence of use in Sweden.⁹⁸

As of august 2020, all of the chosen fentanyl analogues, apart from isobutyrylfentanyl, are listed under the 1961 Single Convention on Narcotic Drugs.⁴⁶ Acetylfentanyl has been implicated in the deaths of 27 people in Sweden,²⁶² while the use of acrylfentanyl and 4F-isobutyrylfentanyl has contributed to 43 and 16 deaths in Sweden, respectively.^{217,220,263} No deaths associated with isobutyrylfentanyl have been reported in Sweden.

Metabolism studies of acetylfentanyl, acrylfentanyl and 4F-isobutyrylfentanyl using urine samples and hepatocyte incubations have been carried out by various groups.^{191,194,195} Similar metabolic pathways have been suggested for these fentanyl analogues as were identified in paper IV concerning cyclopropylfentanyl. Furthermore, Kanamori *et al.* made use of reference standards to identify the 4'-OH, β -OH and 4'-OH-3'-OMe metabolites of acetylfentanyl and fentanyl in hepatocyte incubations.¹⁹¹ These metabolic motifs

were also identified in paper IV for cyclopropylfentanyl, further suggesting that there are shared patterns in the metabolism of fentanyls. Therefore, the same seven metabolic motifs, as were described in paper IV, were chosen for this study.

To make potential patterns in the metabolism of fentanyls more evident, fentanyl and cyclopropylfentanyl were also included in this study. In total, seven potential metabolites across five fentanyl analogues and fentanyl were synthesized. Hepatocyte incubations of the fentanyls were carried out and the resulting metabolite mixtures were analyzed together with the synthesized reference standards by LC-QTOF-MS. Moreover, Watanabe *et al.* have previously matched metabolites of acetylfentanyl, acrylfentanyl and 4F-isobutyrylfentanyl formed by hepatocyte incubations with metabolites identified in urine samples.¹⁹⁴ The findings in the hepatocyte incubations of the current study could therefore be tentatively matched with that urine sample data.

4.5.2. RESULTS AND DISCUSSION

The potential metabolites were generally synthesized using the same synthetic routes as were established in paper IV (Scheme 15). For brevity, small variations in reaction time, temperature and presence of Cs_2CO_3 between the reaction conditions were omitted from Scheme 15.

Scheme 15. (i) 4-(2-bromoethyl)phenol, Cs_2CO_3 , MeCN, 60 °C, 1 h, 11-58%; (ii) 3-(2-bromoethyl)phenol, MeCN, 60 °C, overnight, 8-60%; (iii) 2-methoxyphenethyl bromide, Cs_2CO_3 , MeCN, 60 °C, 72 h; BBr_3 , DCM, rt, overnight, 15-43% over two steps; (iv) phenacyl bromide, Cs_2CO_3 , MeCN, 60 °C, 2 h; NaBH_4 , EtOH, rt, 2 h, 11-71% over two steps; (v) 3,4-dimethoxyphenethyl bromide, Cs_2CO_3 , MeCN, 60 °C, overnight; BBr_3 , DCM, rt, overnight, 41-80% over two steps; (vi) 4-(2-bromoethyl)-6-methoxyphenol, MeCN, rt, 72 h, 34-80%; (vii) 3-(2-bromoethyl)-6-methoxyphenol, MeCN, 60 °C, overnight, 47-83%.

However, one notable exception to the synthetic route was applied in the synthesis of compounds **C7** and **C8** (Scheme 16). Instead of preparing the bromides **50** and **53** (Scheme 14), compound **54** was treated with BBr_3 . The use of BBr_3 is a well-known method for demethylation of aryl methyl ethers.²⁶⁴ The hypothesis was that the use of one equivalent of BBr_3 would result in the

demethylation of one methyl ether, which would yield a mixture of compounds **C7** and **C8** (Scheme 16). Contrary to that hypothesis, both methyl ethers were demethylated and compound **C6** was the exclusive product. However, compound **C6** could be treated with NaH, MeI and 18-crown-6 to afford a mixture of compounds **C7** and **C8** in yields of 3% and 7% respectively. Given the low yields and the additional work of needing to separate and characterize compounds **C7** and **C8** from their mixture, the preparation and use of bromides **50** and **53** is a better route (Scheme 14).

Scheme 16. (i) BBr₃, DCM, rt, overnight, 63%; (ii) NaH, MeI, 18-crown-6, THF, rt, overnight, **C7**: 3%, **C8**: 7%.

The 42 synthesized reference standards were analyzed by LC-QTOF-MS together with the metabolite mixtures generated by hepatocyte incubations of the six fentanyl. By comparing the retention times, accurate masses and fragmentation patterns of the target metabolites in these sets of samples, the exact structures of twenty metabolites identified in the hepatocyte incubations could be determined (Table III).

Table III. Metabolite abundances normalized to the most abundant metabolite with a single hydroxyl group on the phenethyl moiety. Adapted from Wallgren *et al. J. Anal. Toxicol.* 2020, with permission.²⁶⁵

Hepatocyte metabolite abundance (average, n=3)						
	Fentanyl	Acetyl	Acryl	Cyclopropyl	Isobutyryl ^a	4F-IBF
Nor	486%	215%	877%	906%	355%	366%
4'-OH	43%	100%	82%	33%	7%	7%
3'-OH	nd	3%	nd	nd	nd	nd
2'-OH	nd	nd	nd	nd	nd	nd
β-OH	100%	33%	100%	100%	100%	100%
4'-OH-3'-OMe	3%	12%	5%	2%	1%	1%
3'-OH-4'-OMe	nd	nd	nd	nd	nd	nd
3',4'-DiOH	nd	1%	nd	nd	nd	nd

Urinary metabolite abundance (Median of all samples detected)				
	Acetyl	Acryl	Cyclopropyl	4F-IBF
Nor	86%	182%	357%	1632%
4'-OH	100%	100%	100%	100%
3'-OH	nd	nd	nd	8%
2'-OH	nd	nd	nd	nd
β-OH	13%	6%	24%	94%
4'-OH-3'-OMe	100%	42%	52%	104%
3'-OH-4'-OMe	33%	1%	4%	5%
3',4'-DiOH	68%	43%	37%	24%

Urinary data was adopted from Watanabe *et al.* and Vikingsson *et al.*^{194,261} The data was normalized to the most abundant metabolite with a single hydroxyl group on the phenethyl moiety. ^a n=2

The most abundant metabolites across all fentanyls in the hepatocyte incubations were the nor-metabolites (Table III). Among the monohydroxylated metabolites, the β-OH metabolites were the most abundant metabolites for all fentanyls apart from for acetylfentanyl, where its corresponding 4'-OH metabolite (**C2**) was most abundant. These findings are in line with the results of studies of acetylfentanyl and fentanyl by Kanamori *et al.*¹⁹¹ Interestingly, for isobutyrylfentanyl and 4F-isobutyrylfentanyl the 4'-OH metabolites (**E2** and **F2**) were minor when compared to the other fentanyls. Monohydroxylation of the phenethyl moiety was found to be almost exclusive to positions 4' and β.

Among the metabolites with the 3'-OH motif (A-F3), only a small amount was identified in the hepatocyte incubations of acetylfentanyl. This was also the case among the 3',4'-diOH metabolites. Moreover, the respective 4'-OH-3'-OMe metabolites (A-F7) were identified across all fentanyls, albeit in small amounts, whereas its respective isomer (A-F8) was not present in any of the hepatocyte incubations. Kanamori *et al.* also identified the same motif for acetylfentanyl and fentanyl in hepatocyte incubations.¹⁹¹ Overall, there were clear patterns in which metabolites that were formed in the hepatocyte incubations. Furthermore, there were also similarities in the signal ratios of the different metabolic motifs across all studied fentanyls apart from for acetylfentanyl (Figure 39).

Figure 39. Chromatograms of the metabolites with a single hydroxyl group on the phenethyl moiety across the six fentanyls. The signals of the metabolites produced in hepatocyte incubations (dark trace) were compared with the signals of the synthesized reference standards (light trace). *Adapted from Wallgren et al. J. Anal. Toxicol. 2020, with permission.*²⁶⁵

The urinary data used for comparison with the synthesized potential metabolites was adopted from previous studies.^{194,261} Unfortunately, no comparisons could be made with fentanyl and isobutyrylfentanyl as no urinary data or urine samples of them were available. Based on the comparisons with the urinary data, it was found that position 4' was the position most prone to monohydroxylation ahead of the β -position (Table III). However, 4F-isobutyrylfentanyl was an exception since it exhibited a ratio between the two positions of almost one-to-one. Furthermore, the respective 3',4'-diOH and 4'-OH-3'-OMe metabolites were prominent across all fentanyl analogues. The respective 3'-OH-4'-OMe metabolites were also identified in urine samples of all the fentanyl analogues, albeit in lower abundances compared to its respective isomer. In summary, all the fentanyl analogues produced similar metabolic patterns also in urine.

When comparing the metabolic profiles of the fentanyls in urine samples with their metabolic profiles in hepatocyte incubations, there were many similarities but also distinct differences (Table III). Firstly, the preferred position for hydroxylation was the 4'-position in urine while it was the β -position in hepatocyte incubations. Kanamori *et al.* reported the CYP3A4 enzyme to be involved in the formation of the β -OH metabolite while CYP2D6 was suggested to be associated with the oxidation of the 4'-position.²⁰⁸ Hence, the preference towards the different positions for oxidation could be explained by differences in enzymatic activity in the hepatocyte model compared to *in vivo*. Furthermore, the more oxidized metabolites, the 3',4'-diOH and the 4'-OH-3'-OMe metabolites, were much more abundant in the urine samples. These differences suggest that the hepatocyte model's ability to replicate the *in vivo* metabolism is limited.

In summary, the most abundant metabolites in urine of the fentanyls in this study were found to be the 4'-OH, 3',4'-diOH and 4'-OH-3'-OMe metabolites. Given the patterns in metabolism among the studied fentanyls it is likely that other structurally similar fentanyls will produce metabolites with the same motifs. To make the production of reference standards of novel fentanyl analogues more efficient, three new scaffolds with the metabolic motifs already incorporated are suggested (Figure 40).

Figure 40. Chemical structures of three proposed scaffolds for the synthesis of potential metabolites of novel fentanyl analogues.

Additionally, the presence of the β -OH metabolites, while minor in most of the urine samples, is particularly interesting. β -Hydroxyfentanyl is not only a metabolite of fentanyl but also an NPS, which has been abused and placed under international control.^{46,91,214} β -OH metabolites of other fentanyls could therefore also be active. Thus, it is feasible that they could contribute to the pharmacological effects of their respective parent drugs.

4.5.3. CONCLUSION

The metabolism of five fentanyl analogues and fentanyl were investigated to identify potential metabolic patterns. 42 potential metabolites across the six fentanyls were synthesized. These standards were analyzed and compared with metabolites formed through hepatocyte incubations. Furthermore, the results were cautiously compared with metabolites identified in urine samples from previous studies conducted by our research group.

Similar metabolic motifs were identified in all the studied fentanyls, demonstrating patterns in their metabolism. In hepatocyte incubations, the most abundant motifs were found to be the β -OH, 4'-OH and 4'-OH-3'-OMe motifs. While in urine, metabolites with the 4'-OH, 4'-OH-3'-OMe and 3',4'-diOH motifs were identified as major. Consequently, there are limitations of the hepatocyte incubation model in its ability to replicate the *in vivo* metabolism.

With six different fentanyls showing similarities in their metabolic profiles, it is probable that novel fentanyl analogues with similar structures will also metabolize comparably. To make use of these identified patterns and to meet the demand for reference standards, synthetic routes to produce new scaffolds should be developed. These scaffolds should have the identified metabolic motifs incorporated in their structures to facilitate efficient synthesis of metabolites of novel fentanyl analogues.

5. CONCLUSIONS AND FUTURE PERSPECTIVES

In this thesis, structural elucidation of urinary metabolites of synthetic cannabinoids and fentanyl analogues using synthesized reference standards is presented. What follows is a description of the characteristic workflow of the metabolism studies carried out in this research.

Firstly, synthetic targets were chosen based on the results from previous metabolism studies carried out by our research group. These synthetic targets typically contained hydroxyl groups and other oxidized species compared to their respective parent compound. Furthermore, the pentyl side chain, adamantane and phenethyl moieties of the studied NPS were identified as moieties particularly prone to metabolic oxidation. Secondly, by employing scaffold synthesis, synthetic routes were developed to produce numerous potential metabolites of various synthetic cannabinoids and fentanyl analogues. Subsequently, the synthesized potential metabolites were analyzed by LC-QTOF-MS alongside either (i) metabolites generated by hepatocyte incubations, (ii) metabolites in urine samples or (iii) the two in combination. Finally, by comparing the retention times, accurate masses and fragmentation patterns of the metabolites from the different sample sets, the exact structures of many major urinary metabolites could be determined across the studied new psychoactive substances.

Among these major metabolites, several of them are also uniquely formed from their respective parent drugs, which make them fit the criteria for being used as urinary biomarkers for drug detection. Examples of such metabolites are the 3-OH pentyl side chain metabolite and the metabolite with a hydroxyl group on a secondary carbon of the adamantane moiety of the synthetic cannabinoid AKB-48. For cyclopropylfentanyl, acetylfentanyl, acrylfentanyl and 4F-isobutyrylfentanyl, their respective 4'-OH, 3',4'-diOH and 4'-OH-3'-OMe metabolites fit the aforementioned criteria.

Additionally, a concise synthetic route to produce potential synthetic cannabinoid metabolites with the 4-OH-5F pentyl side chain motif was developed and demonstrated for STS-135, MAM-2201, AM-2201 and XLR-11.

Biomarkers with this motif can unambiguously identify drug intake of synthetic cannabinoids with a terminally fluorinated pentyl side chain.

In conclusion, by using synthesized reference standards, structural elucidation of metabolites whose structures could previously only be tentatively identified was accomplished. The identified key metabolites and the developed synthetic routes can be used to provide forensic toxicology laboratories with reference standards of urinary biomarkers for drug detection. Furthermore, pharmacological studies of such metabolites can be carried out to explore their role in the toxicity of their respective parent drugs.

Judging by the rate at which novel NPS enter the recreational drug market, there is seemingly no end in sight to the NPS phenomenon. Thus, new urinary biomarkers will need to be identified and to accomplish this, more metabolism studies will need to be conducted. However, the pentyl side chain, adamantane and phenethyl moieties studied in this thesis are recurring building blocks among NPS. Hence, it is plausible that the metabolic profiles and tendencies identified among the NPS studied in this thesis can be utilized in predicting major metabolites of NPS which have yet to emerge on the drug market. Therefore, for efficiency, new synthetic routes should be developed to create scaffolds containing the identified metabolic motifs. From these scaffolds, different analogues with these metabolic motifs can be afforded. This approach should increase the level of preparedness and aid in the fight against the unrelenting NPS.

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