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Per Ole M. Gundersen

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Norwegian University of Science and Technology
Thesis for the Degree of
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Utvikling av strategier for påvisning av nye psykoaktive substanser og deres metabolitter i biologiske prøver med LC-QTOF-MS-teknologi.

Påvisning av nye psykoaktive rusmidler i biologiske prøver.

Antallet nye psykoaktive substanser (NPS) som lanseres på det illegale markedet er en kontinuerlig utfordring for kliniske og toksikologiske laboratorier. NPS fremstilles syntetisk som alternativ til etablerte| rusmidler for blant annet å omgå narkotikalovgivningen i ulike land. Ved for eksempel å ta utgangspunkt i strukturen til det psykoaktive virkestoffet i cannabis er et stort antall varianter kalt syntetiske cannabinoider utviklet. På samme måte utvikles det stadig nye rusmidler basert på strukturen til blant annet opioider og benzodiazepiner. Det er praktisk talt umulig å ha analytiske metoder som til enhver tid kan påvise alle de nyeste rusmidlene i biologisk materiale. En bekymring er derfor at man går glipp av betydningsfulle funn av NPS i blod eller urin fra pasienter eller avdøde. Visse grupper av NPS, som for eksempel fentanyl-analogene, skaper ekstra bekymring fordi giftigheten er så stor. Dermed er de dosene som inntas tilsvarende små, og følgelig må også analysemetodene være svært følsomme. Man må også ha kunnskap om hvilke nedbrytningsprodukter som er tilstede i kroppen for å kunne påvise inntak. I denne avhandlingen har jeg vist tre eksempler på bruk av en avansert massespektrometrisk teknologi, LC-QTOF-MS, i påvisning av NPS og deres nedbrytningsprodukter i biologisk materiale.

I studie I utviklet jeg en sensitiv metode for påvisning og mengdebestemmelse av metabolittene til de mest brukte syntetiske cannabinoidene i urin. Prepareringen av urinprøvene fram til analyse på LC-QTOF-MS var automatisert og metoden ble validert i henhold til internasjonale retningslinjer. Metoden ble benyttet til å analysere 1000 urinprøver fra personer som var under rusavvenning. En eller flere metabolitter ble kvantifisert og bekreftet i 2,3 % av prøvene.

I studie II ble levende leverceller fra mennesker benyttet for å undersøke nedbrytningsproduktene til tre fluorinerte derivater av det syntetiske opioidet fentanyl, orto- meta- og para-fluorfentanyl. Levercellene ble inkubert med forbindelsene, og deretter analysert med LC-QTOF-MS. Ved å tolke datafilene fra analysen kunne vi vise at de viktigste metabolittene var norfluorfentanyl samt ulike hydroksylerte varianter, et oksid, dihydrodiol-metabolitter og en hydroksymetoksy-metabolitt. Urin fra en person med påviselig inntak av orto-flurofentanyl ble også analysert, og tre av metabolittene fra levercellestudien ble påvist.

Studie III var en systematisk gjennomgang av datafilene fra alle obduksjonsprøver som ble analysert med LC-QTOF-MS ved Avdeling for klinisk farmakologi ved St. Olavs hospital i perioden 2013 til 2018. Analysen gir data der man i prinsippet kan søke etter alle substanser. Vi søkte gjennom 1314 filer på nytt på jakt etter NPS som var ukjente på det tidspunktet prøvene opprinnelig ble analysert. Fem nye funn ble gjort med stor grad av sikkerhet.

Det er store fordeler med LC-QTOF-MS sammenliknet med den instrumenteringen som vanligvis brukes ved kliniske og toksikologiske laboratorier. Disse er blant andre muligheten til enkelt å legge til nye komponenter i metodene, å identifisere ukjente rusmidler og å søke etter nye forbindelser uten ny analyse. Alle disse fordelene ble demonstrert i avhandlingen.

Per Ole M. Gundersen
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Finansieringskilde: Laboratoriemedisinsk klinikk, St. Olavs hospital

Ovennevnte avhandling er funnet verdig til å forsvares offentlig
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Digital disputas finner sted
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Summary

New psychoactive substances (NPS) are emerging in the illegal drug market, which has led to major challenges for analytical laboratories. Keeping screening methods up to date with all relevant drugs is hard to achieve and the risk of missing important findings in biological samples is a matter of concern. Certain groups of NPS, e.g., synthetic opioids including fentanyl analogues, are of special concern due to their high potency. This indicates the possibility of low drug concentrations in vivo and calls for sensitive analytical methods and identification of the most appropriate analytical targets. In this thesis, three studies were carried out to demonstrate the application of liquid chromatography quadrupole time-of-flight mass spectrometry (LC-QTOF-MS) technology in the detection of NPS and their metabolites.

In **Study I**, a sensitive and quantitative screening method in urine with metabolites of synthetic cannabinoids that were frequently seized in Norway in the current time period (AB-FUBINACA, AB-PINACA, AB-CHMINACA, AM-2201, AKB48, 5F-AKB48, BB-22, JWH-018, JWH-073, JWH-081, JWH-122, JWH-203, JWH-250, PB-22, 5F-PB-22, RCS-4, THJ-2201, and UR-144) was developed. The samples were treated with ß-glucuronidase prior to extraction and solid-phase extraction was used. Liquid handling was automated using a robot. Each sample was initially screened for identification and quantification, followed by a second injection for confirmation. The concentrations by which the compounds could be confirmed varied between 0.1 and 12 ng/ml. Overall, the validation showed that the method fulfilled the set criteria and requirements for matrix effect, extraction recovery, linearity, precision, accuracy, specificity, and stability. One thousand urine samples from subjects in drug withdrawal programs were analysed using the presented method. The metabolite AB-FUBINACA M3, hydroxylated metabolite of 5F-AKB48, hydroxylated metabolite of AKB48, AKB-48 *N*-pentanoic acid, 5F-PB-22 3-carboxyindole, BB-22 3-carboxyindole, JWH-018 *N*-(5-hydroxypentyl), JWH-018 *N*-pentanoic acid, and JWH-073 *N*-butanoic acid were quantified and confirmed in 2.3% of the samples. The method was proven to be sensitive, selective, and robust for routine use for the investigated metabolites.

In **Study II**, the in vitro metabolism of ortho-, meta-, and para-fluorofentanyl—three fluorinated derivatives of fentanyl—was investigated using human hepatocytes and compared to the results from an authentic urine sample from a human individual with a confirmed intake. The three fluorofentanyl isomers were incubated with pooled human hepatocytes at 1,

3, and 5 h. LC-QTOF-MS operating in data-dependent mode was used to analyse the hepatocyte samples, as well as the authentic urine sample. Data were analysed by a targeted approach with a database of potential metabolites. The major metabolite formed in vitro was the *N*-dealkylation product norfluorofentanyl. In addition, various hydroxylated metabolites, an *N*-oxide, dihydrodiol metabolites, and a hydroxymethoxy metabolite were found. In the authentic urine sample, the hydroxymethoxy metabolite, norfluorofentanyl, and a metabolite hydroxylated on the ethylphenyl ring were detected. The study showed that the metabolic pattern for ortho-, meta-, and para-fluorofentanyl was close to those previously reported for other fentanyl analogues. The hydroxymethoxy metabolite and the metabolite hydroxylated on the ethylphenyl ring stand out as the metabolites that should be investigated further to determine the most appropriate marker for intake of fluorofentanyl derivatives in urine drug screening for human subjects.

In **Study III**, a strategy for extended retrospective data analysis of data files acquired by LC-QTOF-MS was developed. Diagnostic fragment ions from synthetic cannabinoids (n=251), synthetic opioids (n=88), and designer benzodiazepines (n=26) were obtained from the crowdsourced database HighResNPS.com and converted to a personalized library in a format compatible with the analytical instrumentation. Data files from the analysis of 1314 forensic post-mortem samples performed at the Department of Clinical Pharmacology from January 2014 to December 2018 were retrieved and retrospectively processed with the new personalized library. Potentially positive findings were grouped into category 1 (most confident) and category 2 (less confident) depending on the information available in the files. Five new findings of category 1 were identified: flubromazepam in two data files from 2015 and 2016, respectively, phenibut (4-amino-3-phenylbutyric acid) in one data file from 2015, fluorofentanyl in one data file from 2016, and cyclopropylfentanyl in one data file from 2018. Retention time matches with reference standards further strengthened these findings. One category 2 finding of phenibut was considered plausible after the checking of retention times and signal-to-noise ratios, whereas 34 other potentially category 2 findings were refuted after a closer evaluation. This study showed that new compounds can be detected retrospectively in data files from LC-QTOF-MS using an updated library containing diagnostic fragment ions.

Acknowledgments

The work of this thesis was built on three studies, conducted at the Department of Clinical Pharmacology, St. Olavs hospital, Trondheim University Hospital, and the Department of Clinical and Molecular Medicine, Faculty of Medicine and Health Sciences, Norwegian University of Science and Technology (NTNU) from 2015 to 2020. The work was funded by St. Olavs hospital´s Clinic of Laboratory Medicine, and the Department of Clinical Pharmacology provided laboratory facilities and resources.

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I wish to express my sincere thanks to the other co-writers of the published papers. Dr. Sebastian Broecker for constructive discussions on instrumental issues through the years, and especially for his contributions to the methodology and development of the software solution applied in study III. Professor Henrik Gréen, Dr. Svante Vikingsson and Anna Åstrand for making research facilities at Linköping University and Swedish National Board of Forensic Medicine available to me, and for sharing their knowledge in drug metabolism and structure elucidation. Professor Lars Slørdal for providing me guidance in the field of forensic science, and thus critical contributions to study III.

I thank all my colleagues for making the Department of Clinical Pharmacology a great place to work, and for giving me space to focus on my studies. A special thanks to the Head of Department, Trond Oskar Aamo.

Last but not least, I thank my mother, father and sister with her family for encouragement and support.

Abbreviations

CID	Collision-induced dissociation
CYP	Cytochrome P450
DDA	Data-dependent acquisition
DIA	Data-independent acquisition
EMCDDA	European Monitoring Centre for Drugs and Drug Addiction
ESI	Electrospray ionization
FWHM	Full width at half maximum
HR-MS	High-resolution mass spectrometry
IUPAC	The International Union of Pure and Applied Chemistry
KRIPOS	Norwegian National Criminal Investigation Service
LC	Liquid chromatography
LC-MS/MS	Liquid chromatography tandem quadrupole mass spectrometry
LC-QTOF-MS	Liquid chromatography quadrupole time-of-flight mass spectrometry
LLE	Liquid-liquid extraction
LOC	Limit of confirmation
LOI	Limit of identification
LOQ	Limit of quantification
m/z	Mass-to-charge ratio
ME	Matrix effect
MRM	Multiple reaction monitoring
MS	Mass spectrometry
NPS	New psychoactive substance(s)
oa	Orthogonal acceleration
PCDL	Personal compound database and library
pKa	Acid dissociation constant
ppm	Parts per million
Q1	1st quadrupole

q2	Collision cell
QC	Quality control
QTOF	Quadrupole time-of-flight
RE	Recovery
REK	Regional Committee of Medical and Health Research Ethics
RT	Retention time
SC	Synthetic cannabinoid(s)
SPE	Solid phase extraction
SRM	Selected ion monitoring
THC	Δ9-tetrahydrocannabinol
TOF	Time-of-flight
UHPLC	Ultra-high-performance liquid chromatography
UN	United Nations
UNDOC	United Nations Office on Drugs and Crime

List of papers

Paper I

Per Ole M. Gundersen, Olav Spigset, Martin Josefsson

Screening, quantification, and confirmation of synthetic cannabinoid metabolites in urine by UHPLC-QTOF-MS

Drug Testing and Analysis 11(1) (2019) 51-67. https://doi.org/10.1002/dta.2464

Paper II

Per Ole M. Gundersen, Anna Åstrand, Henrik Gréen, Martin Josefsson, Olav Spigset, Svante Vikingsson

Metabolite profiling of ortho-, meta- and para-fluorofentanyl by hepatocytes and high-resolution mass spectrometry

Journal of Analytical Toxicology 44(2) (2020) 140-148. https://doi.org/10.1093/jat/bkz081

Paper III

Per Ole M. Gundersen, Sebastian Broecker, Lars Slørdal, Olav Spigset, Martin Josefsson

Retrospective screening of synthetic cannabinoids, synthetic opioids and designer benzodiazepines in data files from forensic post mortem samples analysed by UHPLC-QTOF-MS from 2014 to 2018

Forensic Science International 311 (2020) [Epub ahead of print]. https://doi.org/10.1016/j.forsciint.2020.110274

1. Introduction

1.1. New psychoactive substances

1.1.1. Definitions and background

New psychoactive substances (NPS) represent a broad range of drugs. According to the definition from the United Nations Office on Drugs and Crime (UNODC), "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" (1). They share structural and toxicological/pharmacological similarities with psychoactive compounds like morphine, amphetamine, and Δ9-tetrahydrocannabinol (THC) (2, 3). NPS can be classified based on their chemical structures in the following groups: synthetic cannabinoids (SC), cathinones, phenethylamines, opioids, benzodiazepines, arylcyclohexylamines, and other substances (4). An alternative classification is based on the pharmacological action of the different substances: opioids, cannabinoid receptor agonists, dissociatives, classic (serotonergic) hallucinogens, sedatives/hypnotics, and psychostimulants (5).

The history of drug discovery is filled with examples of substances synthesized for medicinal purposes but that ended up as illegal drugs. LSD (D-lysergic acid diethylamide) was first synthesized in 1938 by Albert Hofmann, who intended to obtain an analeptic (circulatory stimulant). In 1943 he continued the work with the substance and, in the process, was accidently intoxicated, which he described as "…remarkable but not unpleasant (…) characterized by extraordinarily intense stimulation…" (6). In the 1950s, LSD was marketed as a psychiatric panacea. The drug became popular also beyond the intended use—so much so that it was scheduled by the United States Government in 1967 and by the UN (United Nations) in 1971. MDMA (3, 4-methylene-dioxymethamphetamine) was synthesized by Merck in 1912 in the process of making vasoconstrictive and styptic drugs (6). The structure was patented but little happened with MDMA until 1976, when Alexander Shulgin resynthesized the compound and reported its psychoactive properties. The consumption of MDMA emerged and it became one of the most popular drugs of abuse. In 1986, MDMA was added to the UN convention as a Schedule I controlled substance (7). Heroin (diacetylmorphine) was first synthesized in 1874 by C.R. Alder Wright and replicated two decades later by a chemist at German AG (6). The compound was marketed as a more

effective and less toxic alternative to codeine and as a substitute for morphine. In the early 1900s, heroin began being abused in Europe and the US and was scheduled in the Single Convention on Narcotic Drugs of 1961 (8).

Many NPS have similar histories, being synthesized and then re-discovered and introduced on the drug market several years later, as illustrated by the SC JWH-018 and designer benzodiazepine flubromazepam in Fig. 1. The term "new" does not necessarily mean that the compound was first synthesized or discovered (as natural compounds) recently but, rather, reflects a recent appearance on the illegal drug market or use as a recreational drug. Alexander Shulgin described synthesis and self-experiments on several phenethylamines and tryptamines in the 1990s (9, 10). Many of these have found their way to the drug market and were consequently categorized as NPS.

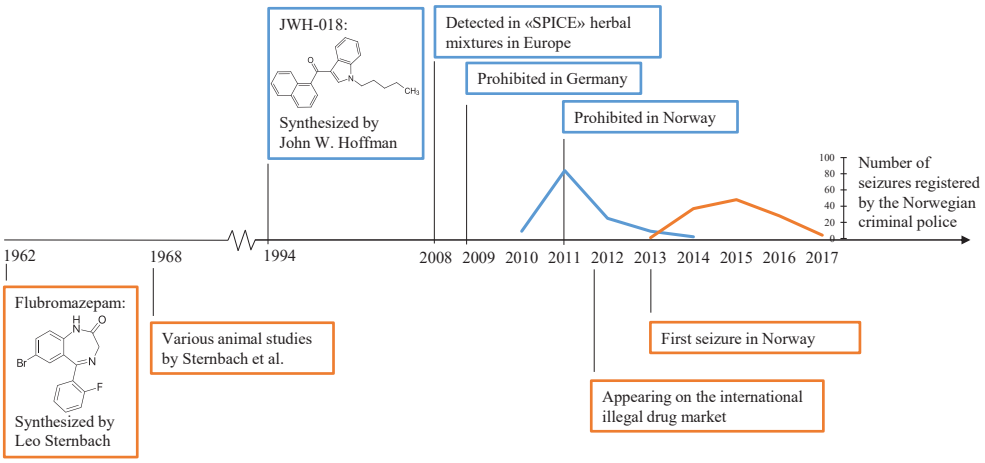


Fig. 1: Timeline showing the appearance, emergence, and decline of JWH-018 (11-13) and flubromazepam (14) on the illicit drug market.

Over the last decade, there has been a significant increase in the number of these compounds, as globalisation and improved information technology have allowed the manufacture and use of NPS to spread quickly around the world (15). An important reason for this increase is that NPS were not scheduled in the Conventions of 1961 or 1971 and the legal situation has been up to each country. Traditionally, regional and national drug control legislation has been based on the scheduling of specific substances (together with their stereoisomers, esters, and salts) (16). However, the inclusion of new substances is often a lengthy process that requires, in most cases, a health risk assessment (based on scientific data and human experience data

that, in the case of NPS, is often scarce), followed by legislative amendments that usually take several months to complete. Fig. 1 shows a typical appearance, emergence, and decline of NPS from the market, illustrated by JWH-018 before and after scheduling. Some countries, e.g., Norway, have responded by adopting more proactive policies that control broader classes of substances (generic legislation) (13). In Norway, legislation was updated with the first general structures in February 2013. It now holds 12 general structures from which all derived compounds are considered illegal. However, an approach like this has limitations, as, over time, new compounds within a specific drug class that deviates from any of the general structures have been introduced (e.g., new generations of SC). The drug packaging is often labelled as “research chemicals” and “not for human consumption” to convey that the products not intended for recreational use (17), or they are referred to as “legal highs” not controlled by the current legislation (18).

The number of newly available NPS every year steadily increased in Europe up to 2014 (101 new reported that year), as shown in Fig. 2A, but in the last years these annual numbers of substances reported for the first time have declined (51 and 55 new substances in 2017 and 2018, respectively) (4). In contrast to the general trend since 2015, there has been an increase in the number of synthetic opioids (Fig. 2B). These are numbers based on reports to the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) Early Warning System, which plays a crucial role in rapidly detecting, assessing, and responding to the public health and social threats that these substances cause. The number of seizures reported to the EMCDDA Early Warning System peaked in 2015, at more than 70,000 (4). There was a decline in the following years.

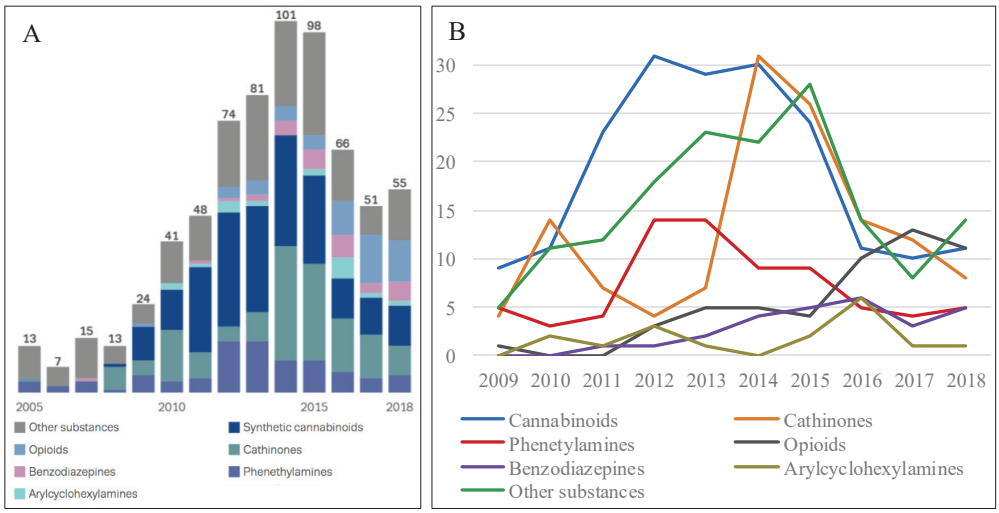


Fig. 2: (A) Number and categories of new psychoactive substances (NPS) reported to the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) Early Warning System for the first time, 2005-2018 (Source: European Monitoring Centre for Drugs and Drug Addiction, European Drug Report 2019 (4)). (B) Number of NPS reported to EMCDDA for the first time, sorted into groups. (Source: European Monitoring Centre for Drugs and Drug Addiction, Health responses to new psychoactive substances, 2016 (19). The numbers from 2016 to 2018 are estimated from Fig. 2A.

1.1.2. Synthetic cannabinoids

Synthetic cannabinoids (SCs) constitute the group of NPS with the highest number of new compounds introduced to the market over the last 10 years. These compounds act as cannabinoid receptor agonists in the complex human endocannabinoid system, similar to the psychoactive ingredient THC in cannabis. The term synthetic cannabinoid receptor agonists is also commonly used for this group of NPS. The first generation of SCs was synthesized to study the receptor mechanism of THC and search for new drugs, especially analgesics (20). John W. Huffman, a Clemson University medicinal chemist, originally synthesized the most extensive series of SC (JWH series) while studying cannabinoid receptor pharmacology (11). In 2008 some of these were first detected in seized herbal products after reports of marihuana-like effects (12). These products were called “Spice” and contained plant material with synthetic cannabinoids added. SC give similar but more intensive physiological and psychoactive effects then THC, resulting in medical and psychiatric emergencies (21). Two factors are likely to be important in explaining this: (A) Studies have shown that many SCs are more potent than THC, as they activate the cannabinoid receptor CB1 more extensively

(22, 23). (B) The potential high doses are caused by the process of mixing the synthetic cannabinoids with the plant material. The many steps and uncertainties in this process result in toxic concentrations as well as the inhomogeneous distribution of the drug in the material (24). Both factors make it difficult to control the dose. The different pharmacodynamic properties of the SC, as well as their propensity for being administered in overdoses, explain the excessive toxicity as compared to THC. These toxicities include serious cardiovascular toxicity, coma/the rapid loss of consciousness, respiratory depression, seizure and convulsions, hyperemesis, delirium, agitation, psychosis, and aggressive and violent behaviour (21, 25-27). The low knowledge about the toxic effect among users and which symptoms to expect, and the reduced awareness of these, increase the risk of fatalities even more. There has been an evolution in SC structures probably driven by changes in legislation. **Studies I** and **III** both involve the identification of SC.

1.1.3. Synthetic opioids and fentanyl analogues

Synthetic opioids are another rapidly expanding group of NPS. Fentanyl analogues are compounds within this class. Fentanyl itself was first synthesized by Janssen Company in Beers, Belgium in 1960 and has since then become one of the world's most important opioid analgesics (28). Pethidine, which was the starting point for the synthesis, was easy to manipulate and new analogues were produced. MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), a synthesis by-product found in the production of the pethidine analogue MPPP (1-methyl-4-phenyl-4-propionoxypiperidine), leads to irreversible precipitation of Parkinsonism in users exposed to it (29). The example illustrates the vulnerable situation to which users of NPS are exposed when they buy products and have very little knowledge of the actual content. Several fentanyl analogues have been widely used in human and veterinary medicine. The main effect of opioids, including fentanyl and its analogues, is binding to and the activation of the μ-opioid receptor in the central nervous system. Fentanyl is estimated to be about 100 times more potent than morphine, whereas the fentanyl analogue carfentanil is estimated to be about 10,000 times more potent than morphine (30). The potency, as well as dependence potential, have put 21 fentanyl analogues in the UN international drug control system. Since 2012, 28 new fentanyl analogues have been identified on the drug market in Europe. Reports to the EMCDDA of fatal poisonings have also increased (4). Fentanyl analogues have been found in different physical forms, most often as powders, tablets, and liquids (e.g., in nasal spray). They have also been detected in mixtures with one or more other substances. Non-pharmaceutical fentanyl and analogues are increasingly found in heroin and

other drugs, presumably reflecting an effort to make these drugs more potent, easier and cheaper to manufacture, and possibly easier to sell. On some occasions, illicit fentanyl analogues have been sold in fake tablets looking like the original medicines. One example of this was a fatal poisoning in Trondheim, where cyclopropylfentanyl was detected in a tablet marked Xanax (originally a pharmaceutical product containing the benzodiazepine alprazolam) (31). The counterfeit tablets (Fig. 3) had the same dimensions and colour as the original. As users can be unaware of this, the risk of life-threatening poisonings is increased. The detection of metabolites from the fentanyl analogue fluorofentanyl and the identification of synthetic opioids is the focus of **Studies II** and **III**, respectively.

Fig. 3: Counterfeit tablets labelled "XANAX" seized in Trondheim and submitted to the Department of Clinical Pharmacology. Analysis showed that they contained the fentanyl analogue cyclopropylfentanyl. Photo: Andreas Westin.

1.1.4. Designer benzodiazepines

Another group of NPS is designer benzodiazepines. The development of benzodiazepines started in the mid-1950s by Hoffmann-La Roche chemist Leo Sternbach, who was searching for better tranquilizers. The first compound, chlordiazepoxide, entered the market in 1960; it was followed by diazepam in 1963 and they quickly replaced barbiturates in the treatment of anxiety and insomnia (32) and overtook meprobamate as the leading tranquilizers (6). Various benzodiazepines are now widely used for these indications as well as for withdrawal treatment, preoperative sedation in anaesthesia, and epileptic seizures. The first benzodiazepines that were more typically used recreationally than therapeutically were phenazepam and etizolam (from 2007 and 2011, respectively). Since 2012 several new benzodiazepines have been detected through seizures; these compounds are called designer benzodiazepines and are either 1) drug candidates that were never approved for medical use

or 2) simple structural modifications or active metabolites of approved drugs. Currently, the EMCDDA is monitoring 28 designer benzodiazepines. During 2018 more than 2.4 million tablets containing these substances were reported in seizures to the EMCDDA Early Warning System (4). Their prevalence of use among subjects driving under the influence of drugs and the number of seizures related to them in Norway have recently been shown to be relatively high compared to other groups of NPS (33). **Study III** covers the identification of designer benzodiazepines.

1.1.5. Other NPS groups

Synthetic cathinones are used mainly as substitutes for cocaine, amphetamine, and other scheduled stimulants. This made them the quantitatively dominant group of seized material in 2016 (increasing every year from 2009). Phenethylamines are a group of monoamine alkaloids with stimulant properties. Amphetamine and MDMA are examples of traditional recreational drugs within this group. Neither synthetic cathinones, phenetylamines, nor any other group of NPS are subject to attention in the studies included in this thesis. The synthetic cathinones were left out mainly because of complex instrument-related methodological issues (including multiple isomers and difficult identification).

1.2. Drug metabolism

Most psychotropic drugs are, by nature, quite hydrophobic and only a small fraction is freely dissolved in blood plasma and accessible to the kidney for excretion. Consequently, the elimination of unmodified drugs in urine is very low. The body has several effective systems for transforming hydrophobic xenobiotics and endogenous substances into more water-soluble moieties. Cytochrome P450 (CYP) is a large superfamily of enzymes that catalyse the metabolism of these substances (34). Examples of reactions catalysed by CYP enzymes are carbon oxidation, heteroatom oxidation, dealkylation, and epoxide formation. These are examples of phase 1 metabolism, characterized by relatively small chemical modifications. In contrast, phase 2 metabolism involves reactions that attach charged species to a xenobiotic and endogenous substance or a phase 1 metabolite of these, thereby further increasing water solubility. Examples of phase 2 reactions are glucuronidation, acetylation, and sulfate conjugation. Hydrophobic drugs like SCs and synthetic opioids are excreted mainly as phase 1 and 2 metabolites and only a small fraction of the parent substance can be detected in urine (35, 36). To detect the intake of these substances through the chemical analysis of urine,

knowledge of the metabolic pathways of the drugs in question and which metabolites are the most abundant in urine is essential. The close structural resemblance observed within a group of NPS poses a challenge if an unambiguous determination as to which specific drug was ingested based on a urine sample. One such example is AM-2201 and JWH-018, both of which have the major metabolites JWH-018 *N*-pentanoic acid and JWH-018 *N*-(5-hydroxypentyl). Nevertheless, the specific markers AM-2201 *N*-(4-hydroxypentyl) and AM-2201 *N*-(6-hydroxyindole) of AM-2201 and JWH-018 *N*-(4-hydroxypentyl) of JWH-018 are also formed and can be used to distinguish between the intake of these two (37, 38). A careful selection of target metabolites is, therefore, required. New SCs that are biotransformed to metabolites identical to a drug's metabolite that is already covered by a method can be introduced to the market. Consequently, the exact intake cannot be confirmed without updating the method with newly available unique markers. The introduction of AMB-FUBINACA, which gives the same metabolite as AB-FUBINACA, is an example of the latter (39).

1.3. Liquid chromatography quadrupole time-of-flight mass spectrometry

Chemical identification and quantification of drugs in biological samples is essential for clinical and toxicological laboratories. Mass spectrometry (MS)-based techniques have been available for this purpose for decades. MS instruments detect compounds based on mass-to-charge ratio (*m/z*). The introduction of the large number of similar compounds has created the need to adopt instrumentation with increasing selectivity. High-resolution MS (HR-MS) is capable of distinguishing compounds with a small difference in mass and, in this way, offers more selectivity compared to unit resolution MS instrumentation. Quadrupole time-of-flight (QTOF) and Orbitrap are examples of HR-MS instrumentation. In the majority of bioanalytical applications, HR-MS is coupled to a chromatographic system.

1.3.1. Liquid chromatography

A separation technique like liquid chromatography (LC) in front of the MS separates components in a complex biological sample that improves the detection and identification of drugs. When performing analysis with LC, the sample is solved in a mobile phase which is pumped through a column containing a stationary phase. In reverse-phase LC, among the most-utilized chromatographic principle in modern chemistry, the stationary phase is a nonpolar coating of porous particles packed in a column, while the mobile phase is a polar

mixture of water plus organic solvent (40). The analyte molecule solved in the mobile phase interacts with the stationary phase and is retained. The time it takes for the analyte molecule to elute from the chromatographic column is its retention time (RT). The chemistry of the stationary phase and mobile phase composition are the main factors controlling the selectivity, while the length of the column and the size of the particles affect the system's capacity to separate compounds, which is referred to as column efficiency. Increasing length and decreasing particle size result in a higher resolving capacity and greater efficiency. Instruments that can handle increased back-pressure, which is a result of smaller particle size (< 2 μm), have been introduced to the market (41). Such systems are called ultra-high-performance LC (UHPLC). In **Studies I-III**, different column dimensions have been used but all with the same stationary phase chemistry (see Table 4 in paragraph 3.4). The high selectivity achieved by HR-MS may lead to an undermining of the importance of chromatography, but the high efficiency of the LC system is essential to decreasing the number of co-eluting peaks and detecting all compounds of interest. This is of special importance in screening methods covering NPS that typically have very small differences in chemical structure.

1.3.2. Electrospray ionization

For detection by MS, drugs in the sample eluting from the LC must be ionized, charged, and transferred from atmospheric pressure into the high vacuum zone of the MS instrument. This process takes place in the ion source. Electrospray ionization (ESI) is a universal ion source suitable for analysing both small and large molecules of various polarity in complex biological sample mixtures (42, 43). Generation of molecular ions occurs when the liquid from the LC is exposed to a strong electric field under atmospheric pressure while forming a spray at the end of a capillary. An electric field with a potential difference of 2-6 kV between the capillary and the counter-electrode is produced, which results in the accumulation of charges at the surface of the droplets (42). As the solvent evaporates, the droplets will shrink and their charge-to-volume ratio will increase; ultimately, desorption of ions from the surface occurs. The best ion formation efficiency is achieved when the pH in the mobile phase is adjusted below acid dissociation constant (pKa) for bases and above pKa for acids. Most drugs have a proton accepting functional group integrated into the molecule. Ionization is secured by adjusting the pH in the chromatographic system minimum 2 pH units below the pKa of the proton accepting group. When ESI is operating in positive mode (positive

potential), positively charged ions are transferred to the MS, while in negative mode negatively charged ions are transferred.

1.3.3. High-resolution mass spectrometry

HR-MS instrumentation offers both high resolution and high mass accuracy. In the The International Union of Pure and Applied Chemistry (IUPAC) definition, mass resolution is expressed as m/Δm, where m is the mass of the ion and Δm is the width of the peak or spacing between two peaks of equal intensity with a valley between them no more than a fraction (e.g., 10%) of the height of the smallest peak (10% valley definition) (44). A common standard is to estimate resolution based on 50% of the peak height (full width at half maximum, FWHM). Specifying the fraction of peak height used in the estimation of resolution is important to be able to compare instruments and methods. An instrument that achieves a resolution > 10,000 by the FWHM definition is considered to be an HR-MS instrument (45), though there is no absolute boundary. In time-of-flight (TOF) instruments, the resolution is constant across the mass range. Mass accuracy is the difference between measured mass and the theoretical mass, often expressed as parts per million (ppm). Mass accuracy is maintained by periodic external and continuous internal mass calibration (46).

TOF-MS was first described in 1946, and the first commercial instrument was introduced in 1955 (45, 47, 48). A renewed interest in this technique in the late 1980s was shown when progress in electronics made the handling of the data flow easier. The basic principle in a TOF analyser is that ions with *m/z* are separated according to their velocity when drifting in a field-free region after being accelerated by an electric field. The first systems were of linear design, meaning that the traveling direction of the ions is linear through the instrument. Linear design is a suitable configuration for techniques in which ions are generated in pulses, e.g., matrix-assisted laser desorption ionization. When the ions are generated continuously, as with chromatographically based systems, the beam of ions must be transformed into a pulsed process.

Poor mass resolution was a disadvantage of the first TOF analysers (45). A longer flight-tube will increase the resolution, but a too-long flight path will decrease the performance. The mass resolution is also affected by factors resulting in flight time deviation among ions with the same *m/z*. These factors are time distribution, space distribution, and kinetic energy distribution. Delayed pulsed extraction corrects the energy dispersion by transferring more

energy to the ions that remained in the source for a longer time. Introducing the electrostatic reflector creates a retarding field acting as a mirror by deflecting the ions and sending them back through the flight tube. This will correct kinetic energy dispersion, as the faster ions will spend more time in the reflector and, consequently, reach the detector at the same time as slower ions with the same *m/z*, resulting in an increased resolution.

Continuous ion generation requires a modification to the linear TOF configuration. The first approach was to sweep the ion beam over a slit to form an axial pulse. This resulted in a loss of ions and a low duty cycle. A more efficient solution was to build the flight tube orthogonal to the ion beam coming from the source, known as orthogonal acceleration (oa) (46). The ions formed in the source are focused by the ion optics and travel to the orthogonal accelerator. These ions fill the space of the ion accelerator before they are pushed by an electric field in the orthogonal direction of their original direction into the flight tube (45). In the time during which the ions complete the flight, new ions have re-filled the accelerator and are ready to be pushed into the flight tube. This flight cycle occurs at a frequency of several kHz. The flight time is considerably longer than the time needed to fill the accelerator, which results in a duty cycle below 100%. (Only the ions passing through the accelerator at the "push" ultimately reach the detector. The duty cycle can be defined as the number of ions that are subject to mass analysis relative to the number of ions formed in the ionization step.) Because of the rapid cycle time, hundreds of consecutive pushes are summarized to produce a single averaged spectrum. All ions pushed into the flight tube are detected, giving a full scan spectrum at every time point. The resolution of modern TOF instruments is superior to that of triple quadrupole instruments.

The oaTOF can be combined with another mass analyser in a hybrid configuration offering tandem mass spectrometry experiments. QTOF is the most common of these instruments and consists of a quadrupole (Q1) and a collision cell (q2) in front of the oaTOF (48). A schematic illustration of the instrument is given in Fig. 4. The mass range of these instruments is limited by the quadrupole analyser, typically m/z 3-4,000. All the analytical work in this thesis was conducted on Agilent 6540 or 6550 LC-QTOF systems. The 6550 model differs from the 6540 model primarily by an altered interface region, iFunnel technology. In this technology, the capillary responsible for sampling ions from ESI to MS is shortened and has six parallel bores, which allows more ion-containing gas into the MS (49). In addition, two ion funnels transmit the ions to the optics and, at the same time, ensure the removal of gas and neutral

particles. According to the producer of the instruments, this results in an increase in the analyte-signal on average compared to older instruments without the iFunnel technology (50). From experience, a corresponding rise in noise has been registered. Consequently, an improved signal-to-noise ratio for all compounds cannot be expected when 6550 is used, as compared to 6540.

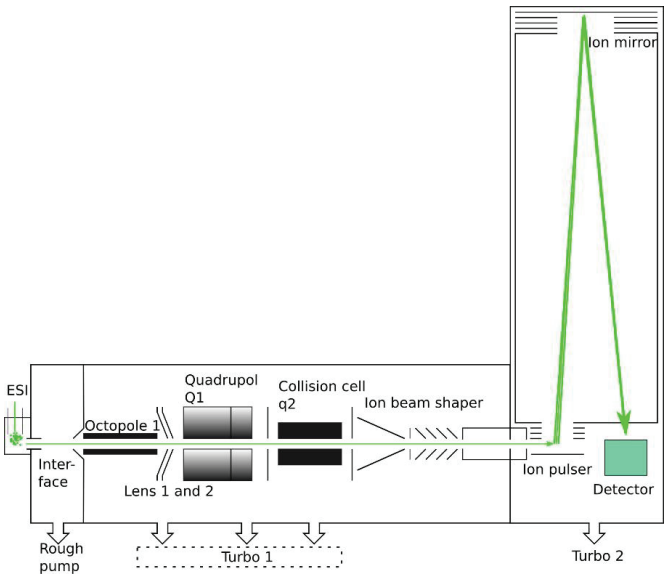


Fig. 4: Schematic representation of QTOF-MS for coupling with liquid chromatography. Green line illustrating the ion path.

1.3.4. Data acquisition by LC-QTOF-MS

The QTOF can be operated in different modes, either with or without active Q1 and q2 (45). In MS-only mode, the Q1 and q2 act as ion guides allowing all ions to reach the TOF. The result is a data file containing only full spectra MS. The best instrument sensitivity can be achieved in this operational mode, as no analysis time is lost while Q1 and q2 are activated and deactivated. The QTOF can also perform “in space” collision-induced dissociation (CID) experiments in MS/MS mode where Q1 is used as an ion filter allowing selected precursors through to the q2, where they undergo fragmentation induced by a collision with a gas (typically nitrogen or argon). The fragment ions and remaining precursor ion are then analysed by the TOF, giving the MS/MS spectrum. CID fragmentation in QTOF instruments is utilized primarily for identification in complex mixtures and structure elucidation. The instrument can acquire CID fragment data from precursors with or without predefined criteria.

In data-dependent acquisition mode (DDA), MS/MS spectra are acquired from several precursor ions selected in real time from an MS-only scan, either automatically based on predetermined rules (nontargeted acquisition) or based on a predefined list (targeted acquisition) (51, 52). In the nontargeted mode (Auto MS/MS in Agilent instruments), the instrument constantly cycles between MS-only and MS/MS mode. Precursor ions from the MS spectrum which are above an intensity threshold are ranged by the instrument based on relative abundance, and an MS/MS spectrum from a specified number of precursors (often three) is acquired before the next MS spectrum is acquired. After the MS/MS spectrum from one precursor is acquired, this specific precursor is excluded from selection for a time (exclution time) of typically half the width of a chromatographic peak. The maximum number of co-eluting compounds whose precursor ions can be isolated and MS/MS spectra acquired is given by the acquisition frequency, number of precursors selected per cycle, and exclusion time. The risk of discrimination of low abundant precursor ions is present and, as a consequence, this strategy is not optimal for retrospective identification of real unknowns. On the other hand, MS/MS spectra are acquired from precursor ions with a narrow Q1 m/z isolation window, typically 1-3 Da. This results in selective MS/MS spectra with minimal interferences present.

In targeted mode (Targeted MS/MS in Agilent instruments), a list of m/z values representing the target analytes instructs the instrument to isolate and fragment a pre-selected precursor in a given time interval (51). An MS/MS spectrum is, therefore, generated only when a preselected precursor is detected within its expected RT window. This makes the targeted mode very sensitive but not suited for retrospective analysis or for identifying real unknown compounds. In both Targeted and Auto MS/MS, MS-only data is always available.

In data-independent acquisition (DIA) mode, Q1 functions as an ion guide; the instrument operates by switching q2 between low-energy (0 eV) and high-energy MS scans (typically in the range of 10-40 eV). When a high-energy scan is acquired, all precursor ions are exposed to nonspecific CID, which provides fragment information that can be used for identification or structure elucidation purposes (53). The high-energy scans can be very complex and contain fragments from all co-eluting compounds and the background matrix, which makes interpretation more complicated. Instrument producers have worked on solutions to reduce the complexity of these spectra, e.g., by isolating the mass window in selected sequences. With this approach, a full low-energy MS scan is followed by a series of high-energy MS scans

with different isolation mass ranges before the next full MS scan (54). This requires a very high acquisition frequency to acquire data from rapid eluting compounds.

1.3.5. Compound identification

There are several workflows for processing and interpreting HR-MS data depending on the instrument platform and data acquisition mode. Broad coverage of these is outside the scope of this text. In Agilent processing software, however, the workflow can be separated into two parts: find compounds ("peak picking") and define the identity of the compounds by applying a database or library. Three main choices are available for finding compounds for further identification:

1. "Find by formula" takes a list of formulas, calculates their monoisotopic masses and isotope pattern, and searches through the MS-only spectra of the data files. Ion chromatograms are extracted and integrated, and peak spectra are generated. A mass match score based on accurate mass, isotope abundance pattern, and isotope spacing is calculated for every detected ion (Fig. 6). The software can filter out compounds below a certain threshold score. RT agreement can be applied as an additional criterion. "Find by formula" can also compare reference MS/MS spectra with MS/MS spectra acquired from the compounds of the precursors. This can be used to confirm identity.

2. "Find by Auto MS/MS" and "Find by Targeted MS/MS" are software features applied to data containing the MS/MS spectra. The MS/MS spectra acquired close in time to, and originating from, the same precursor ion are grouped to form a compound.

3. "Molecular feature extraction" works by extracting the elution profile of each mass and grouping those masses that are related by the same elution profile. The compound contains the mass of the protonated molecule $[M+H]^+$, all its adducts, and the MS/MS spectra, if available.

Compounds are identified by searching a database or library. Throughout, this text uses "database" and "library" according to Agilent's definitions. Database is a list of compound names with theoretical identification information (formula, monoisotopic mass, etc.) and RT if available. Library is the sum of compounds in a database containing MS/MS spectra (Fig. 5).

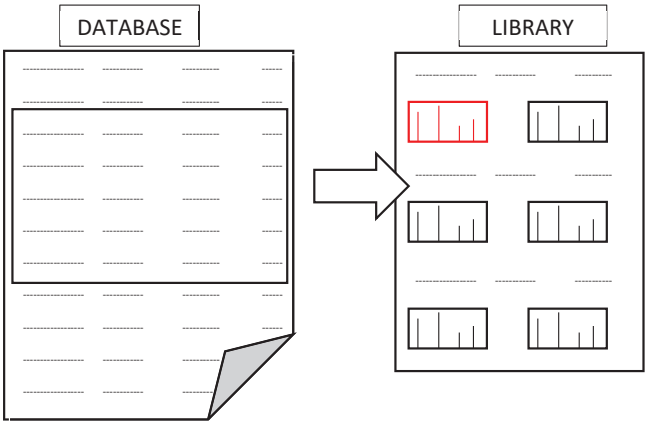


Fig. 5: Illustration of a library containing MS/MS spectra (highlighted in red) in relation to a database.

In a library search, MS/MS spectra from data are compared with a spectral library holding reference spectra. The numbers of matching and non-matching fragments and the mass accuracy of the fragments are criteria in the identification of the compound. The comparison is done by both reverse search (the peaks in the library are compared to the acquired MS/MS spectra) and forward search (the peaks in the acquired MS/MS spectra are compared to the library).

To differentiate the level of confidence of a compound identified from data acquired by HR-MS instruments, various procedures have been proposed. Performance criteria with respect to MS have been defined by the European Commission in relation to pesticide residue detection (55). This directive presents a system of identification points that are given based on the MS technique used and the acquisition method applied. To confirm the presence of a substance, a minimum of four identification points is required. All HR-MS techniques are valued so that measuring one diagnostic ion (the molecular ion, characteristic adducts of the molecular ion or characteristic fragment ions) gives two points. Measuring a fragment ion in addition to the molecular ion will give four points. This is the same number of identification points as is given for a method using liquid chromatography tandem quadrupole mass spectrometry (LC-MS/MS) with unit resolution operated in selected reaction monitoring (SRM) or multiple reaction monitoring (MRM) mode where one precursor and two daughter fragments are monitored.

Another approach based on the information available from the data acquired by HR-MS instruments has been suggested by Schymanski et al. (56). In this five-level approach, level 5 through level 1 require increasing information from the MS signal to MS/MS spectra and RT (Table 1). When reference material is not available, a definite confirmation of structure cannot be done. However, even with reference material available for comparison, very small differences in retention between possible positional isomers might require optimized chromatographic separation or special chromatographic techniques to achieve the specificity required to identify the correct structure.

Table 1: Level of confidence and required identification parameters according to the criteria suggested by Schymanski et al. (56).

Level of confidence	Identification parameter	Comments
Level 1: Confirmed structure	MS[1], MS/MS[2], RT[3] from reference standard	The highest degree of confidence.
Level 2: Probable structure		When no RT comparison is available, closely related positional isomers with identical fragmentation patterns are indistinguishable.
a) By library spectrum match	MS, MS/MS, Library MS/MS	
b) By diagnostic evidence	MS, MS/MS, Experimental data	
c) By RT match	MS, RT from reference standard	
Level 3: Tentative/putative candidate(s) Possible structure, substituent, class	MS, MS/MS, Experimental data	The acquired MS/MS data give evidence for possible structure(s) but are insufficient for exact identification.
Level 4: Unequivocal molecular formula	MS with isotope distribution and adducts	-
Level 5: Exact mass of interest	MS	Information about isotope pattern is lacking, e.g., because of low signal intensity.

[1]MS-only data
[2]MS/MS spectra or diagnostic fragment ions from high-energy spectra
[3]Retention time

Fig. 6 illustrates the information that LC-QTOF-MS can provide in order to identify a compound (exemplified by flubromazepam). The RT agreement between the compound and reference standard, the mass match score, and the MS/MS spectra match with the library give the identification parameters that are sufficient to confirm the structure (Level 1). The calculation of the mass match score is done automatically by the processing software, and the accuracy, isotope distribution, and isotope spacing are weighted (w), normally with the factors 100, 60, and 50 respectively.

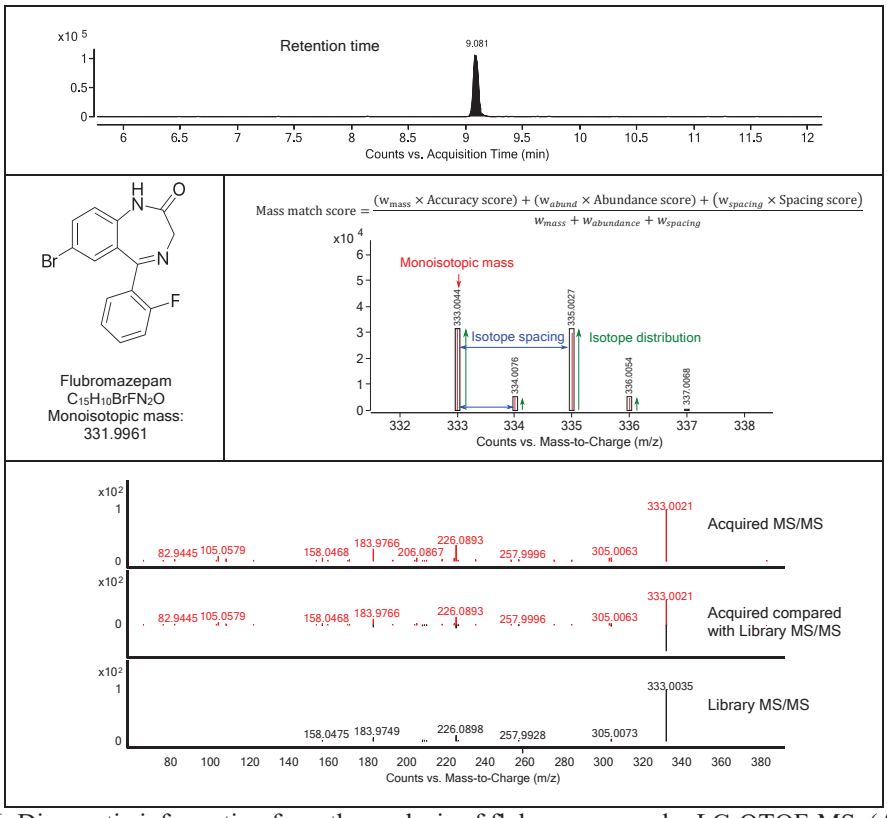


Fig. 6: Diagnostic information from the analysis of flubromazepam by LC-QTOF-MS. (A) Mass match score, (B) Retention time, and (C) Library match.

1.4. LC-QTOF-MS in the analysis of NPS in biological samples

Rapid changes in the illicit drug market create significant challenges for the laboratories within clinical and forensic toxicology. To address this challenge, laboratories must have the capacity to develop methods covering a broad spectrum of chemical compounds and could easily be updated with new drugs. Unit resolution MS, in particular LC-MS/MS, has been widely used in the screening of NPS (57). These techniques are a good choice in the analyses of a definite number of compounds due to their robustness, sensitivity, and selectivity. Several published papers have demonstrated the use of LC-MS/MS in quantitative methods for NPS in general and separate groups in both blood (58-61) and urine (62-70). The disadvantage of using these techniques is that the target must be known in advance and in-depth knowledge

about the drug market and current situation of emerging compounds is crucial. Immunological methods have also been introduced for the detection of NPS. However, due to rapid changes that have been observed in the market in recent years, it is not feasible to create new immunological methods rapidly enough and they are becoming less useful compared to HR-MS-based methods that more easily can be adjusted to current demands (71).

HR-MS, e.g., LC-QTOF-MS, on the other hand, acquires full spectrum data and is not limited by scan/dwell times; additionally, introducing new compounds to the method will not affect the detection of those already included. It will require only the availability of the reference standard. HR-MS has been used mainly for qualitatively targeted and non-targeted methods (71-77). However, also for quantitative purposes, HR-MS instrumentation has been shown to be highly applicable (78). Methods based on HR-MS depend on a database or library for the identification of NPS.

Databases or libraries with NPSs can be developed "in-house" from reference compounds (72, 75, 76). However, rapid changes in NPS require access to a high number of well-defined reference compounds to maintain an "in-house" database or library. The procurement of reference standards is costly, particularly if a database should be up to date with as many new and relevant compounds as possible. Also, commercial databases and libraries are available, developed by the different instrument manufacturers, though their use is often restricted to the specific instrument software (e.g., the Forensic Toxicology Personal Compound Database and Library from Agilent), and users are dependent on the frequency of new releases and/or additions being up to date. Free online sources of HR-MS identification data are also available, e.g., mzCloud[1] developed in collaboration with Thermo Fisher Scientific. However, this data is not easily implementable in the workflow of other HR-MS instruments than Orbitrap, manufactured by Thermo Fisher Scientific. Another opportunity is crowdsourced databases with information submitted by global HR-MS users. One such example is HighResNPS.com (79). When CID is performed on a certain compound, different instrument configurations tend to generate the same diagnostic fragment ions even though the relative abundance may vary. Thus, fragment data acquired on one instrument can be used as identification across platforms (79-81). In principle, the same is true for a crowdsourced

[1] http://www.mzcloud.org

database with diagnostic fragments acquired by instruments from different manufacturers, provided that the added fragment masses are converted to theoretical values.

In contrast to LC-MS/MS methods based on SRM or MRM, HR-MS full-spectrum data remain available and permit the identification of non-target compounds and retrospective analyses, also called post-target analyses. In data from HR-MS instrumentation with fragmentation capabilities, e.g., QTOF-MS or the linear ion trap Orbitrap, fragmentation data are also available. In principle, all compounds are available for investigation at a certain level, though the available data are limited by sample extraction recovery, chromatographic selectivity, and the degree of ionization and fragmentation. Depending on which acquisition mode is used, the QTOF-MS data also can contain fragment ions originating from the molecular ions generated in the ion source. A limited number of studies apply this retrospective approach in a forensic or clinical toxicology setting (82-84). Retrospective analysis of urine samples has been used to detect metabolites of pesticides (85) and to detect drugs and pesticides in other matrices including sewage water, surface water, and food (86-90). Based on new knowledge, a post-targeted analysis of data can lead to a new finding in a specific toxicological or clinical sample and, ultimately, change the conclusion of the case. A retrospective study is also important as an internal quality check for the laboratory to assess whether the screening repertoire used is comprehensive and relevant. In addition, a trend in the abuse of a specific drug or specific drugs can be identified, as exemplified in a study by Kriikku et al. in which the toxic lifespan of U-47700 was explored (91).

Potential disadvantages of the use of HR-MS are the higher cost compared to LC-MS/MS and immunological instrumentation and the large size of data files generated. In addition, efficient processing and interpretation of the data requires powerful computers and highly trained chemists with MS experience (57). A summary of the advantages and disadvantages of a selection of different analytical techniques appears in Table 2.

Table 2: Advantages and disadvantages of different analytical techniques (+ is least advantageous and +++ is most advantageous).

Feature	Immunology/immunoassay	LC-MS/MS[1]	LC-QTOF[2]
Specificity	+ Cross-reactivity of the antibody with compounds other than the analyte of interest. Confirmation by MS is needed.	++	+++
Sensitivity	+ High cut-offs and risk of false negatives.	+++	++
Dynamic repertoire	+ Not dynamic. Limited by the producer.	++	+++
Instrument cost	++	++	+
Ease of operation and complexity of data interpretation	+++	++	+

[1]Liquid chromatography triple quadrupole
[2]Liquid chromatography quadrupole time-of-flight

1.5. Choice of sample matrix

To detect drugs, including NPS, for clinical and forensic toxicology purposes, both the choice of sample type and the availability are important factors. For purposes of detecting drugs of abuse, a urine sample has several advantages, such as non-invasive sampling and an expanded detection window (see Table 3) (92, 93). However, many NPS, such as SCs, synthetic opioids, and designer benzodiazepines, undergo extensive metabolism. Consequently, a screening method in urine must cover the most abundant and unique metabolites originating from the target drug. The close structural similarities seen within the same group of NPS often result in several drugs forming identical main metabolites, but in many cases, unique less abundant metabolites are also produced. Another challenge is the availability of synthesized reference materials of the preferable drug metabolite. There is often a time-consuming and resource-demanding process from the time a new drug is introduced to the market to the point at which the selected metabolites have been synthesized and can be included in a new or updated method.

Blood is currently the preferred sample type when the degree of intoxication should be determined as the blood concentration reflects the amount of drug present in the body at the time of sampling. The availability of reference materials of the parent drug is better compared to that of urine. The risk of adulteration is also relatively low. Oral fluid is comparable to

blood with regards to concentration and detection time, although large variations between compounds are observed (94).

Hair is superior to other sample matrices when one considers detection time. This is obviously limited by the length of the hair, as it grows at a rate of roughly one cm per month (95). On the other hand, relating the level of drug to the degree of intoxication is not possible. Distinguishing a single intake from chronic use is also very challenging, as a hair sample is usually cut in length segments prior to analysis. The analytical result then represent any drug use within the growth period of the segment.

Table 3: Advantages and disadvantages using different sample types for drug detection (+ is least advantageous and +++ is most advantageous) (92, 93).

Feature	Blood	Urine	Hair
Ease of collection	+	+++	+++
Non-invasive sampling	++	+++	+++
Detection time	+	++	+++
No adulteration	+++	+	+
Reflect intoxication/impairment	+++	+	0/-
Importance of quantitative result	+++	++	+

1.6. Sample preparation

The complexity of a biological sample and the trace amount of drugs and their metabolites present most often require a certain sample preparation before analysis by LC-MS. This preparation can be simple and unspecific, like dilution or precipitation, or more extensive and specific. It can be performed either as a separate procedure or on-line the LC-MS system. The most basic preparation techniques, like filtration and dilution, can be applied if the matrix is less complex (low content of protein and fat) and the concentration of the analyte is relatively high.

Protein precipitation is a generic sample preparation applied mostly in the analysis of blood, serum, or plasma. In this technique, the proteins in the sample are precipitated by an organic solvent, acid, base, high salt concentration, or a combination of these. The efficiency of the precipitation depends on the reagent used and the sample/reagent ratio. If a polar organic solvent is used, precipitation of proteins is caused mainly by a significant decrease in the dielectric constant of the aqueous solution (96). In general, ionic compounds are more soluble

in solvents with high dielectric constants, e.g., water with 80 at 20°C. As the dielectric constant decreases through the addition of, e.g., acetonitrile with a dielectric constant of 37.5 at 20°C, the solution becomes a poorer solvent for the proteins. Through its polar groups, the organic solvent interacts with the polar groups of the proteins in competition with water. In addition, the hydrophobic groups may disrupt the intramolecular hydrophobic interactions. Finally, a large volume of organic solvent compared to water leaves only a small amount for hydration of the proteins. Upon dehydration by the organic solute, protein molecules attract each other to a sufficient degree by van der Waals forces and, thus, become insoluble in the organic-water mixture. Using ice-cold solvents or freezing the mixture can increase the efficiency of the precipitation.

Extraction techniques can offer a more specific sample clean-up. Liquid-liquid extraction (LLE) is based on the transfer of a compound from one liquid phase to another liquid phase according to the solubility. In solid phase extraction (SPE), a compound's distribution between a solvent and the surface of a solid phase (sorbent) is utilized. Unlike chromatographic separation, the compound is either completely retained or completely unretained by the SPE material. The principles used in SPE are either reverse phase exchange, ion exchange extraction, or normal phase exchange. The first two can also be used in combination to achieve an even more specific extraction and, ultimately, a cleaner extract. The reverse phase exchange typically consists of a silica base with hydrophobic groups or a polymeric material. The polymeric material has the advantage of being ready to use without the need for conditioning. A generic procedure involving reverse phase SPE consists of the following steps (97):

1. Conditioning with polar organic solvent if a silica-based material is used.
2. Applying the sample (pH adjusted if necessary).
3. Washing with an aqueous solvent.
4. Elution with an acidic/basic polar organic solvent.

1.7. LC-QTOF-MS in the study of drug metabolism

In general, metabolites of illicit drugs are formed by complex enzymatic processes. Studying authentic human urine samples from individuals with a known or suspected intake of the drug of interest is the preferred approach. However, due to the limited access to such samples, as well as limited information about the intake itself (e.g., purity of the drug, time from intake,

identity), in vitro model systems of drug metabolism have emerged as important supplementary tools. In these experiments, the drug of interest is added to drug-metabolizing enzymes and, after incubation, the extracts are subjected to chemical analysis by, e.g., LC-QTOF-MS, to identify the metabolites formed. Both human liver microsomes and human hepatocytes have been used in studies of NPS (98-100). Hepatocytes have the advantage of being living cells with an intact cell membrane that has active transport functions, complete metabolic pathways, and metabolic enzymes and cofactors at physiologically relevant concentrations (101). However, due to extrahepatic drug metabolism and transport, as well as inter-individual differences, the in vitro results may differ from those obtained in vivo. LC-QTOF-MS can be used for two important tasks in drug metabolism studies:

1. Identifying possible candidate compounds based on previous knowledge of metabolic pathways.
2. Conducting a first elucidation of the structure of these compounds by investigating the acquired MS/MS spectra.

As several positional isomers are usually formed, different variants must be synthesized and the actual metabolites can be confirmed by RT agreement if the LC method achieves sufficient separation.

2. Aims of the thesis

The aim of this work was to explore the use of LC-QTOF-MS in the study of NPS by developing a screening method, identifying metabolites in vitro and retrospectively reviewing data files from post mortem samples. The specific aims were as follows:

Study I

To develop a high throughput quantitative screening method for SCs in urine, using LC-QTOF-MS and automated sample preparation. We also aimed to describe our experience and results from analyzing 1,000 consecutive routine urinary samples sent to our laboratory where screening for SCs had been requested, to evaluate the feasibility of the method in clinical practice.

Study II

To investigate the metabolism of ortho-, meta-, and para-fluorofentanyl using human hepatocytes and elucidate the structure of the metabolites using LC-QTOF-MS. A secondary aim was to investigate whether the exact position of the fluorine atom had any influence on the degree of formation of the various metabolites.

Study III

To develop a strategy for re-processing data files of forensic post mortem samples analyzed by LC-QTOF-MS from January 2014 to December 2018 in search for NPS in the subgroups synthetic cannabinoids, synthetic opioids and designer benzodiazepines after creating a new library based on a crowdsourced database.

3. Materials and Methods

3.1. Chemicals and reagents

All compounds used in validation, quantification, confirmation, and metabolite experiments were purchased and shipped with a certificate. In addition, three 5F-AKB-48 metabolites synthesized at the University of Linköping were kindly donated for identification of an unknown metabolite detected during **Study I**. The chemicals utilized in buffer preparation and chromatographic analysis were of LC-MS quality.

3.2. Samples and sample preparation

3.2.1. Study I

In **Study I**, 1,000 urine samples were analysed. The urinary screening method consisted of commercially available and assumed relevant metabolites of the SCs most frequently used in Norway at the time the method was developed, based upon reports from institutions analysing these groups of compounds in biological samples and seizure statistics from the Norwegian National Criminal Investigation Service (KRIPOS). Metabolites of the SCs included were AB-FUBINACA, AB-PINACA, AB-CHMINACA, AM-2201, AKB48, 5F-AKB48, BB-22, JWH-018, JWH-073, JWH-081, JWH-122, JWH-203, JWH-250, PB-22, 5F-PB-22, RCS-4, THJ-2201, and UR-144. A complete list of the metabolites, including formulas, monoisotopic masses, CAS numbers, IUPAC names, and structures, is given in Table S1 of Paper I.

All pipetting operations were performed using a Tecan Freedom Evo pipetting robot (Tecan, Männedorf, Switzerland). A urine sample, calibrator, or quality control (QC) in aliquots of 600 µL was pipetted into a 2 ml 96-well plate together with the internal standard solution, ammonium acetate, and β-glucuronidase. The plate was incubated for 1 h at 60°C and the sample was then transferred to a Waters Oasis® HLB PRiME 30 mg HLB 96-well plate (Waters, Wexford, Ireland) SPE. A positive pressure processor (Waters, Milford, MA, USA) was used to gently push the sample and the following reagents through the packing material. The SPE material was washed with water and 10% methanol (v/v) in water in sequence following elution twice with 10% methanol (v/v) in acetonitrile. The eluate was collected in a rack of 96 glass vials in a tray with a well plate footprint (J.G. Finneran Associates Inc., Vineland, NJ, USA) and then evaporated and reconstituted (preconcentration factor of 1.5).

The method was applied to a total of 1,000 consecutive routine urinary samples originating from subjects in whom intake of SCs was suspected. The samples were collected in 2014 and the first half of January 2015. Upon arrival at the laboratory, these samples were principally analysed with a routine targeted LC-MS/MS method described previously (102). The collection and storage of the samples selected for subsequent analysis with the present method were approved by the Regional Committee of Medical and Health Research Ethics (REK) in Mid Norway (approval No. 2014/2281).

3.2.2. Study II

In **Study II**, extracts from the incubation of human hepatocytes and one urinary sample were analysed by UHPLC-QTOF-MS. The incubation of human hepatocytes with para-fluorofentanyl (*N*-(4-fluorophenyl)-*N*-[1-(2-phenylethyl)-4-piperidinyl]propanamide) and ortho-fluorofentanyl (*N*-(2-fluorophenyl)-*N*-[1-(2-phenylethyl)-4-piperidinyl]propanamide and meta-fluorofentanyl (*N*-(3-fluorophenyl)-*N*-[1-(2-phenylethyl)-4-piperidinyl]propanamide) was performed in accordance with the protocol used by Åstrand et al. (103). The experiment was performed at the laboratory facilities at the Division of Drug Research, Department of Medical and Health Sciences, Linköping University and the Department of Forensic Genetics and Forensic Toxicology, the Swedish National Board of Forensic Medicine, Linköping. The project was supported by the Vinnova (the Psychomics project, Eurostar Project ID 10628) and Strategic Research Area in Forensic Sciences (Strategiområdet forensiska vetenskaper, grant number 2016:7) at Linköping University.

Briefly summarized, the in vitro protocol consisted of preparing the cryopreserved pooled human hepatocytes (LiverPool™ from BioreclamationIVT (Baltimore, MD, USA)), ending up with a solution containing $2.0x10^6$ cells/mL in William medium E (Thermo, Stockholm, Sweden). The concentration of viable cells was evaluated with Trypan blue (0.4% v/v) exclusion dye method. Each fluorofentanyl isomer solved in William medium E was then incubated with 10^5 cells in separate wells in a 96-well plate. The incubations were stopped after 1, 3, and 5 h by adding ice-cold acetonitrile spiked with internal standards (amphetamine-d8, phenobarbital-d5, diazepam-d5, and mianserin-d3) to each well. A negative control (only cells) and a degradation control (only drug) were incubated for 5 h and positive controls were incubated for 0 and 5 h. The positive control contained a mix of CYP substrates (caffeine, bupropion, diclofenac, omeprazole, dextromethorphan, chlorzoxazone, and midazolam). The controls incubated for 0 h were prepared by adding acetonitrile to the drug

immediately before the cells were added. The plates were vortexed and left at -20°C before centrifugation. Finally, the supernatant was transferred to a new 96 well-plate.

A urinary sample from a patient intoxicated with ortho-fluorofentanyl was used for metabolite profiling and comparison with the in vitro results. The sample was analysed with and without enzymatic hydrolysis. For the hydrolysed sample preparation, urine was incubated with β-glucuronidase and diluted with mobile phase mixture. No pH adjustment was done. The non-hydrolysed sample was diluted only with a mobile phase mixture. Both samples were filtered using a syringe filter with a polytetrafluoroethylene membrane (VWR, Radnor, PA, USA) before injection.

3.2.3. Study III

The data files investigated in **Study III** originated from forensic post mortem samples of legal autopsies in the period 2014 to 2018. Samples from a total of 1314 cases were analysed in this period. Whole blood was used in all cases when available, and, if not, spleen tissue was used. The samples were prepared in weekly batches by the same procedure throughout the period. Briefly, the sample was thawed to room temperature and 200 mg was weighed into a microtube. Internal standards (codeine-d3, morphine-d3, benzoylecgonine-d3, and griseofulvin) and ice-cold acetonitrile were added for protein precipitation. The tube was then mixed and centrifuged before the supernatant was transferred to a 96-well plate, evaporated to dryness, and reconstituted (preconcentration factor of 2.5). In the cases in which only spleen was available, sample preparation was adjusted according to the condition of the tissue. Blood-like material was handled as a blood sample. In the other cases, tissue material was homogenized with an equal volume of H_2O and further processed like a blood sample.

Permission to re-process the data files was given by REK in Mid Norway (approval No. 2018/2157). The data files were anonymized and the analyst was blinded to the original findings when doing the re-processing. According to the permission granted, re-analysis of the sample specimens as such could not be performed.

3.3. Method development

The method optimization in **Study I** aimed to develop a general method that could detect the relatively diverse group of SC metabolites and also include new, similar metabolites as they became available. Different sample preparation techniques, LC conditions, and MS settings

were explored, and the optimization process revealed several methodical issues and challenges. The chromatographic conditions achieving the best separation of isomers with identical fragmentation patterns, such as AKB48 *N*-(5-hydroxypentyl) and AKB48 *N*-(4-hydroxypentyl), as well as separating as many of the analytes as possible from endogenous compounds, were found by testing three different columns—C18, phenyl-hexyl, and biphenyl—in combination with different mobile phase setups and gradients. A C18 column and a linear gradient were eventually chosen (see Table 4). In general, urine as a matrix results in high background and potential interferences. These interferences could potentially affect the continuous measurement of lock masses maintaining the high degree of mass accuracy achieved by the UHPLC-QTOF-MS system. Interference was observed close to m/z 121.0509, which is monitored together with m/z 922.0098 as lock masses to control mass accuracy. This resulted in a high mass error in certain spectra. Instead of using high-resolution mode, which compromises the dynamic range, an alternative lock mass, m/z 118.0863 from trimethylglycine ($[M+H]^+$), was chosen.

The instrumental settings in **Study II** were adopted from the previously published studies by the group of Henrik Gréen at Linköping University and the Swedish National Board of Forensic Medicine (103, 104). Two different LC gradients were tested on para-, meta-, and ortho-fluorofentanyl: (*i*) from 0 to 40% mobile phase B the first 13 min and (*ii*) from 0 to 65% mobile phase B the first 13 min. Gradient *i* gave the best retention and was therefore expected to give the best separation for the presumably more polar metabolites.

LC and QTOF-MS settings were already selected for the original analyses on which **Study III** was based; therefore, method development in this study was limited to the post-acquisition part.

3.4. Instrumentation

The LC module Infinity 1290 UHPLC from Agilent (Santa Clara, CA, USA) was used in all three studies. The most important settings are summarized in Table 4. Two different QTOF-MS models, both from Agilent (Santa Clara, CA, USA), were applied in the detection of drugs and metabolites in the three studies: 6550 (**Studies I** and **II**) and 6540 (**Study III**).

High sensitivity was an important factor in the development of the method in **Study I**. Therefore, all samples were first analysed using the MS-only mode. Presumably, positive

samples were injected a second time with a higher injection volume, 4 GHz detector state (improved resolution), and targeted MS/MS mode. Collision energy of either 10, 20, or 40 eV was applied to each precursor based on previous experiments in order to collect MS/MS spectra containing fragments and traces of the precursor. The targeted MS/MS method was used to confirm the findings from the MS-only method. The confirmation was done by comparing the MS/MS spectra with a library (see paragraph 3.5.2).

Auto MS/MS was used in both **Studies II** and **III**. This is a DDA mode in which the instrument cycles between MS and MS/MS mode. This method differs from the method in **Study I** in the way that the instrument selects three precursors from the MS-only full mass spectrum, ranks them by abundance, and isolates and fragments them one at a time. Collision energies calculated based on the mass of the precursors were applied. After one MS/MS spectrum was acquired, this specific precursor was excluded from further fragmentation in approximately the time representing a half chromatographic peak width (0.03 min). A MS/MS spectrum was applied for structure elucidation in **Study II** and for the identification of compounds via library comparison in **Study III** (see paragraph 3.5.2). Details of the settings in all QTOF methods are presented in Table 5.

Table 4: Liquid chromatography settings chosen in the **Studies I, II,** and **III**.

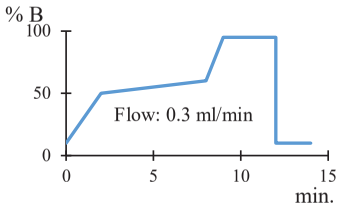
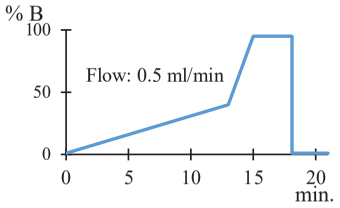
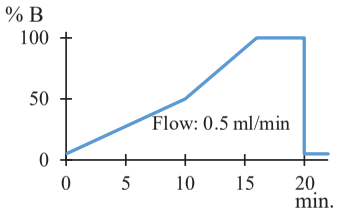
Study	Mobile phase A and B	Column	Gradient profile	Injection volume	Temperature
I	A: 0.1% formic acid in water B: 0.1% formic acid in acetonitrile	Zorbax Eclipse Plus C18 Rapid Resolution HD column (2.1x100 mm, 1.8 µm), Agilent		1st injection: 5 µl 2nd injection: 10 µl	Auto sampler: 10°C Column: 60°C
II	A: 0.05% formic acid in 10 mM ammonium formate B: 0.05% formic acid in acetonitrile	Acquity HSS T3 column (2.1x150 mm, 1.8 µm), Waters		Hepatocytes: 1 µl Authentic sample: 5 µl	Auto sampler: 7°C Column: 60°C
III	A: 0.025% formic acid in 10 mM ammonium formate B: 0.05% formic acid in acetonitrile	Acquity HSS T3 column (2.1x100 mm, 1.8 µm), Waters		2 µl	Auto sampler: 10°C Column: 50°C

Table 5: QTOF-settings chosen in the **Studies I, II,** and **III**.

Study	QTOF model	Electrospray ionization		iFunnel settings		MS mode and mass range	Scan rate and detector state
I	Agilent 6550	Polarity	Positive	Exit DC[1]	40 V	1st injection:	1st injection:
		Fragmentor voltage	375 V	RF HP[2]	150 V	MS-only	2 Hz
		Capillary voltage	3,500 V	RF LP[3]	100 V	50-1,000 m/z	2 GHz
		Gas temperature	150°C				
		Gas flow	15 L/min			2nd injection:	2nd injection:
		Nebulizer pressure	20 psig			Targeted MS/MS	6 Hz (MS and MS/MS)
		Sheath gas temp	380°C			50-1,000 m/z	4 GHz (high-resolution)
II	Agilent 6550	Polarity	Positive	Exit DC[1]	50 V	Auto MS/MS	MS: 5 Hz
		Fragmentor voltage	375 V	RF HP[2]	150 V	MS: 100-950 m/z	2 GHz
		Capillary voltage	3,500 V	RF LP[3]	60 V		
		Gas temperature	150°C				
		Gas flow	18 L/min			MS/MS: 50-950 m/z	MS/MS: 10 Hz
		Nebulizer pressure	50 psig				2 GHz
		Sheath gas temp	380°C				
III	Agilent 6540	Polarity	Positive	Not available		Auto MS/MS	MS: 6 Hz
		Fragmentor voltage	120 V			MS: 50-1,000 m/z	2 GHz
		Capillary voltage	3,500 V				
		Gas temperature	320°C				
		Gas flow	8 L/min			MS/MS: 50-1,000 m/z	MS/MS: 6 Hz
		Nebulizer pressure	40 psig				2 GHz
		Sheath gas temp	380°C				

[1]Exit direct current
[2]Radio frequency high pressure
[3]Radio frequency low pressure

3.5. Post-acquisition method

3.5.1. Creating databases and libraries

For the library used in **Study I**, CID spectra were added to the in-house library according to Broecker et al. (51). This procedure involved diluting individual stock solutions of SC metabolites in methanol and injecting them on a guard column with an isocratic flow using 0.1% formic acid in water and 0.1% formic acid in acetonitrile (50:50) as the mobile phase. Three CID spectra of the protonated compound using collision energies of 10, 20, and 40 eV were acquired. The acquired CID spectra were then transferred to the library file using Agilent MassHunter Qualitative software (Qual) B.07.01 and MassHunter PCDL Manager B.07.01 (Agilent, Santa Clara, CA, USA). In this process, the fragment masses in every spectrum were corrected to their theoretical masses. Fragments with intensities lower than 1% of the most abundant mass in each spectrum were deleted.

In **Study II**, no library was used. Instead, a database with molecular formulas of expected metabolites was used for the detection of compounds. These expected metabolites included mono-, di-, and trihydroxylations, carbonylation, dihydrodiol formation, methylation, carboxylation, defluorination, N-dealkylations (including loss of the fluorophenyl group), amide hydrolysis, glucuronidation, and combinations of these.

The library in **Study III** consisted primarily of synthetic cannabinoids, synthetic opioids, and designer benzodiazepines listed in the free, online, spreadsheet-format, crowdsourced HR-MS database HighResNPS (highresnps.com) as of May 2019. The Agilent MassHunter processing software is not capable of using the diagnostic fragments directly in the identification of compounds in a workflow based on DDA. To apply the information from the database on our workflow, diagnostic fragments had to be transformed into “synthetic” MS/MS spectra in the personal compound database and library (PCDL) format. The software tool “Spectrum Generator”, created by Broeckers Solutions (Berlin, Germany), was used to convert the text-based information of diagnostic ions from the HighResNPS database into the Agilent .cef file format, which allows for an import of library spectra for each PCDL entry. Using this approach, the diagnostic fragment ions were stored as a library spectrum. The relative abundance of the ions was not taken into account, though this would have been possible with the software “Spectrum Generator”. The collision energy of the library spectra was chosen by the software as 20 eV simply to have a value in the PCDL.

3.5.2. Data processing and identification

Different processing workflows were chosen in the identification of compounds in **Studies I, II,** and **III**. The workflow in **Study I** was as follows: In data from the first injection, compounds were identified based on accurate monoisotopic mass and RT (ID criteria I) in the Agilent MassHunter Quantitative B.07 software (Santa Clara, CA, USA). This signal was also used for the quantification of the compounds. Samples with compounds fulfilling ID criteria I were processed using the "Find by Formula" algorithm in Agilent MassHunter Qual. Samples with compounds identified with a mass match score ≥ 80 (ID criteria II) were injected a second time using the targeted MS/MS mode. The acquired data were processed with the library of CID spectra. MS/MS spectra from the positive sample were compared to CID from the library. A positive match confirmed the presence of the compound (ID criteria III).

In **Study II**, the "Find by formula" algorithm was used with the database previously described. Only compounds with a mass error within 15 ppm and a peak area above $1x10^4$ were regarded as potential metabolites. In addition, the fragment ions in the MS/MS spectrum associated with the precursor ion of the compound were evaluated. Potential metabolites detected in the negative control, 0 h samples, or degradation controls were excluded.

The processing workflow in **Study III** was based on the algorithm "Find by formula" together with a library search, both using the HighResNPS subset PCDL. The "Find by formula" search led to positive findings that were based on MS-only data. Compounds with a mass error of less than ± 5 parts per million (ppm) and a score above 80 were retained. A filter in the software was applied to distinguish between compounds with MS/MS spectra (category 1) and without MS/MS spectra (category 2). A visual evaluation of the spectra from category 1 compounds was undertaken to evaluate whether the fragments in the acquired spectrum were among the significant fragments in the library spectrum and not only fragments of low abundance, e.g., from contaminants. For category 2 compounds, no MS/MS data had been acquired and fragment confirmation could not be done, which means that only the MS signal could be used to evaluate the quality of the findings. A mass accuracy limit of ± 10 ppm, a mass match score above 80, and a peak area threshold of $5x10^4$ were first applied (criterion a). This was tested with 42 random data files and produced 74 findings. After investigation of the results and the filtering out of findings due to interferences and background signal, only compounds with a mass accuracy better than ± 5 ppm and a mass match score above 95 were left. These two thresholds were consequently used as criterion b (three findings in the 42 data

files). Finally, a third factor was added to criterion b, an RT restriction of 1.5 min., as the compounds in the groups under investigation are highly likely to elute after this time period (criterion c). Criterion c gave one finding in the data files. A compound appearing in several data files in the same batch was considered an isomer originating from the chemicals used or as endogenous molecules with equal theoretical masses. The risk of accepting false positives is higher for category 2 findings than for category 1 findings, especially if thresholds and limits are set too wide.

Any new finding was further evaluated by comparing acquired MS/MS spectra with other sources (e.g., mzCloud) or by analysing the reference standard available at the laboratory. If consistency in fragments or RTs was observed, the finding was reported to a person with access to the original case report. If a presumably novel moiety was identified and a reference standard was available, this standard was purchased and analysed, and RTs and MS/MS spectra were compared.

3.6. Method validation

3.6.1. Study I

The screening method in **Study I** was validated according to guidelines for forensic applications (105). Parameters tested were limit of quantification (LOQ), linearity, selectivity, RT stability, carry-over, matrix effects, recovery, precision, accuracy, and stability. LOQ was defined as the lowest concentration level giving reproducible results when spiked urine was analysed at 10 days with precision (CV) < 20% and accuracy within 80 – 120% of the theoretical value. The linear range of every compound was explored by using the analysed calibrators from the first four days of validation (all days within a week) at six calibration levels. $R^2 \geq 0.990$ was regarded as accepted. The selectivity of the method was evaluated by spiking 10 different blank urines with a mix of drugs of abuse or their corresponding metabolites commonly observed in the samples sent to the laboratory for screening for drugs of abuse. Limit of confirmation (LOC) was defined as the minimum concentration in spiked negative samples which fulfilled the most stringent criterion (ID criteria III).

The stability of RT and relative RT (ratio of analyte RT to internal standard RT) was monitored through an analytical sequence of a minimum of 14 h on three random validation days. RT deviation of ≤ 1% throughout an analytical sequence up to 14 h was accepted. The carry-over from a high-concentration sample to the next was determined by injecting blank

urine after a sample containing a concentration equal to its highest calibration level or at least 125 ng/mL. A carry-over of less than 20% of LOQ was accepted.

The matrix effect (ME) was estimated as the signal of analyte spiked in blank urine extracts (B) relative to mobile phase (A) (see equation 1). Values in the interval 75 – 125% were regarded as acceptable. Recovery (RE) was estimated by comparing the signal in six blank urines fortified with all compounds after extraction (B) to the signal in the same samples fortified to the identical concentration level before extraction (C) (see equation 2). RE ≥ 75% was regarded as acceptable for quantification.

$$ME\ (\%) = \frac{B}{A} \times 100 \qquad (1)$$

$$RE\ (\%) = \frac{C}{B} \times 100 \qquad (2)$$

The intra-day precision was determined by analysing 10 parallels of two concentration levels in the same sequence. The inter-day precision was calculated by analysing one sample at two different concentration levels at 10 different days over a period of five weeks. The acceptance criterion of intra- and inter-sequence precision at both concentration levels was a CV ≤ 15%. The average value of the inter-day data was used to calculate the accuracy expressed as the deviation from theoretical. The acceptance criterion of accuracy was the values in the interval of 85 – 115%. The stability of the compounds was tested at 4°C and 25°C in spiked QC samples stored in glass tubes at one concentration level. In addition, the stability of extracted samples in the autosampler at 10°C was tested.

3.6.2. Study II

The analytical method in **Study II** was not validated, but has been proven fit for purpose in several previously published studies of similar compounds (103, 104, 106, 107).

3.6.3. Study III

Study III is a retrospective targeted method, and the high number of new compounds covered makes a full validation insuperable. Instead, several compounds were selected from each group of NPS and a limited validation was performed. The compounds selected were:

1. Synthetic cannabinoids: MDMB-CHMICA, AB-CHMINACA, BB-22, JWH-018, PB-22, and THJ-018

2. Synthetic opioids: Fentanyl, remifentanil, cyclopropylfentanyl, para-fluorofentanyl, furanylfentanyl, and acetylfentanyl
3. Designer benzodiazepines: Deschloroetizolam, diclazepam, etizolam, flubromazepam, flubromazolam, pyrazolam, and meclonazepam

The validation parameters were sensitivity, ME, and RE. To estimate the limit of identification (LOI), blood samples were spiked at 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, 10, and 20 ng/ml in triplicates and analysed as described for the post mortem samples. LOI was defined as the minimum concentration at which a compound was identified and at least one MS/MS spectrum was acquired for library search in all three parallels. ME was calculated from the peak area in B and in neat standard solution (A) (see equation 1). RE was calculated from peak areas in pooled whole blood samples spiked before (C) and after (B) the extraction to a final concentration of 0.1 µg/ml (see equation 2).

4. Results

4.1. Study I

4.1.1. Validation

The validation parameters were within the set criteria and requirements for the majority of analytes (see Tables 1 and 2 in Paper I). However, high matrix effects and insufficient recoveries call into question the ability to accurately quantify 14 of the 35 investigated analytes. Therefore, the method must be considered semi-quantitative for these compounds. The compounds that failed to meet the criteria were as follows.

PB-22 *N*-(4-hydroxypentyl) could not be baseline separated from the isomer PB-22 *N*-(5-hydroxypentyl). PB-22 *N*-(4-hydroxypentyl) which eluted first and is a more specific marker of PB-22 intake was kept, whereas PB-22 *N*-(5-hydroxypentyl) was excluded from the calibrators. As baseline separation was not achieved, this must be regarded as semi-quantification. The correlation coefficients were above 0.990 except for RCS-4 *N*-(4-hydroxypentyl)phenol, AB-FUBINACA M3, AM-2201 *N*-(5-hydroxyindole), JWH-018 *N*-(5-hydroxypentyl), THJ-2201 *N*-pentanoic acid, JWH-210 *N*-(5-hydroxyindole), JWH-210 *N*-(5-hydroxypentyl), and JWH-210 *N*-pentanoic acid. The acceptance criterion of inter-sequence precision of ≤ 15% was achieved for all analytes except JWH-210 *N*-(5-hydroxyindole) (17%), JWH-210 *N*-pentanoic acid (19%), and THJ-2201 *N*-pentanoic acid (17%) at low concentration. The accepted accuracy of 85% – 115% was achieved for all compounds except AB-FUBINACA M2 (84%), BB-22-3-carboxyindole (79%), JWH-210 *N*-pentanoic acid (131%), and JWH-210 *N*-(5-hydroxyindole) (119%) at low concentrations, AB-PINACA pentanoic acid (119%), AB-CHMINACA M1A (117%), and AM-2201 *N*-(5-hydroxyindole) (121%) at high concentrations, and AB-FUBINACA M3 at both low and high concentrations (119% and 135%, respectively).

MEs from 57% to 262% were observed. The compounds showing the highest degree of ion suppression were AB-CHMINACA M1A (57%), PB-22 *N* pentanoic acid (64%), PB-22 *N*-(4-hydroxypentyl) (63%), and RCS-4 *N*-(4-hydroxypentyl)phenol (74%). The compounds showing the highest degree of ion enhancement were AM-2201 *N*-(5-hydroxyindole), AB-FUBINACA-M2, and THJ-2201 *N*-pentanoic acid (220 – 262%). JWH-122 *N*-(5-hydroxypentyl, AB-PINACA COOH, AM-2201 *N*-(4-hydroxypentyl), AB-FUBINACA-M3,

and AB-CHMINACA 3-carboxyindazole had somewhat less ion enhancement (133 – 175%). The remaining 23 compounds were within the acceptance criterion.

4.1.2. Results of authentic samples

In 21 of the 1,000 samples analysed, one or more metabolites were quantified and confirmed, while in two additional samples, metabolites were quantified and identified with ID criteria II, giving a frequency of positive findings of 2.3%. A total of seven different metabolites were confirmed and two identified with ID criteria II. Additionally, two metabolites were subsequently identified based on new reference substances. A summary of the findings, with suggestions of which drug(s) had been ingested in each case, is given in Table 3 in Paper I.

4.2. Study II

4.2.1. Identification of the metabolites in vitro

For each fluorofentanyl analogue, 14 potential metabolites were detected in the in vitro experiment. Ortho-, meta-, and para-fluorofentanyl produced metabolites by the same principal metabolic pathways, but with certain differences in RT and absolute chromatographic peak areas. The compounds eluted from 4.75 to 12.81 minutes, and all had a mass error of less than 4.52 ppm (see Table 1 in Paper II). The chromatographic separation of the metabolites of ortho-fluorofentanyl formed in vitro is given in Fig. 2A in Paper II). *N*-dealkylation at the piperidine ring resulting in the loss of the phenetyl moiety and forming norfluorofentanyl was observed to be the main metabolite in vitro. A compound corresponding to hydroxylation of this metabolite was also detected. Four different hydroxylated metabolites were detected: One with the hydroxy group at either the piperidine ring or at the ethyl linker, one with the hydroxylation on the amide alkyl chain, one hydroxylated at the phenetyl moiety, and the last being an *N*-oxide metabolite. Five di-hydroxylated metabolites and two compounds corresponding to dihydrodiol metabolites were also detected. Finally, a compound corresponding to a hydroxylated and methoxylated metabolite was detected.

4.2.2. Metabolites detected in an authentic urine sample

Three of the metabolites found in vitro were also detected in the authentic ortho-fluorofentanyl urine sample. These were norfluorofentanyl, hydroxy-fluorofentanyl

hydroxylated at the phenyl moiety, and the metabolite hydroxylated and methoxylated. The chromatographic separation of the metabolites of ortho-fluorofentanyl in the authentic urine sample is shown in Fig. 2B in Paper II.

4.3. Study III

4.3.1. Validation of the original analytical method

LOI was estimated for a representative group of synthetic cannabinoids, synthetic opioids, and designer benzodiazepines. LOI is unknown for new compounds but the experiment indicated that synthetic cannabinoids could be detected if present above approximately 10 to 20 ng/ml, synthetic opioids above 1 ng/ml, and designer benzodiazepines above 10 ng/ml. Major differences were observed in the estimated RE (%) of the synthetic cannabinoids, with values ranging from 32% (THJ-018) to 91% (AB-CHMINACA) (Table 2 in Paper III). The remaining compounds had RE (%) above 82%. All compounds showed an ME between 69% and 127%, which shows that both ion suppression and ion enhancement occur. See Table 2 in Paper III for the complete result set. The relatively high ME values for the studied compounds are acceptable and indicate that severe ion suppression is unlikely for other compounds in these groups. The instrument response and RT variation over time were expressed by plotting the peak area and RT of the internal standards extracted from one calibrator from each analytical run. This not only reflects the variation in instrument response but also the variation in extraction efficiency and matrix effects over time. This provides a more relevant expression compared to a direct injection of a neat performance test sample.

4.3.2. Retrospective data file analysis

A total of 1314 data files (242, 252, 273, 242, and 305, respectively, from the years 2014 to 2018), was processed with the new PCDL. The retrospective analysis revealed six new findings of category 1 in addition to two compounds (fluorofentanyl and cyclopropylfentanyl) that had been reported when the data files were processed with the original method, but first after seized material had become available (Tables 3 and 4 in Paper III). The six new findings were flubromazepam in two data files from 2015 and 2016, respectively, phenibut (4-amino-3-phenylbutyric acid) in a data file from 2015, JWH-167 in a data file from 2014, tilidine in a data file from 2015, and methoxyacetylfentanyl in a data file from 2016. The last three were refuted after RT comparison with reference material.

In addition, there were 35 possible findings of category 2. Further evaluation of RT, signal-to-noise ratio, and chromatographic peak shape for every finding was done and only one finding of phenibut remained (Table 5 in Paper III). As no MS/MS spectra was available for library comparison, this finding was, however, not possible to confirm to the same degree of confidence as those in category 1.

5. Discussion

The three studies included in this thesis deal with the detection and metabolite profiling of NPS using LC-QTOF-MS. The sharp increase of NPS on the illicit drug market in the last decade has led to major challenges for clinical and toxicological analytical laboratories that strive to keep their drug screening methods up to date. From 2008, when the first SC was identified in Europe, there was a steep increase in the number of different compounds within this group (4). **Study I** presents a urine screening method based on LC-QTOF-MS suited to tackle a diverse and changing drug market, as the analytical repertoire can easily be updated. NPS are introduced to the market with little or no knowledge of pharmacology and pharmacokinetics (108). In the analysis of urine, careful selection of analytical targets is essential to determining which drugs were taken in. **Study II** demonstrates that in vitro assays using human hepatocytes modelling human metabolism in combination with LC-QTOF-MS for metabolite identification can serve as a powerful means of gaining such knowledge. The possibility of retrospectively searching for compounds in previously generated data files is an important advantage of LC-QTOF-MS. This can provide valuable information about the presence of new drugs in the population and offer an important internal quality check that the laboratory can use to assess whether the screening repertoire used is comprehensive and relevant. **Study III** presents a strategy in which a crowdsourced database of NPS is integrated into a retrospective workflow screening old data files for SCs, synthetic opioids, and designer benzodiazepines.

5.1. Study I

5.1.1. Method validation

A screening method capable of quantifying and confirming a variety of SC metabolites at concentrations relevant for clinical and toxicological investigations was developed and validated according to guidelines for forensic applications (105). Satisfactory recovery and selectivity, linearity, precision, and accuracy within accepted limits were demonstrated for a majority of the investigated metabolites. For those metabolites not meeting the acceptance criteria, the method was regarded as semi-quantitative (marked with *SEMI* in Table 1 in Paper I). A further discussion of the causes of these limitations of the method is given in paragraph 5.1.3.

5.1.2. Authentic samples

Several synthetic cannabinoids share identical metabolites. This makes the interpretation of analytical findings challenging, and the selection of optimal targets requires a well-considered approach (35). An example is JWH-018 *N*-pentanoic acid, JWH-018 *N*-(5-hydroxypentyl), and JWH-073 *N*-pentanoic acid, all of which can be the result of the consumption of both JWH-018 and AM-2201. JWH-018 *N*-(4-hydroxypentyl) is formed after JWH-018 consumption, though small amounts of JWH-018 can be produced during the smoking of AM-2201, which may result in trace levels of JWH-018 *N*-(4-hydroxypentyl) (37, 38). To substantiate the intake of either JWH-018 or AM-2201, a reference standard of JWH-018 *N*-(4-hydroxypentyl) was analysed in retrospect. Acceptable chromatographic separation from the 5-OH isomer was achieved. When samples positive for JWH-018 *N*-(5-hydroxypentyl) were re-investigated, JWH-018 *N*-(4-hydroxypentyl) was also confirmed by the RT and MS/MS spectrum. JWH-018 *N*-(4-hydroxypentyl) was not quantified but the peak areas were similar to those of JWH-018 *N*-(5-hydroxypentyl) in the same sample. The peak areas in the positive samples show that the two metabolites were formed in similar amounts, indicating that JWH-018 and not AM-2201 was the drug of origin.

Another example was the pentanoic acid metabolite of AKB48 that was detected in six samples. The specific metabolite of 5F-AKB48 hydroxylated at the pentyl chain (5F-AKB48 *N*-(4-hydroxypentyl)) was not detected in any of the samples, suggesting that our findings originated from AKB48 and not the 5-fluoro analogue. However, the seizure statistics from KRIPOS indicate that the use of 5F-AKB48 was more frequent than that of AKB48 at the time of sample collection. Previous studies have shown that both AKB48 and 5F-AKB48 are metabolized to AKB-48 *N*-pentanoic acid and AKB48-*N*-(5-hydroxypentyl) (98, 109). Therefore, our initial findings could not unambiguously determine which compounds were ingested by these individuals. A retrospective search in the data files from the positive AKB48 *N*-(5-hydroxypentyl) for the general formula of hydroxylated 5F-AKB48 ($C_{23}H_{30}FN_3O_2$) revealed a peak three minutes earlier than 5F-AKB48 *N*-(4-hydroxypentyl) in five out of the six positive samples. Through investigation of the acquired MS/MS spectra of this compound, the fragmentation pattern could be compared to the literature (98, 109) and reveal the structure (Fig. 7). The detection of the fragments m/z 151.1117 and 133.1012 corresponding to a hydroxylated adamantyl cation $[C_{10}H_{15}O]^+$ and water loss, and not the m/z 135.1168 that dominates the spectra during fragmentation of the metabolite hydroxylated at

the pentyl chain, strongly suggested that the metabolite was hydroxylated at the adamantyl group. Sample # 10 (sample # in accordance with Table 3 in Paper I) had the lowest concentration of AKB48 *N*-pentanoic acid, indicating that the absence of a detected hydroxylated metabolite was sensitivity related. To confirm the structure, three synthesized metabolites of 5F-AKB48 hydroxylated at the adamantyl group (hydroxyl group in position 3 and both axial and equatorial orientation in position 4), which were kindly donated by the Department of Forensic Genetics and Forensic Toxicology, National Board of Forensic Medicine (Linköping, Sweden), were analysed. Chromatographic separation was achieved and the RT and fragmentation pattern of the equatorial positioned structure was congruent with the peak detected in the samples.

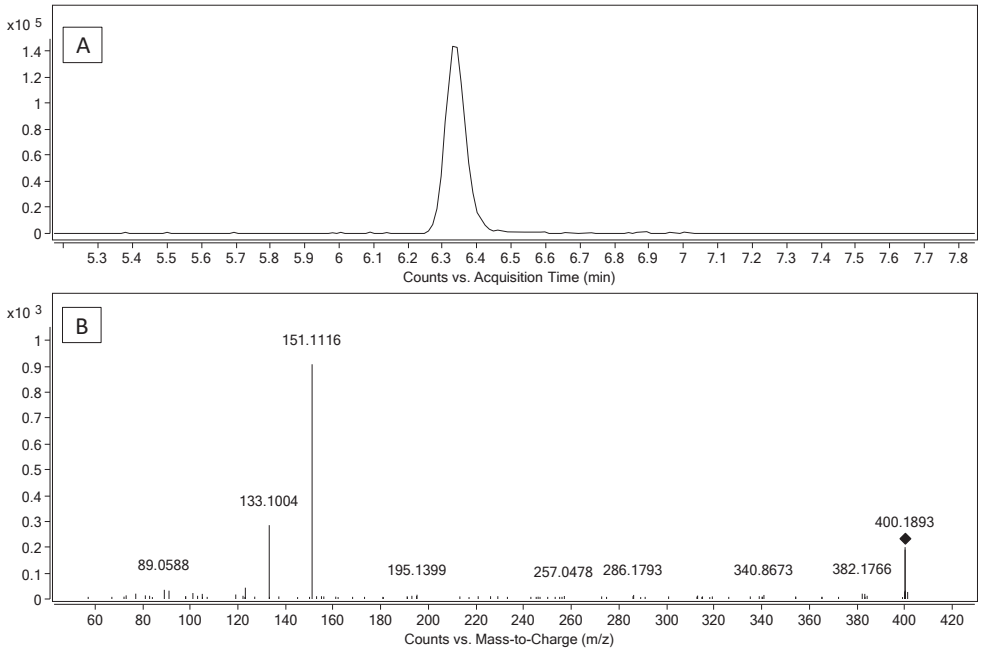


Fig. 7: Extracted ion chromatogram of $[C_{23}H_{30}FN_3O_2 + H]^+$ (A) and a MS/MS spectrum of the precursor at a collision energy of 20 eV (B).

The detected AKB48-OH metabolite in samples # 3, 4, and 8 eluted slightly earlier than AKB48 *N*-(5-hydroxypentyl), though baseline separation was not achieved. The MS/MS spectra of the precursor ($C_{23}H_{31}N_3O_2$, a mono-hydroxylated metabolite of AKB48) at this RT showed a fragmentation pattern typical of the AKB48 metabolite hydroxylated at the adamantyl group, while the MS/MS spectra produced at the RT of AKB48 *N*-(5-hydroxypentyl) confirmed the presence of this metabolite as well (Fig. 8). The concentration

estimation of the metabolite in these samples was based on the calibration curve of AKB48 *N*-(5-hydroxypentyl). The hydroxylated metabolite in samples # 5 and 6 was confirmed to be AKB48 *N*-(5-hydroxypentyl), indicating individual differences in the metabolic pathways. The original choice of AKB48 and 5F-AKB48 metabolites was not sufficient for purposes of deciding the specific consumption of these drugs. The method allowed for a retrospective investigation of metabolites outside of the original panel, which allowed us to confirm that the drug of origin was 5F-AKB48. The absence of AKB48 *N*-(4-hydroxypentyl) in any of the samples supports the theory that AKB48 was not the drug of origin in any of the cases. Sample # 10 was the only sample of these in which it was not possible to distinguish between the intake of AKB48 or 5F-AKB48.

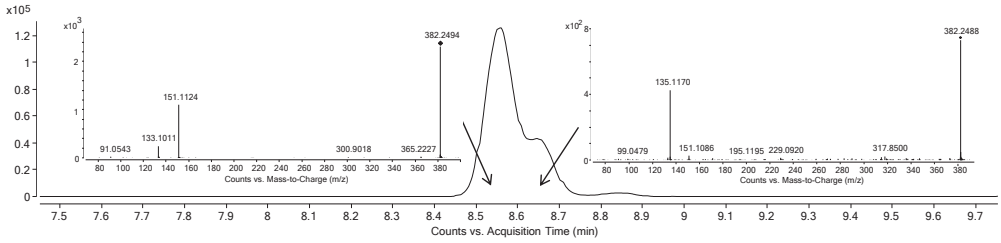


Fig. 8: Extracted ion chromatogram of hydroxylated AKB48 $[C_{23}H_{31}N_3O_2 + H]^+$ and an MS/MS spectrum acquired from the precursor from the beginning of the peak and an MS/MS spectrum from the shoulder of the peak. Both MS/MS spectra have a collision energy of 10 eV.

The AB-FUBINACA M3 metabolite was semi-quantified in six samples with a concentration range of 1.35 to 2,300 ng/mL. AB-FUBINACA M3 is formed by oxidation of the primary amide producing a carboxylic acid, while AB-FUBINACA M2 is formed by oxidation at the oxobutane moiety. The M3 has, in contrast to M2, previously been demonstrated to be one of the top three markers of AB-FUBINACA (99, 110). Having AB-FUBINACA M2 as an analyte in the panel and not detecting it serves as additional proof to the studies cited above of this being an unsuitable marker. AB-FUBINACA itself was not included in the method, but a retrospective search for the formula of this compound returned a positive finding in samples # 3, 4, and 8 (not detected in # 5, 7, and 10), which were also the samples with the highest concentrations of FUBINACA M3. This method was, to the best of the author's knowledge, the first published comprehensive screening method containing AB-FUBINACA M3. The results show that including this marker is essential to detecting AB-FUBINACA. It must be emphasized, though, that the methyl ester analogue AMB-FUBINACA (also known as MMB-

FUBINACA) and the ethyl ester analogue EMB-FUBINACA also can result in AB-FUBINACA M3 (39). A method published after our article (Paper I) included both AB-FUBINACA M3 and a second metabolite with a carboxyl group on the indazole (AB-FUBINACA M4) (111). AB-FUBINACA M3 was detected in 92 samples, whereas the second metabolite was detected in 21 of the same samples, which confirms that a screening method should contain the M3 metabolite to secure optimized conditions for the detection of AB-FUBINACA, AMB-FUBINACA, and EMB-FUBINACA.

In five of the six samples containing AB-FUBINACA M3, at least one metabolite of 5F-AKB48 was also detected. This can be the result of a concomitant intake of either AB-FUBINACA, AMB-FUBINACA, or EMB-FUBINACA and 5F-AKB48 from two different products, or of the intake of a product containing both drugs, either sold as a mix or with one being a contamination of the other. Information from KRIPOS showed that in only one out of 11 AB-FUBINACA seizures was 5F-AKB48 detected in the same product. In two out of 11 seizures of AB-FUBINACA, a seizure of 5F-AKB48 was made in the same case. As our samples were anonymized before analysis, we could not determine whether some of them were from the same individual(s) or the same geographical area. A corresponding situation was seen with JWH-073, which was always detected when any of the metabolites of JWH-018 were present. Demethylation of JWH-018 to JWH-073 and further oxidation to JWH-073 *N*-pentanoic acid has previously been hypothesized and cannot be ruled out (112).

5F-PB-22 3-carboxyindole could not be confirmed with the spectral library in the two samples in which a concentration below the LOC (5 ng/mL) was observed. The second injection, however, provided MS spectra that strongly indicated the presence of the compound at a concentration > 2.5 ng/mL, even though the concentration was too low to be confirmed with ID criteria III. Neither 5F-PB-22 3-carboxyindole nor BB-22 3-carboxyindole is a specific marker of 5F-PB or BB-22 intake, respectively. 5F-PB-22 3-carboxyindole can originate from 5F-MDMB-PICA (113), and a biotransformation of MDMB-CHMICA to BB-22 3-carboxyindole can take place (114). Other specific markers were not available as certified reference materials. In the case of BB-22, the absence of specific metabolites for MDMB-CHMICA and AMB-CHMICA in biological samples must be documented to prove the intake of this substance (115).

In statistics provided by KRIPOS regarding seizures in Norway in 2014 (the year when the samples were submitted), 5F-AKB48 was at the top with 43 seizures, followed by 5F-PB-22, BB-22, AB-FUBINACA, and AM-2201 with 15, 15, 11, and 10 seizures, respectively. JWH-210, PB-22, UR-144, AKB48, JWH-018, JWH-073, AB-CHMINACA, JWH-122, and JWH-081 were reported in five or fewer seizures. With the present method, metabolites of 5F-AKB48 were found in six samples. In addition, the metabolites of five other SCs or their closely related analogues were found.

5.1.3. Strengths and weaknesses

With a generic sample preparation and the analytical methodology presented here, the continuous addition of new analytes is relatively straightforward, with a limited number of validation experiments depending on whether the analyte is added for qualitative or quantitative purposes. Qualitative validation should include experiments to determine the LOC, selectivity, retention time, stability, carry-over, and stability of the new compound. For quantitative purposes, additional experiments to determine LOQ, ME, recovery, precision, accuracy, and linearity should be conducted. In both cases, access to certified standards is a prerequisite. Statistics from KRIPOS gathered after the publication of Paper I showed only single seizures of some of the SCs representing the metabolites in the method during 2017. In the first six months of 2018, no seizures were registered of these SCs. Recent data were not available at the time of publication of this thesis. Frequent revisions of the analytes covered by the method are required with the addition of new compounds if reference standards for relevant metabolites become available.

Certain limitations to the method must be acknowledged. Although the chromatography covered a wide polarity range, in particular, early eluting polar compounds suffered from more pronounced MEs, higher LOQs and LOCs, and less precise quantification. The majority of compounds showed MEs and recoveries within the acceptance criteria. A general sample preparation, which was chosen here, can be used for the extraction of analytes with a broad spectrum of physico-chemical properties, though a high ME—and, thereby, an unfavourable influence on the analytical quality—was observed for some compounds. Choosing a sample preparation method that removes the matrix more effectively may most likely decrease the MEs but also potentially reduce the recoveries of many of the analytes. LLE using a suitable buffer for pH adjustment and an as-small-as-possible volume of organic solvent could have been an alternative in an automated setting if the robot is equipped with suitable exhaust to

reduce exposure to the surroundings. However, compromises must be made when a method covering this high number of components with a broad range of chemical properties is developed. The measured ME outside the accepted range indicates that both ion suppression and ion enhancement occur. Quantifications with corresponding internal standards for all analytes would potentially compensate for the ME. However, in a screening method, this is not easily achieved and a compromise on the analytical quality for certain analytes must be accepted. Moreover, a tendency towards lower recovery for the compounds which are more retained on the analytical column indicates that these compounds also are stronger adsorbed on the SPE sorbent. This must be taken into account when new compounds are introduced to the screening method. As a consequence of high MEs, low recoveries, and the absence of dedicated isotopically labelled internal standards, the method must be regarded as semi-quantitative for the following analytes: AB-CHMINACA M1A, AB-CHMINACA 3-carboxyindazole, AB-FUBINACA-M2, AB-FUBINACA-M3, AB-PINACA COOH, AM-2201 *N*-(4-hydroxypentyl), AM-2201 *N*-(5-hydroxyindole), JWH-122 *N*-(5-hydroxypentyl), JWH-210 *N*-(5-hydroxyindole), JWH-210 *N*-(5-hydroxypentyl), JWH-210 *N*-pentanoic acid, PB-22 *N*-pentanoic acid, PB-22 *N*-(4-hydroxypentyl), RCS-4 *N*-(4-hydroxypentyl)phenol, and THJ-2201 *N*-pentanoic acid.

Due to the poor quality of MS/MS spectra acquired for a few analytes, relatively high concentrations were needed to achieve acceptable library-search scores, with correspondingly high LOCs. Co-eluting isomeric species suppressing or contaminating the MS/MS spectra by introducing additional fragment masses or poor ionization and fragmentation of the precursor can cause these problems. Limited data are available on the expected concentrations of the different metabolites in urine after recreational use, though a relatively broad range of concentration levels, from less than one to up to hundreds of ng/mL, has been reported (68, 69). Most of the analytes have a LOC at or below 1 ng/mL, which will be sufficient to confirm them at their presumable levels in urine. The time window of detection after intake will obviously be narrower if the LOC is higher. LOCs of AB-PINACA pentanoic acid, RCS-4 *N*-(4-hydroxypentyl)phenol, RCS-4 *N*-pentanoic acid, AB-FUBINACA M2, PB-22 3-carboxyindole, and BB-22 3-carboxyindole were up to 50 times higher compared to LOQs presented using LC-MS/MS-based methods (69, 102, 116, 117). Most of these elute early (RTs < 4 min) and are more prone to MEs as they co-elute with matrix components. LOC values higher than LOQ values were expected, as the LOC is based on a more stringent identification criterion. The LOQ is, in most methods, based on the signal-to-noise ratio of the

quantifier transition together with the accuracy of the concentration measurement. In the presented method, the instrument is acquiring both MS and MS/MS and is, therefore, spending time switching between modes, which compromises sensitivity. Other compounds, like AKB48 *N*-(4-hydroxypentyl), AKB48 *N*-(5-hydroxypentyl), AKB48 *N*-pentanoic acid, AM-2201 *N*-(4-hydroxypentyl), JWH-018 *N*-(5-hydroxypentyl), JWH-203 *N*-pentanoic acid, JWH-018 *N*-pentanoic acid, JWH-210 *N*-pentanoic acid, JWH-250 *N*-pentanoic acid, UR-144 *N*-5-hydroxypentyl, UR-144 *N*-pentanoic acid, and UR-144 *N*-(4-hydroxypentyl), had LOCs at the same level or even below the LOQ achieved in methods with a comparable panel of analytes based on LC-MS/MS (64, 66-69, 118).

The lack of reference material of SC metabolites available is a significant restriction in the pursuit of an updated screening method in urine. Recently, a new qualitative approach was presented to overcome the need for metabolite reference standards (119). Here, the SC metabolites were produced in vitro by human liver microsome assays and identified by LC-QTOF-MS. The resulting fragmentation information was included in an "in-house" library. The library was applied to an LC-MS/MS urine screening method of 75 SCs and 339 of their metabolites in urine. An alternative to MS methodology for screening is based on the biological activity of SC metabolites, using a cannabinoid receptor activation assay (120). However, this method requires that the SC metabolites exhibit receptor binding activity. Furthermore, this technique is not widely available and confirmation with MS must be done to identify the specific substance.

5.2. Study II

5.2.1. Identification of the fluorofentanyl metabolites

Extracts from the incubation of human hepatocytes with the three fluorofentanyl isomers were analysed with LC-QTOF-MS, and molecular formulas of potential metabolites were searched for with a database using the *Find by Formula* algorithm. To examine the potential metabolites further, diagnostic fragment ions in their MS/MS spectra were compared to the fragmentation patterns of the parent compounds. All three parent compounds contained the fragment ions of m/z 84.0808, 105.0702, 134.0965, 150.0710, 188.1438, 234.1291, and 299.1912, exemplified by the MS/MS spectrum of ortho-fluorofentanyl in Fig. 9. Elucidation of MS/MS spectra from the metabolites was based on recognition of these fragment ions or fragments corresponding to the addition of the mass from typical biotransformations. When

the molecular position for the specific biotransformation step could be proposed, the metabolite was given a name in correspondence with the letter- and number-based system given in Fig. 9. The metabolites were named using O (ortho), M (meta), or P (para) and were numbered 1 to 14, corresponding to the RT order. An example of the elucidation and proposed structure is presented in Fig. 10 for the metabolite O7/M7/P7. The other identified metabolites are shown in Fig. S1 in the supplementary information of Paper II.

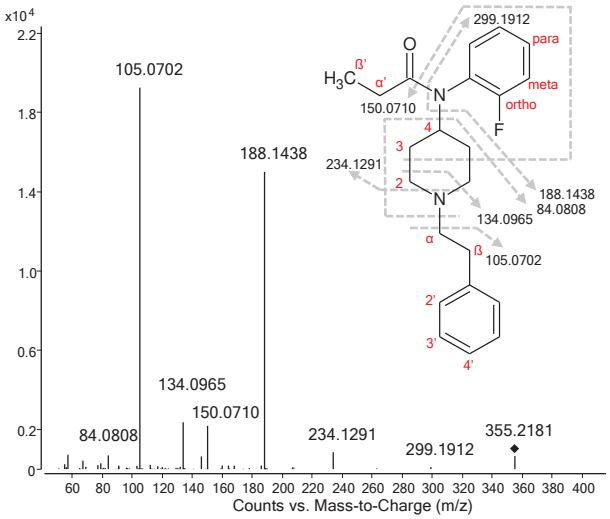


Fig. 9: MS/MS spectrum of ortho-fluorofentanyl, the suggested explanation of fragmentation, and a positional system indicating the position of substituents.

N-dealkylation at the piperidine ring resulting in the loss of the phenetyl moiety and forming norfluorofentanyl (O2/M2/P2) was observed to be the main metabolite in vitro. Several metabolite studies of fentanyl analogues with similar structures support this finding (103, 107, 121-123). The MS/MS spectra showed one abundant fragment ion at m/z 84.0815, indicating that the piperidine ring was intact in these metabolites. The enzyme CYP3A4 has previously been shown to be responsible for the *N*-dealkylation of fentanyl (16). A compound (O1/M1/P1) corresponding to hydroxylation (addition of 15.9949 u, i.e., +O, when compared to the mass of the parent) of the *N*-dealkylated metabolite was also detected. The fragment ion m/z 84.0805 also dominated these spectra, suggesting hydroxylation at the amide alkyl chain or N-phenyl ring.

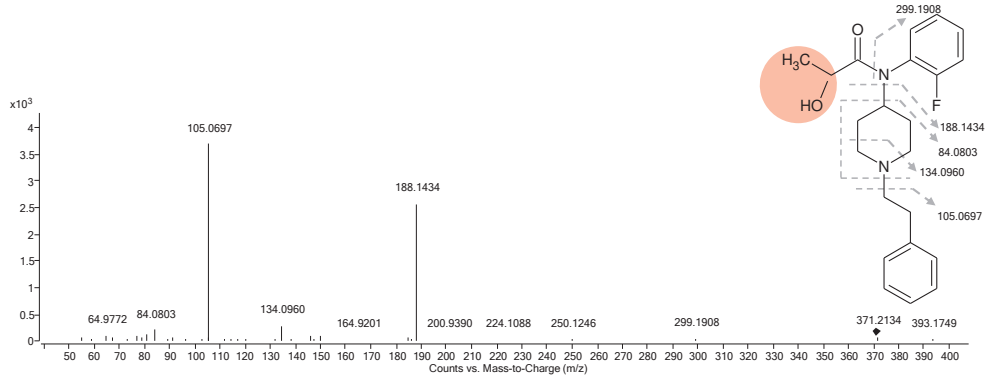


Fig. 10: Fluoro fentanyl metabolite O7 with its MS/MS spectrum and purposed structure. The highlighted part of the molecule indicates the possible position of hydroxylation.

Four different hydroxylated metabolites (addition of 15.9949 u, i.e., +O, when compared to the mass of the parent) with the protonated molecular ion [$C_{22}H_{27}FN_2O$ +H] and m/z 371.2131 were detected eluting from 9.31 to 12.81 min. The most abundant of these metabolites was O12/M12/P12. An exact structure could not be elucidated. Fragment ions m/z 204.1380 (addition of an oxygen to fragment ion m/z 188.1438) and m/z 186.1277 (from water loss) and m/z 150.0710 and 164.0864 indicate hydroxylation in the 2-position. At the same time, fragment ion m/z 353.2020 can correspond to an elimination of H_2O from the metabolite which favors an interpretation towards aliphatic hydroxylation, and the fragment ion m/z 105.0695 may have been formed from a β-hydroxy metabolite. Therefore, we propose that O12/M12/P12 is a monohydroxy metabolite with the hydroxyl group at either the piperidine ring or the ethyl linker. The second most abundant monohydroxylated metabolite was O7/M7/P7. Fragment ion m/z 188.1434 corresponds to an intact phenetylpiperidine structure and, together with m/z 299.1908, indicates hydroxylation on the amide alkyl chain (Fig. 10). Traces of this metabolite were detected in the 0 h sample of meta-fluorofentanyl. Monohydroxylated metabolite O8/M8/P8 was hydroxylated at the phenetyl moiety as indicated by the fragment ions m/z 121.0646 (m/z 105 +O). Hydroxylation at the phenyl ring (position 2', 3', or 4') or a β-hydroxy at the *N*-alkyl chain is most probable, as α-hydroxyl metabolites are not known to exist (intermediate to the *N*-dealkylation pathway). The last monohydroxylated metabolite O14/M14/P14 elutes after the parent drug, which is unexpected for a more polar compound. These late-eluting metabolites have been described previously for *N*-oxide metabolites of fentanyl analogues (7, 10) and in an in vitro study of the metabolism of nicotine (17). Based on its RT, O14/M14/P14 is assumed to be an *N*-oxide. Also, fragment

ions m/z 105.0700 and m/z 164.0868 indicate an unchanged phenethyl moiety and *N*-phenyl ring, which is consistent with an *N*-oxide, as shown with similar compounds in previous studies (7, 13).

Five di-hydroxylated metabolites (addition of 2x15.9949 u, i.e., $+O_2$, as compared to the mass of the parent) with the protonated molecular ion [$C_{22}H_{27}FN_2O_3$ +H] and m/z 387.2078 were detected per parent in vitro. The most abundant O13/M13/P13 with fragment ion at m/z 164.0867 and lack of m/z 188.1434 indicates an unchanged *N*-phenyl ring and that hydroxylation has occurred at the amide alkyl chain, phenethyl moiety or at the 2-position of the piperidine ring. The fragment ion at m/z 207.1279 can be formed by the loss of the amide alkyl chain and the phenetyl moiety (with one carbon left; $C_{12}H_{16}FN_2$). This is open to a second interpretation that includes a monohydroxylated *N*-oxide or a water loss from an *N*-oxide with dihydrodiol. O11/M11/P11 shows a fragment of m/z 186.1276, which indicates that the first hydroxylation is at position 2 at the piperidine ring (after water loss similar to O12/M12/P12) and the fragments with m/z 105.0700 and 164.0868, suggesting an intact *N*-phenyl ring and phenetyl moiety and, therefore, the second hydroxylation at the amide alkyl chain. The presence of a fragment ion at m/z 207.1247 means that the first oxidation can be an *N*-oxide (as shown for O13/M13/P13). The third di-hydroxylated metabolite O10/M10/P10 was detected, and the fragment ion of m/z 121.0643 indicates hydroxylation on the phenetyl moiety. The location of the second hydroxyl group could not be determined by the MS/MS spectrum, though 164.0863 indicates position 2 at the piperidine ring. The MS/MS spectrum of O4/M4/P4 and O6/M6/P6 did not contain any diagnostic fragments which could help interpret the position of the hydroxyl groups.

Two compounds corresponding to dihydrodiol metabolites were detected: O3/M3/P3 and O5/M5/P5. Theoretically, a dihydrodiol can be located on the ethylphenyl ring or the *N*-phenyl ring. The common fragment ion of m/z 164.0864/164.0875 suggests that the *N*-phenyl ring is unchanged in both compounds and that the ethylphenyl ring is the target of the biotransformation. The absence of the expected fragment ion m/z 139.0754 and the presence of fragment ions m/z 121.0640/121.0633 can be explained by water loss from one of the dihydrodiol hydroxyl groups, which leads to re-aromatization, as shown by Watanabe et al. (106). The exact position of the dihydrodiol hydroxyl groups on the ethylphenyl ring could not be determined from the fragmentation pattern. Finally, a compound corresponding to a hydroxylated and methoxylated metabolite with the protonated molecular ion [$C_{23}H_{29}FN_2O_3$

+H] was detected: O9/M9/P9. The fragment ion at m/z 151.0749 suggests the presence of hydroxyl and a methoxy group at the phenethyl moiety (4'-hydroxy-3'-methoxy-fluorofentanyl). The presence of m/z 119.0487 is caused by the loss of methanol.

5.2.2. Metabolites detected in an authentic urine sample

Three of the metabolites found in vitro were also detected in the authentic ortho-fluorofentanyl urine sample. These were norfluorofentanyl (O2), 2', 3', or 4'-hydroxy-fluorofentanyl (O8) and either 4'-hydroxy-3'-methoxy-fluorofentanyl or 3'-hydroxy-4'-methoxy-fluorofentanyl (O9). A comparison of the MS/MS spectra of metabolites detected in both urine and in vitro samples is shown in supplementary information Fig. S2 in Paper II. In urine, O9 had the highest abundance, in contrast to the in vitro experiment, where this metabolite was of low abundance compared to the other metabolites. Norfluorofentanyl (O2) and 2', 3', or 4'-hydroxy-fluorofentanyl (O8) were the second and third most abundant metabolites in the authentic urine sample.

5.2.3. Differences between metabolites of ortho-, meta-, and para-fluorofentanyl

Some notable differences were found when the RTs and chromatographic peak areas of the corresponding metabolites from the three fluorofentanyl isomers were compared. The three parent compounds eluted within 0.06 min of each other, with para-fluorofentanyl eluting first (Table 1 in Paper II). Such a small difference could be the result of instrumental instability, though the two internal standards (amphetamine-d8 and midazolam-d3) showed excellent RT stability (within 0.01 min) between the samples, indicating that the difference between the parent compounds is real. The retention order varied from metabolite to metabolite (Table 1 in Paper II) and the RT difference varied from 0.02 min (O12/M12/P12) to 0.37 min (O11/M11/P11). The position of the fluorine atom obviously affects the RT more in some metabolites than in others. There were also differences in peak areas between isomers, though without any clear pattern. Metabolites from all three parent compounds were formed by the same principal pathways, even though the difference in peak areas for some of the metabolites was considerable; e.g., for O13/M13/P13, it was 10-fold. As relative peak areas between metabolites probably vary between individuals, they most likely cannot be used as an identification parameter.

5.2.4. Metabolic pathways

The general metabolic pattern for the three fluorofentanyl isomers could be suggested as shown for ortho-fluorofentanyl in Fig. 3 in Paper II. Metabolites were formed through *N*-dealkylation and/or oxidations. In addition, a pathway of hydroxylation and methylation via dihydrodiol forming O9/M9/P9 could be proposed. The same pathway was shown by Watanabe et al., which suggested that the dihydrodiol compound can be metabolized by the enzyme cathechol-O-methyltransferase to a metabolite containing a hydroxyl group and methylation of a second hydroxyl group giving O9/M9/P9, possibly through an intermediate catechol compound (106). However, such a di-hydroxylated precursor of O9/M9/P9 could not be detected among the metabolites. The main in vitro metabolites norfluorofentanyl (O2/P2/M2) and hydroxy fluorofentanyl (O7/P7/M7, O8/P8/M8, O12/P12/M12, and O14/P14/M14) were consistent with findings in previous studies on both fentanyl and some other fentanyl analogues (106, 122-124). Glucuronidated metabolites were not observed. This was consistent with previous in vitro studies of fentanyl analogues using hepatocytes. Watanabe et al. detected only one glucuronidated metabolite from the fentanyl analogues acetylfentanyl, acrylfentanyl, furanylfentanyl, and 4-fluoro-isobutyrylfentanyl (106). Carboxylated metabolites have been detected in previous studies of fentanyl analogues, e.g., 2,2,3,3-tetramethyl-cyclopropylfentanyl (103) and crotonylfentanyl (125). No metabolites of this type were detected for fluorofentanyl. The amide hydrolysis product, fluoro-4-anilino-*N*-phenylpiperidine, was detected in the degradation control, 0 h sample and with declining peak areas throughout the experiment. This finding indicates that the compound is not formed in vitro, which is in contrast to other studies of similar fentanyl analogues, in which amide hydrolysis is a significant metabolic pathway (106, 123, 126). The relatively low number of metabolites detected in the authentic urine sample compared to the in vitro study can at least partly be attributed to the low drug concentration in the urine sample and the simple dilute-and-shoot sample preparation.

Norfluorofentanyl (O2/P2/M2) may be a suitable marker of drug intake, as it was the most abundant metabolite found in vitro and was also detected in the authentic urine sample. However, norfluorofentanyl might not be specific enough for use as a single analytical target. Future fentanyl analogues could potentially produce this metabolite as well; a more specific marker will be necessary to unambiguously identify the ingested compound. O8/P8/M8 includes the whole structure of its parent drug and, therefore, might be a better candidate.

Unfortunately, though it is abundant in vitro, only traces were detected in the authentic urine sample. O9/P9/M9 is another specific marker and, according to the results of the authentic urine sample, is likely to be in relatively high abundance.

The elimination half-lives of ortho-, meta-, and para-fluorofentanyl are not studied, though it is reasonable to believe that they would be similar to fentanyl itself and, thus, to be somewhere between 3 and 12 h (127). Detection times in urine might be extended through use of O9/P9/M9 and/or O8/P8/M8 as markers, though this must be confirmed by analyses of several positive samples.

5.2.5. Strengths and weaknesses

This in vitro model chose human hepatocytes over human liver microsomes because they are living cells and contain all endogenous enzymes, cofactors, drug transporters, and drug-binding proteins essential for human drug metabolism (128). However, due to extrahepatic drug metabolism and transport, as well as inter-individual differences, the in vitro results may differ from those obtained in vivo. Though only one authentic urine sample was available, the results indicate that there are differences in both the range of metabolites and the number detected. As ortho-fluorofentanyl was a new compound and, obviously, the prevalence was low, the availability of authentic samples was limited. Having one authentic human sample provides far more information than not having any sample for comparison purposes. However, the availability of only one authentic urine sample is clearly a weakness of this study; thus, a definite recommendation regarding the most appropriate marker to choose in the analysis of human urine cannot be given.

Compounds corresponding to the hydroxylated metabolites of diclofenac, omeprazole, and midazolam were detected in the positive control samples incubated for 5 h, showing that the cells were functional. No glucuronidated metabolites were detected in the in vitro study or in the authentic sample, which may be due to a very limited formation of these and/or a lack of detection capacity with the analytical instrument used. The similar peak areas observed for the same metabolite in the hydrolysed and non-hydrolysed authentic urine sample indicates that glucuronidation is not taking place, at least not to any significant extent. However, again, caution should be exercised in the interpretations, as only one sample was available. To the best of the author's knowledge, no additional research on the metabolism of ortho-, para-, or meta-fluorofentanyl was published in the period between the publishing of Paper II and the

completion of this thesis. However, new research on the metabolism of other fentanyl analogues has been published (104, 121, 129, 130). A study of the metabolism of buturylfentanyl with hepatocytes found nor-buturylfentanyl to be the major metabolite, followed by four hydroxy metabolites (121). Liver microsomes were used to study the metabolism of cyclopropylfentanyl, and the major metabolites were formed via *N*-dealkylation (nor-metabolite), monohydroxylation, and *N*-oxidation, while the nor-metabolite was found to be the most abundant in vivo (129). Another study of cyclopropylfentanyl in-vivo identified seven metabolites, with the nor-metabolite as the most abundant. In addition, the exact structures of three hydroxy metabolites were found (104). In a study of fentanyl and the five analogues acetylfentanyl, acrylfentanyl, cyclopropylfentanyl, isobutyrylfentanyl, and 4F-isobutyrylfentanyl, several metabolites were first synthesized and used in the identification and structure elucidation of the metabolites found in vivo and in vitro by hepatocytes (130). The nor-metabolites were the most abundant for all analogues. The ß-OH metabolite (corresponding to O12/M12/P12 in this study) was the most abundant hydroxy metabolite in vitro for all analogues except acetylfentanyl, for which the 4'-OH metabolite was most abundant. The available in vivo data showed that the 4'-hydroxy-3'-methoxy metabolites were abundant in urine, which is in agreement with the finding of this thesis (corresponds to compound O9).

LC-QTOF-MS or other comparable HR-MS instrumentations are well-suited to acquiring identification data from in vitro experiments, as accurate mass and MS/MS spectra can be used to tentatively elucidate the structures. However, when identification relies only on MS/MS elucidation, the differentiation between some metabolite isomers is difficult or impossible. Therefore, metabolites are identified, but their exact structure remains unclear. This corresponds to a level 2 identification according to Schymanski et al. (56). To confirm the structure, a series of proposed metabolites can be synthesized and analysed to compare the MS data (monoisotopic mass, retention time, and MS/MS spectra) to the results from in vitro extracts and authentic urine samples (104, 121, 130), at least when the synthesized compounds and in vitro extracts and/or urine samples are analysed with the same chromatographic conditions.

5.3. Study III

5.3.1. Validation of original analytical method

When the post mortem samples were analysed by LC-QTOF-MS upon arrival in the period from 2014 to 2018, the original analytical method had obviously not been validated for any of the analytes included in the new PCDL. To estimate the LOI and RT variation that could be expected for the analytes in the retrospective data analysis, a limited validation was performed for a selection of compounds. By following the internal reference standards in samples from the data set, a variation in peak areas during the period due to, e.g., instrument condition and periodic maintenance was observed. How this, in turn, affected the LOIs is difficult to determine, as the value is a result not only of signal intensity but also the automatic selection of precursor ions based on the DDA settings. Though the signal intensity is low, the compound will likely be identified if the precursors are among those selected for fragmentation.

5.3.2. Retrospective data file analysis

From the MS information available in the data files, the findings were placed into two groups: category 1 (eight findings) and category 2 (35 findings). Category 1 findings included two data files with flubromazepam. Both had a mass match score higher than 95, a mass accuracy better than 3.46 ppm, and an RT deviation of less than 0.07 min. The mass match can be visualized by the resemblance of the spectrum of flubromazepam and the theoretical pattern indicated by the boxes in Fig. 11.

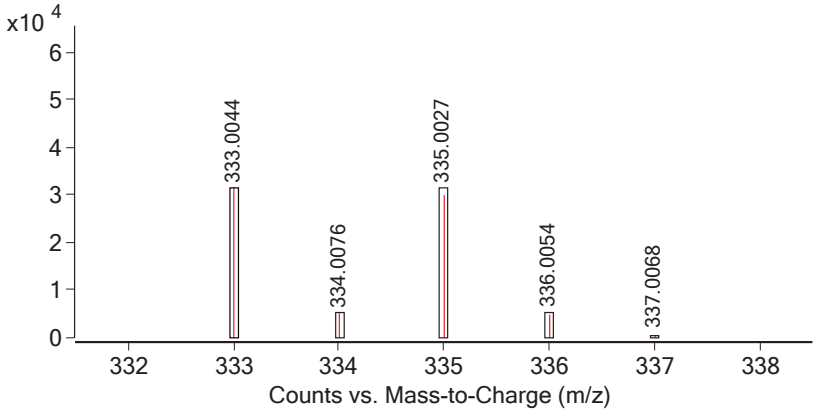


Fig. 11: MS spectrum of flubromazepam extracted from a data file with the theoretical isotopic pattern illustrated by the black boxes.

The three diagnostic fragments in the library spectrum were also found in the MS/MS data acquired from the precursor in the two data files (see Fig. 12A). An additional comparison of the MS/MS spectra from the data file and the analysis of a reference standard showed good agreement for additional fragment masses (see Fig. 12B). Flubromazepam was first described in 1962 and is a highly potent and incompletely evaluated benzodiazepine structurally related to phenazepam (14, 131). Flubromazepam started to emerge in online shops in Europe in 2012. In Norway, it was detected in seized material by KRIPOS for the first time in 2013.

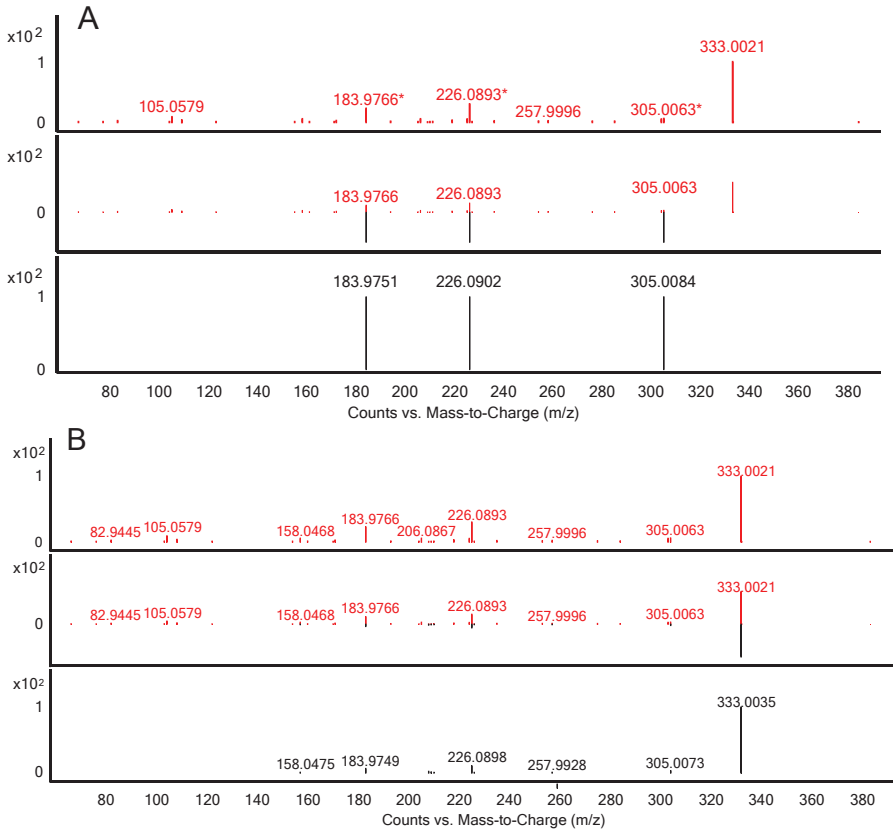


Fig. 12: (A) Acquired MS/MS-spectrum of flubromazepam with diagnostic fragments marked with an asterisk (at the top), library spectrum from PCDL (at the bottom), and a comparison (in the middle). (B) Acquired MS/MS spectrum (at the top), full MS/MS spectrum from the reference standard (at the bottom), and a comparison (in the middle).

Phenibut was identified in one data file from 2015. This category 1 finding showed a mass match score higher than 85, a mass accuracy of −1.63 ppm, and an RT deviation of 0.12 min. compared to a reference standard analysed in 2018. Evaluation of the RT over time showed that a deviation up to 0.5 min. could be expected due to a change of the analytical column lot

and tubing. Phenibut is a neuropsychotropic drug with possible cognition-enhancing effects that was discovered and introduced into clinical practice in the Soviet Union during the 1960s (132). The drug is widely used in Russia and is claimed to have various clinical effects, e.g., to relieve tension and anxiety and to improve sleep. Phenibut was relatively unknown outside Russia until 2011, when a quantity was seized in Sweden (133). Due to the concern of misuse, the drug was reported to EMCDDA and later classified as an NPS by UNODC. The availability of the drug online has increased and reports on misuse, intoxication, and dependency have been published (134-137). In Norway, phenibut is not scheduled or classified as a medicinal drug and is not available for legal sale. Private import is prohibited by law. KRIPOS did not detect phenibut in any cases before 2019. Our laboratory reported the detection of phenibut in seized material and biological samples for the first time in 2016. Since then, it has been part of the routine analytical repertoire at our laboratory.

Fluorofentanyl was detected in one data file from 2016, with a mass match score higher than 97, a mass accuracy of -0.21 ppm, and good agreement in the diagnostic ions. Analysis of reference material showed an RT deviation of less than 0.05 min. Moreover, a compound with molecular formula $C_{23}H_{28}N_2O$ was detected in a data file from 2018 with a mass match score higher than 96 and mass accuracy of 2.87 ppm. The diagnostic fragments of m/z 105.0699 and 188.1434 showed that the compound most probably was a fentanyl analogue and the software suggested either cyclopropylfentanyl, methacrylfentanyl, or crotonylfentanyl. These three compounds share the same formula and diagnostic fragments. Consequently, they cannot be distinguished from each other based on category 1 criteria only, though analysis of reference material showed good RT agreement (deviation 0.01 min.) with cyclopropylfentanyl. In fact, fluorofentanyl and cyclopropylfentanyl had already been confirmed by targeted analysis of the data files based upon information from an analysis of seizures from the scene requested by the police (31, 138). However, as these compounds would not have been detected originally if we had not known which substances to suspect, they are included in the present material.

Identification of flubromazepam, phenibut, fluorofentanyl, and cyclopropylfentanyl (of category 1) was based on the mass match score, the presence of diagnostic fragment ions and, finally, RT agreement. Fulfilment of these criteria provided the highest level of confidence that can be achieved in a retrospective review when re-analysis of the actual specimen was not possible. Detection and confirmation of compounds with HR-MS can be divided into different

levels of confidence based on information available from the data acquisition, as suggested by Schymanski et al. (56). In that approach, level 5 through level 1 requires increasing information from the MS signal to diagnostic fragments and RTs (56). The findings of category 1 in our retrospective method can be compared to a situation close to level 1. Level 1 requires confirmation with a reference standard, which was the case with our new findings. However, as long as the sample and standard are analysed at different times, a definite confirmation is not achieved.

In a retrospective approach, the co-identification of metabolites can further strengthen the confidence in a finding. Searches for the major metabolites of the detected compounds were done in the relevant data files. Metabolites from published in vivo and in vitro studies were selected (14, 103, 107, 139). Neither of the metabolites of fluorofentanyl were detected in the data file containing this compound. In the data file containing cyclopropylfentanyl, the *N*-dealkylated metabolite and two hydroxylated metabolites were detected. The metabolites of flubromazepam found in the literature to be the most abundant (hydroxylated flubromazepam and debrominated flubromazepam) were not detected in any of the two positive samples. The metabolism of phenibut has, to our knowledge, not been studied, and no putative target metabolites have been described in the literature.

After further investigation, three other positive category 1 findings could be refuted (Table 4 in Paper III). For methoxyacetylfentanyl, the RT deviation compared to the reference standard was significant, indicating that the compound was, rather, an isomer of methoxyacetylfentanyl with similar fragmentation patterns. There were no other described fentanyl analogues with an identical molecular formula. However, the presence of fragments of m/z 105.0699 and 188.1434 was a strong indicator that the compound consisted of the piperidine and phenyl moiety characteristic of fentanyl itself as well as many fentanyl analogues. Metabolites of fentanyl hydroxylated at the alkyl or phenetyl moeity have the same monoisotopic mass as methoxyacetylfentanyl, and the diagnostic fragments 105.0699 and 188.1434 will be the same (Fig. 13). Fentanyl was reported in the original analysis of the sample, which explains the presence of a metabolite. Thus, it could be concluded that the finding was caused by fentanyl intake.

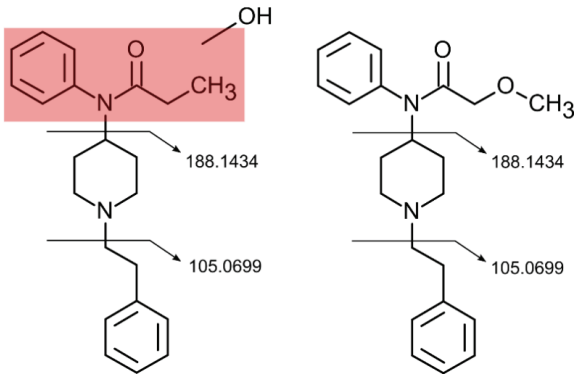


Fig. 13: Structures of hydroxyfentanyl (left) and methoxyacetylfentanyl (right) with their common fragments. The superimposed area indicates the position of the hydroxyl group.

Category 1 findings of JWH-167 and tilidine were detected in one data file each, from 2014 and 2015, respectively. The fragments in the MS/MS spectra were in relatively good agreement with the diagnostic fragments from the library spectrum. In addition, the m/zCloud database was consulted and showed agreement with one additional fragment. Reference standards were acquired to compare RTs, and significant RT differences clearly showed that neither JWH-167 nor tilidine was present. These examples of false-positive results illustrate the importance of having access to the reference substance in order to check RT conformity.

A total of 35 possible category 2 findings (see Table 5 in Paper III) was found when criterion c was applied (i.e., better than 5 ppm mass accuracy, mass match score higher than 95, and RT 1.5 min. or later). Further evaluation of all findings was done. Disproving was due to large RT deviations from reference standards, signal-to-noise ratios ≤ 3, and poor peak shapes. See Table 5 in Paper III for a detailed list of findings and their respective evaluations. Based on the RTs of other SCs analysed with the same chromatographic conditions in the validation, (see Table 2 in Paper III), findings of SCs with RTs less than 5 min. were regarded as highly unlikely and removed from the list. A similar limit of 4 min. was applied for synthetic opioids. A category 2 compound found in one or more data files, and also found with the same RT in other data files having MS/MS spectra acquired but no library match, was likewise rejected. Thus, a review of the 35 suggested category 2 findings, only one finding of phenibut remained. However, as no MS/MS spectra were available for library comparison, this finding could not be confirmed with the same degree of confidence as those of category 1.

5.3.3. Strengths and weaknesses

The PCDL constructed in this study is based on data acquired on instruments from different manufacturers and based on different principles. Previous studies have shown that libraries constructed from data acquired on either Orbitrap or QTOF can be used interchangeably by both instruments, providing that suitable collision energies are applied (140, 141). An essential feature of the PCDL is the mass accuracy of the diagnostic fragments. In HighResNPS, the masses of the fragments are added by either typing the formula, selecting the correct formula from a drop-down list of common fragments, or typing the theoretical mass of the acquired fragment. This ensures that mass errors from the acquisition are not transferred to the database. A second important setting is the choice of collision energy applied during the acquisition of the diagnostic fragments that are added to the database. The collision energy applied can be either discrete (e.g., 10, 20, and 40 eV) or ramped, providing a combined result. Information about the choice of strategy used in the individual entry was not present in the database. In the acquisition method used in this study, the collision energy applied was correlated to the mass of the precursor. This can potentially result in differences in relative abundance when comparing a library spectrum and an acquired MS/MS spectrum. However, the settings in the retrospective reprocessing algorithm ensure a hit even if only one of the diagnostic fragment ions could be found in the acquired spectrum.

The risk of false-negative samples will always be present when one is searching for compounds that have not been subject to specific evaluation of LOI, which is the case for the majority of the compounds in the PCDL. In addition, the instrument response has been shown to fluctuate to some extent during the period of data acquisition. Due to the relatively high LOIs and low recoveries among the synthetic cannabinoids in the validation, the risk of false negatives appears to be more likely in this group. It should also be emphasised that the two large NPS groups—cathinones and phenetylamines—were left out of this study to limit the extent of investigated compounds.

Application of the method on our data files has shown that the identification of molecular ions that were not selected for fragmentation (category 2) clearly requires a manual re-evaluation. The list of category 2 findings was significantly longer than that of category 1 findings; however, 35 potential positives out of 1314 data files is a manageably low number. The peak area threshold of $5x10^4$ was important to keep the number of potential category 2 findings low, but will, at the same time, result in higher detection limits for these compounds

compared to category 1 compounds. A review of the data files from the LOI experiments shows that a peak area of $5x10^4$ generally corresponds to two- or threefold the concentration of the LOI of category 1 substances (see Table S2 in the supporting material). These data also indicate that a mass match score of 95 is achieved for most compounds when a peak area around $5x10^4$ is measured. All except one of the potential category 2 findings could be disproved after a careful evaluation of RTs and signal-to-noise ratios in the chromatogram. The need for manual evaluation of category 2 findings is a limitation of the DDA approach. If DIA had been used, there would have been few presumable findings where the MS-only signal was detected but no fragment ions were available. DIA, on the other hand, is limited by co-eluting compounds being fragmented at the same time, resulting in complicated high-energy spectra. Co-eluting compounds that share the same fragments can further complicate the interpretation. DDA generates MS/MS spectra from a known precursor which minimizes the risk of "contaminating" fragments from co-eluting compounds. On the other hand, there is a limit to the number of co-eluting precursors that can be isolated and fragmented. Both DDA and DIA methods are improved by selective chromatography that can resolve closely eluting compounds. The many category 2 findings also show the importance of having the fragmentation information in order to carry out efficient retrospective analysis. Re-analysis of case samples was not possible in this study due to ethical restrictions. Consequently, the presumable category 2 finding of phenibut could not be confirmed. In real forensic casework, the sample could have been re-analysed with a targeted MS/MS method in which the precursor ion of phenibut is prioritized for fragmentation experiments. If a match with a library spectrum was achieved, the finding would have been of category 1.

5.4. General discussion

In all three studies, the strengths and benefits of LC-QTOF-MS as an analytical tool in drug analysis were demonstrated. A targeted method similar to the screening in **Study I** based on LC-MS/MS would have performed as well as the presented method did when it comes to quantification and specificity. However, there are some advantages to using LC-MS/MS, including more straightforward data processing and interpretation compared to LC-QTOF-MS. This is beneficial in a routine setting with high sample throughput. Moreover, the size of the LC-MS/MS data files generated is small compared to that of LC-QTOF-MS. On the other hand, **Study I** showed that the increased flexibility of LC-QTOF-MS compared to LC-MS/MS made it possible to quickly include new compounds in order to determine which parent drug had been ingested. Generally, an unlimited number of compounds can be searched for in data from LC-QTOF-MS, but the level of confidence of the findings is dependent on what identification information is available. The ability to search data retrospectively was also important in this context.

The strategy in **Study II** express the necessity for HR-MS data for the identification of molecular formulas of the potential metabolites in the extracts and authentic urine sample. High mass accuracy and resolving power are the prerequisites for the identification of compounds by LC-QTOF-MS. Calculating the molecular formula from a measured mass results in several candidates, while including isotopic pattern and isotopic abundance in the algorithm (mass match score) removes > 95% of false candidates (142). Secondly, structure elucidation is far better when performed on MS/MS spectra acquired with HR-MS instrumentation as compared to unit resolution instruments (e.g., LC-MS/MS). The strategy in **Study III** shows that the high-resolution MS and -MS/MS spectra available in the LC-QTOF-MS data files is decisive to carry out the retrospective search. However, the sensitivity of the method might be inadequate for certain drugs occurring at low concentrations.

The three different applications in this thesis produce different levels of confidence for the compounds identified (see Table 6). In **Study I**, level 1 is achieved through the second injection of the sample. In this method, a standard is always analysed together with the unknown samples for RT comparison, and a match with library MS/MS spectrum is required. In forensic analysis, the principle of two separate sample preparations is central and two independent analyses are required. In **Study II**, completely new compounds were detected,

which means that no reference standards were available for comparison. Level 2 is, therefore, the highest level of confidence that can be achieved (see Table 6). The retrospective approach in **Study III** produces two different categories of findings depending on whether or not MS/MS spectra information is acquired. Category 1 findings are of level 2, but if there is RT compliance with a reference standard, the level increases to somewhere between 2 and 1. Category 2 findings can be classified only as level 4.

Table 6: Data processing workflows in the three studies and level of confidence achieved, based on the criteria proposed by Schymanski et al. (56).

Study	Peak picking and identification	Identification information	Level of confidence[1]
I	1st injection: Quantitative method	RT and monoisotopic mass (ID criteria[2] I)	Level 2
	1st injection: Find by Formula	RT and mass match score (ID criteria II)	Level 2
	2nd injection: Find by Auto MS/MS and library search	MS/MS (ID criteria III)	Level 1
II	Find by formula; database of possible metabolites	Manual elucidation of MS/MS spectra	Level 2
III	Find by formula and library search	Mass match score and MS/MS (Category[3] 1) plus RT	Level 2 (1)
		Mass match score (Category 2)	Level 4

Abbreviations: ID = identification; RT = retention time
[1]Level of confidence as explained in Table 1
[2]Identification criteria defined in paragraph 3.5.2
[3]Compounds with different identification information, defined in paragraph 3.5.2

5.5. Application and further perspectives

There has been a decrease in new SCs appearing in Europe every year since 2014, and very few seizures of SCs are currently registered in Norway by KRIPOS. A reason for this can be that the nature of these drugs—including problems with dosing, side effects, and uncertainty of content—makes them unattractive. In a study of a Norwegian internet forum dedicated to sharing knowledge on recreational drug use, a three-phase situation for the SCs over seven years was described (143). The first phase was enthusiastic and the second phase was characterized by growing scepticism; in the third phase, members of the community were rejecting these new drugs based on negative reviews from users. There is, nevertheless, a continuing need for analyses detecting SCs in biological samples in the future. However, having reference material for the correct metabolite available in order to maintain a completely up-to-date urinary screening method, similar to the one applied in **Study I**, is probably not manageable.

Oral fluid (OF) is an alternative matrix to urine that retains the benefits of non-invasive and easy sampling, though its targets are the parent compounds, such as in a blood analysis (144). Relevant reference substances of the parent compound will be available on the market much earlier than their respective relevant metabolites. As the doses of SCs are low due to their high potency, and as the degree of transfer from blood to OF is unknown but most likely limited, low detection limits are needed to avoid false-negative test results. The detection times for drugs in general are also shorter in OF than in urine (92). Several published methods utilize different sampling equipment, preparation techniques, and MS configurations for the analysis of SCs in OF (145-152). If LC-QTOF-MS is used, a crowdsourced database as described in **Study III** can be applied, and enable detection of potentially new compounds for which reference standards are not yet available for purchase.

From 2009 to 2018, EMCDDA registered 49 new synthetic opioids, of which 34 were fentanyl derivatives (4). In Norway, prescribed opioids are the most frequent cause of death in drug-induced cases (153). However, toxicological and clinical laboratories face challenges when attempting to detect these new compounds in different matrices. Only a small fraction of fentanyl is excreted as a parent compound, 85% is excreted within 72 h in feces and urine, mainly as metabolites (154, 155). Similar metabolic patterns are likely for its analogues, and a prudent selection of target metabolites is decisive in order to identify the drug intake. A

comprehensive knowledge base from in vitro studies (like **Study II**) of several similar compounds will reveal patterns that can be important tools for predicting the metabolism of future new drugs analogues. In addition, software that uses pattern recognition techniques together with hand-made rules or machine learning algorithms to identify the points of the molecule that are subject to metabolic changes and the potential products (so-called in silico studies), can be used. The presumed metabolites—and, ideally, their predicted diagnostic fragment ions (in MS)—can be added to databases used in un-targeted screening methods and used for the detection of potential new drugs in urine at an early point.

The strategy developed in **Study III** was carried out using a library of 375 unique compounds from three different classes of NPS. Application of the strategy to a large number of files demonstrated that data processing time was not the time-limiting factor; broadening the search by including even more groups of NPS (e.g., synthetic cathinones and phenethylamines) would be feasible. On the HighResNPS.com website, the complete database (1536 compounds with 783 having fragment data) is now available in PCDL format (accessed April 9, 2020). However, a more extensive library will provide an increased number of tentative findings that have to be evaluated. The retrospective approach is also important as a quality control tool. In a situation in which new compounds are constantly introduced to the drug market, laboratories that perform comprehensive screenings will inevitably have problems continuously updating their methods. Carrying out a study as described here with old data files will provide valuable information about whether the laboratory has been able to keep the screening method up to date. The approach can also be applied to specific cases if new information appears.

Large differences between countries have been observed throughout the years of emerging attention to NPS. In Norway, a dramatic number of 27 deaths associated with the amphetamine-like substance PMMA (paramethoxymethamphetamine) was registered from 2010 to August 2012 (156). Several other NPS have been identified in autopsy cases and are considered to be the cause of death (31, 138, 157-159). The number of NPS-related deaths is still relatively low in Norway as compared to, e.g., Sweden, where the number of cases related in particular to synthetic opioids has been high, with 40 cases of acrylfentanyl in 2016 (160), seven cases of furanylfentnyl from November 2015 to March 2016 (161), and nine cases of AH-7921 in 2013 (162). The actual prevalence of NPS in the population is unknown, as the detection of these drugs in post mortem samples only display a small part. There is also

a challenge in the interpretation of post mortem drug concentrations related to the cause of death, as the potency of the NPS is often unknown (163). It can only be speculated as to why the numbers of cases observed in these two countries differ to such an extent. Does it reflect the true prevalence in the countries or are there significant differences in the analytical methods applied in Swedish and Norwegian forensic laboratories? By performing retrospective studies like the strategy presented in **Study III**, it becomes possible to reveal whether previously missed NPS could be hidden among the numerous masses generated from endogenous and exogenous constituents in the sample, provided the method has sufficient sensitivity.

A data file acquired using LC-QTOF-MS can be thought of as a digitalization of the physical sample, though the results from **Study III** have shown that a definitive confirmation of new drugs is not possible without re-analysing positive samples together with reference standards. For a precise estimation of the concentration, the sample must be analysed in sequence with a series of calibrators and controls. Increased use of HR-MS in forensic settings requires large and secure data storage capacities, such that files can be stored for the period required by the national legislation. The ethical aspects of searching for compounds, both endogenous and exogenous (drugs and other chemicals), not related to the original request must also be addressed.

Artificial intelligence—and, more specifically, machine learning—can offer new potential for untargeted analysis of the large data sets acquired using HR-MS instrumentation. A promising proof-of-concept study has demonstrated that a machine learning model trained with raw data from DIA LC-QTOF-MS analysis were able to classify blank samples and samples containing drugs (164). Though further steps has to be taken to actually identify substances, this can lead to a new approach towards performing untargeted analyses of new drugs in a routine setting.

6. Conclusions

In clinical and toxicological investigations, laboratories seek to identify and quantify all relevant drugs present in humans, including NPS. Synthetic opioids and SCs are potent drugs, and sensitive screening methods are important. To cover a broad range of metabolites of SCs with different physio-chemical properties in **Study I**, relatively universal sample preparations and chromatographic conditions were chosen. This strategy has proven to be beneficial when the panel of drugs included need to be updated. However, compromises in method design that effects analytical performance are inevitable. The validation showed acceptable performance, but for some analytes the matrix effects and recoveries achieved were outside acceptance criteria and the method was thus regarded as semi-quantitative for these. By optimizing the sample preparation and chromatographic conditions, the validations results might have been improved for these compounds but at the expense of others. The method was applied to 1,000 authentic samples from subjects undergoing drug treatment programs, in which metabolites were confirmed in 2.3 %. Interpretation of the analytical results revealed that careful selection of metabolites in the method is decisive if a specific drug within a class need to be determined. This is a challenge as the availability of commercially synthesized metabolites is limited. Interpretation is further complicated by the release of structurally similar compounds that give rise to metabolites identical to those already present in the method. The ability to retrospectively process data files from previously analysed samples may reveal additional important metabolites that later can be confirmed and included in the method. The targets in a method like this have to be adjusted according to the drugs used and the current legislation in order to keep them relevant.

When a previous unknown NPS appears on the market, very little is known about its pharmacology, including its metabolic pathways, and consequently which metabolites could be expected to be present in biological samples, in particular urine. This is of special importance for drugs that undergo extensive metabolism. Metabolite profiling by hepatocytes and LC-QTOF-MS as demonstrated in **Study II**, is a relatively cost-effective and straightforward tool to gain such knowledge. The fentanyl analogues ortho-, meta-, and para-fluorofentanyl each formed fourteen metabolites. These findings were in accordance with previous studies on similar fentanyl analogues and included the *N*-dealkylation product norfluorofentanyl which was the most abundant, an *N*-oxide, hydroxylated and methylated metabolites. The three analogues produced metabolites by the same principal metabolic

pathways. However, there were differences in both absolute chromatographic peak areas and RT for several metabolites, possibly attributed to the location of the fluorine atom. Metabolites discovered in an in vitro study may differ from those formed in humans and the results must therefore, if possible, be supported by findings in authentic urine samples. In this study only one sample from a person with confirmed intake was available. Norfluorofentanyl, a monohydroxylated metabolite and a hydroxymethoxy metabolite were detected. These results are crucial for selection of suitable targets for synthesis of reference standards. Furthermore, molecular formulas and corresponding diagnostic fragment ions can be added to databases for tentative identification in samples analysed with HR-MS instrumentation.

The strategy presented in **Study III** demonstrated that data files from LC-QTOF-MS can be re-evaluated to identify NPS that were unknown or not included in the method applied when the original analyses were performed. Identification information, including diagnostic fragment ions, of SCs, synthetic opioids, and designer benzodiazepines were taken from a crowd-sourced database and integrated in a new library. The re-evaluation of data files from 1314 forensic post mortem samples resulted in five new findings with the highest degree of confidence possible with a retrospective approach, requiring retention time and MS/MS resemblance. The number of new findings was lower than expected and were mainly found in data files from the first half of the time period investigated. This indicates that our laboratory has been able to keep the method fairly up to date throughout the investigated period. Furthermore, the application is not limited to retrospective analyses, but can easily be applied for use as a supplement to the standard screening method.

From the discussions of strengths and limitations of these strategies it can be concluded that LC-QTOF-MS or other equivalent HR-MS based techniques provide essential information to toxicological or clinical investigations of new emerging NPS in biological samples.

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RESEARCH ARTICLE

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Screening, quantification, and confirmation of synthetic cannabinoid metabolites in urine by UHPLC–QTOF–MS

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Abstract

Synthetic cannabinoids are one of the most significant groups within the category new psychoactive substances (NPS) and in recent years new compounds have continuously been introduced to the market of recreational drugs. A sensitive and quantitative screening method in urine with metabolites of frequently seized compounds in Norway (AB-FUBINACA, AB-PINACA, AB-CHMINACA, AM-2201, AKB48, 5F-AKB48, BB-22, JWH-018, JWH-073, JWH-081, JWH-122, JWH-203, JWH-250, PB-22, 5F-PB-22, RCS-4, THJ-2201, and UR-144) using ultra-high pressure liquid chromatography–quadrupole time of flight–mass spectrometry (UHPLC–QTOF–MS) has been developed. The samples were treated with ß-glucuronidase prior to extraction and solid-phase extraction was used. Liquid handling was automated using a robot. Chromatographic separation was achieved using a C18-column and a gradient of water and acetonitrile, both with 0.1% formic acid. Each sample was initially screened for identification and quantification followed by a second injection for confirmation. The concentrations by which the compounds could be confirmed varied between 0.1 and 12 ng/mL. Overall the validation showed that the method fulfilled the set criteria and requirements for matrix effect, extraction recovery, linearity, precision, accuracy, specificity, and stability. One thousand urine samples from subjects in drug withdrawal programs were analyzed using the presented method. The metabolite AB-FUBINACA M3, hydroxylated metabolite of 5F-AKB48, hydroxylated metabolite of AKB48, AKB48 *N*-pentanoic acid, 5F-PB-22 3-carboxyindole, BB-22 3-carboxyindole, JWH-018 *N*-(5-hydroxypentyl), JWH-018 *N*-pentanoic acid, and JWH-073 *N*-butanoic acid were quantified and confirmed in 2.3% of the samples. The method was proven to be sensitive, selective and robust for routine use for the investigated metabolites.

KEYWORDS

high resolution mass spectrometry, synthetic cannabinoids, urine screening

1 | INTRODUCTION

Synthetic cannabinoids (SCs) are a group of cannabinoid receptor agonists produced as alternatives to Δ-9-tetrahydrocannabinol (THC), the main psychoactive compound in cannabis. The first SCs were synthesized to investigate the endogenous cannabinoid system and to explore potential new pharmaceuticals.[1] In 2008, an increasingly popular recreational drug containing the SC JWH-018 [1-naphthyl(1-

pentyl-1H-indol-3-yl)methanone] was identified.[2] Since then, legislation has evolved to criminalize the trafficking and use of this class of compounds in many countries. At the same time, though, these legislative activities have acted as a motive to produce new compounds not covered by the current legislations. In the last decade, this "race" has resulted in an increasing number of new SCs entering the market for recreational drugs. As one of the most important classes of new drugs, the ability to find and determine SCs in biological samples is important on an individual level (abuse, toxicity, law enforcement) as well as a social level (drug market trends, extent of trafficking).

Urinary screening methods of SCs based on immuno assay or chromatography with mass spectrometry (MS) detection, in particular liquid chromatography (LC) with quadrupole tandem-MS (MS/MS) detection, have dominated in the toxicological laboratories.[3] Used for analyses of a definite number of compounds, these techniques are a good choice due to their robustness, sensitivity, and selectivity. However, these methods can only identify the compounds they are designed for, and updates are not easily performed. A number of quantitative screening methods in urine by LC–MS/MS have previously been published.[4-8] High resolution mass spectrometry (HRMS) with quadrupole time of flight (QTOF) instrumentation that acquires full spectrum data is not limited by scan/dwell times, and introducing new masses/formulas to the method will not affect the detection of the previously included ones. In addition, retrospective analysis of previously acquired data can be performed. Few articles have previously been published exploring quantitative screening of SCs using HRMS, although the technique has more frequently been used solely for qualitative targeted and non-targeted methods.[9-11] In a non-targeted method, ideally all MS spectra plus additional MS/MS spectra are acquired for a tentative identification, and can be obtained from findings of interest after sample acquisition. The method presented in this article can be described as a dynamic quantitative and targeted screening method since MS data from the first injection are used for quantification purposes while MS/MS data for confirmation are acquired in a second injection only for confirmation of a definite panel of analytes. By this approach the targets included in the method can be adjusted in accordance to the current drugs of interest. Potential disadvantages using HRMS instrumentation are the higher cost compared to LC–MS/MS and the large size of data files generated. In addition, an efficient processing of the data requires powerful computers.

In comparison with blood, advantages of detecting metabolites of drugs of abuse in urine include the expanded detection window and the non-invasive sampling. Quantification of metabolites can be valuable when a recent intake needs to be distinguished from residual drug excretion from a former intake. This principle is well known after intake of cannabis, and various algorithms have been developed for this purpose.[12-14] For synthetic cannabinoids some data exist on the urinary pharmacokinetics and excretion rate of the metabolites of JWH-018 and JWH-073,[6,15] whereas for other compounds, very little is known. Thus, for synthetic cannabinoids more data are needed before a recent intake can be unequivocally distinguished from residual drug excretion. Nevertheless, gathering data from quantitative analyses of the various metabolites in serial urinary samples is a prerequisite for developing the algorithms needed. Moreover, the access of quantitative methods is crucial in order to carry out pharmacokinetic studies (ie, to estimate half-lives, peak concentrations and detection times in urine). However, the low concentrations of unconjugated metabolites in urine often require cleavage of the glucuronidated metabolites by hydrolysis before analysis. In previously published identification and quantification assays, preparation techniques varying from simple dilution,[6] salting-out liquid–liquid extraction (LLE)[10] and traditional LLE[4] to more complex procedures including supported liquid extraction[9] and solid-phase extraction (SPE)[5] have been used. To simplify sample preparation, automatization of this procedure has become more common.[5,6,10]

All SCs undergo metabolism to a certain extent.[16] Consequently, a screening method for SCs in urine must cover the most abundant and unique metabolites if an accurate determination of the drug taken is necessary. Some SCs that are biotransformed to metabolites which are unique and unambiguously can point out the specific drug ingested. However, compounds with close structural similarities often result in several identical metabolites, but in many cases also unique secondary metabolites are produced. One such example is AM-2201 and JWH-018, both having the major metabolites JWH-018 *N*-pentanoic acid and JWH-018 *N*-(5-hydroxypentyl). Nevertheless, the specific markers AM-2201 *N*-(4-hydroxypentyl) and AM-2201 *N*-(6-hydroxyindole) of AM-2201 and JWH-018 *N*-(4-hydroxypentyl) of JWH-018 are also formed and can be used to distinguish between intake of these two.[17,18] A careful selection of metabolites is therefore required. New SCs that are biotransformed to metabolites identical to a drug that already is covered by a method are frequently introduced. Consequently, the exact intake cannot be confirmed without updating the method with new available unique markers. The introduction of AMB-FUBINACA which gives the same metabolite as AB-FUBINACA is an example of the latter.[19]

Reference standards are necessary for performing quantification. It is both a time-consuming and a resource-demanding process from the time a new drug is introduced on the market to the point when selected metabolites have been synthesized and can be included in a new or updated method. Potential metabolites can be identified by exposing human liver microsomes[20,21] or human hepatocytes[22] to the drug in question, and analyze the residues with MS, together with urinary samples from people with known consumption of the same drug.

The aim of the present study was to develop a high throughput quantitative screening method for SCs in urine, using LC–QTOF–MS and automated sample preparation. To evaluate the feasibility of the method in clinical practice, we also aimed to describe our experience and results from analyzing a total of 1000 consecutive routine urinary samples sent to our laboratory where screening for SCs had been requested.

2 | MATERIALS AND METHODS

The analytes included in this method consisted of commercially available and assumed relevant metabolites of the SCs most frequently used in Norway at the time the method was developed. The seizure statistics from the Norwegian National Criminal Investigation Service (KRIPOS) were used to choose relevant SCs. A complete list of the metabolites included, formulas, monoisotopic masses, CAS numbers, IUPAC names, and structures is given in the Supporting Information (Table S1).

2.1 | Chemicals and reagents

Metabolite reference standards of JWH-018 *N*-pentanoic acid, JWH-073 *N*-butanoic acid, JWH-122 *N*-pentanoic acid, JWH-203 *N*-pentanoic acid, JWH-210 *N*-pentanoic acid, JWH-081 *N*-pentanoic acid, JWH-250 *N*-pentanoic acid, AM-2201 *N*-(5-hydroxyindole), AB-PINACA COOH, AB-FUBINACA M3 and the isotope labeled d4-JWH-250 *N*-pentanoic acid and d4-JWH-018 *N*-pentanoic acid were purchased as solutions from Chiron (Trondheim, Norway). 5F-PB-22 3-carboxyindole, 5F-AKB48 *N*-(4-hydroxypentyl), AB-CHMINACA 3-carboxyindazole, AB-CHMINACA M1A, AB-CHMINACA M2, AB-PINACA *N*-pentanoic acid, AKB48 *N*-(4-hydroxypentyl), AKB48 *N*-(5-hydroxypentyl), AKB48 *N*-pentanoic acid, BB-22 3-carboxyindole, AM-2201 *N*-(4-hydroxypentyl), JWH-018 *N*-(5-hydroxypentyl), JWH-210 *N*-(5-hydroxyindole), JWH-210 *N*-(5-hydroxypentyl), PB-22 3-carboxyindole, PB-22 *N*-(4-hydroxypentyl), PB-22 *N*-pentanoic acid, RCS-4 *N*-(4-hydroxypentyl)phenol, THJ-2201 *N*-pentanoic acid, UR-144 *N*-(4-hydroxypentyl), UR-144 *N*-5-hydroxypentyl, UR-144 *N*-pentanoic acid, and d5-UR-144 *N*-(5-hydroxypentyl) were from Cayman Chemicals (Ann Arbor, MI, USA). LiChrosolve® hypergrade LC–MS quality of acetonitrile and methanol in addition to LiChrosolve® water were from Merck (Darmstadt, Germany). ARISTAR® formic acid was from VWR Chemicals (Oslo, Norway). Ammonium acetate of LC–MS grade was from Sigma Aldrich (St Louis, MO, USA) and β-glucuronidase stock solution (Helix promatia) was purchased from Roche Diagnostics (Mannheim, Germany).

2.2 | Preparation of solutions

Stock solutions of the reference compounds were prepared and further diluted and combined into five different working solutions. One set was prepared for calibrators and one set for quality controls (QCs). Calibrators and QCs were prepared by fortifying blank urine with the working solutions and stored at 4°C. An overview of the calibration levels, QCs, and distribution of metabolites in working solutions are given in the Supporting Information (Table S2). A solution of internal standards was prepared by diluting stock solutions in 20% methanol (*v/v*) in water to a concentration of 100 ng/mL d4-JWH-250 *N*-pentanoic acid and d4-JWH-018 *N*-pentanoic acid and 50 ng/mL d5-UR-144 *N*-(5-hydroxypentyl). The buffer for sample pretreatment of 30.8 g/L ammonium acetate was prepared by dissolving the salt in water. A solution of β-glucuronidase containing 25 000 units/mL was prepared from a stock solution. Needle wash was made from methanol/acetonitrile/isopropanol/water/formic acid (25:25:25:23:2, *v/v*).

2.3 | Authentic samples

The method was applied on a total of 1000 consecutive routine urinary samples sent to our laboratory for which screening for SCs had been requested. These samples originated from subjects in whom an intake of SCs was suspected, mainly patients enrolled in medication-assisted treatment programs for drug dependence and patients undergoing other forms of treatment for drug dependence. The samples were received from all over Norway and were collected through 2014 and in the first half of January 2015. At arrival at the laboratory, these samples were principally analyzed with a routine targeted LC–MS/MS method covering JWH-018 *N*-pentanoic acid, JWH-073 *N*-butanoic acid, JWH-122 *N*-pentanoic acid, JWH-203 *N*-pentanoic acid, JWH-210 *N*-pentanoic acid, JWH-081 *N*-pentanoic acid, JWH-250 *N*-pentanoic acid, and AM-2201 *N*-(5-hydroxyindole). This method has previously been described in a publication but then with focus only on JWH-018 *N*-pentanoic acid and JWH-073 *N*-butanoic acid.[6] The collection and storage of the samples selected for subsequent analysis with the present method was approved from the Regional Committee of Medical and Health Research Ethics in Mid Norway (approval No. 2014/2281). As these samples had to be anonymized prior to analysis in accordance to the approval given by the Ethics Committee we were precluded from comparing the results of these two methods.

In a subsample containing specimens from five patients who had tested positive for JWH-018 *N*-pentanoic acid and/or JWH-073 *N*-butanoic acid by the targeted LC–MS/MS method described,[6] a separate approval from the Regional Committee of Medical and Health Research Ethics in Mid Norway (approval No. 2014/737) and individual consent from each patient made it possible to compare the results from that method with the present. From these patients, originating from the same drug rehabilitation clinic and having their samples collected over a short period of time after suspected drug use,[6] a total of 27 samples were available.

2.4 | Method optimization

The method optimization aimed at developing a general method that could detect the relatively diverse group of metabolites and also include new, similar metabolites as they become available. Different sample preparations techniques, LC conditions, and MS settings were explored and the optimization process revealed several methodical issues and challenges. An extraction based on supported liquid extraction, SLE+ from Biotage (Uppsala, Sweden) and SPE HLB PRiME from Waters were compared. The SPE resulted in better sample clean-up and compound recovery. The HLB solid phase consisted of a water-wettable combined hydrophilic and lipophilic polymer. This sorbent did not require conditioning and equilibrating steps, which resulted in a fast throughput and provided to some degree a more convenient protocol and was therefore chosen.

An evaporation and reconstitution step was required and two evaporation temperatures (30°C or 50°C) and reconstitution solvents (20/80 and 50/50 (*v/v*) mobile phase A/B) were tested to minimize the loss of compounds in these steps. Highest recovery was found with evaporation at 30°C and reconstitution in 20/80 (*v/v*) mobile phase A/B. Initially the eluates were collected in a well plate of plastic but this material introduced contaminants interfering with the analysis. This was most noticeable using ethyl acetate as eluent in the SLE+ process. Contaminants were avoided when plastics were replaced by a well plate consisting of glass vials.

As most SCs undergo phase II metabolism with conjugation, for example to glucuronic acid[16] a hydrolysis step was required before analysis. Hydrolysis efficiency and reproducibility was tested using different conditions: 10, 25, or 30 μL of Helix promatia extract (25,000 units/mL) was added to samples fortified with 500 ng/mL of JWH-018 *N*-pentanoic acid glucuronide and UR-144 *N*-(5-

hydroxypentyl) glucuronide and incubated for one or two hours at 60°C. The efficiency of hydrolysis was determined by measuring the glucuronide and hydrolysis product in treated and untreated samples. Using 25 or 30 µL extract gave the same effective hydrolysis when incubated for 1 hour, and 25 µL was therefore chosen to minimize the contribution of enzyme to the matrix.

The chromatographic conditions achieving the best separation of isomers with identical fragmentation patterns, such as AKB48 *N*-(5-hydroxypentyl) and AKB48 *N*-(4-hydroxypentyl), as well as separating as many of the analytes as possible from endogenous compounds, was found by testing three different columns, C18, phenyl-hexyl and biphenyl, in combination with different mobile phase set-ups and gradients. A C18 column and a linear gradient were chosen.

In general, urine as a matrix results in high background and potential interferences affecting the continuous measurement of two lock masses maintaining the high degree of mass accuracy achieved by the LC–QTOF–MS system. Interference was observed close to *m/z* 121.0509 which is monitored together with *m/z* 922.0098 as lock masses to control mass accuracy. This resulted in a high mass error in certain spectra. Instead of using high resolution mode which compromises the dynamic range an alternative lock mass, *m/z* 118.0863 from trimethylglycine ([M + H]+) were chosen.

2.5 | Sample preparation

All pipetting operations were performed using a Tecan Freedom Evo pipetting robot (Tecan, Männedorf, Switzerland). Urine sample, calibrator, or QC in aliquots of 600 µL was pipetted into a 2-mL 96-well plate. Volumes of 20 µL internal standard solution, 600 µL ammonium acetate and 25 µL β-glucuronidase were added and the plate was incubated for 1 h at 60°C. After cooling to ambient temperature, 1000 µL of the sample was transferred to a Waters Oasis® HLB PRiME 30 mg HLB 96-well plate (Wexford, Ireland) SPE. A positive pressure processor (Waters, Milford, MA, USA) was used to gently push the sample and the following reagents through the packing material. The SPE material was washed with 1000 µL water and 1000 µL of 10% methanol (*v/v*) in water in sequence following elution twice with 500 µL 10% methanol (*v/v*) in acetonitrile. The eluate was collected in a rack of 96 glass vials in a tray with well plate foot print (J.G. Finneran Associates Inc., Vineland, NJ, USA) and dried completely under air at 30°C prior to reconstitution with 400 µL 80/20 mobile phase A/B (*v/v*).

2.6 | Instrumentation

Instrumental analysis was performed using a 6550 QTOF-MS (Agilent, Santa Clara, CA, USA) with electrospray ionization (ESI) and iFunnel interface coupled with a 1290 Infinity UHPLC system from Agilent. Mobile phase A and B consisted of 0.1% formic acid in water and acetonitrile, respectively, and separation was achieved using a Zorbax Eclipse Plus C18 Rapid Resolution HD column (2.1x100 mm, 1.8 µm) from Agilent maintained at 60°C. A linear gradient with a flow of 0.30 mL/min starting at 10% mobile phase B increasing to 50% in 2 minutes, continuing to 60% in the next 6 minutes and further increasing to 95% in 1 minute was employed. This condition was maintained for 3 minutes and before the next injection the initial condition was held for 2 minutes, giving a total cycle time of 14 minutes.

Positive ionization was used with the fragmentor voltage at 375 V, capillary voltage at 3500 V, gas temperature at 150°C, gas flow at 15 L/min, nebulizer pressure at 20 psig and sheath gas temperature at 380°C. The following settings were applied for the iFunnel interface: Exit direct current of 40 V and radio frequency high pressure and low pressure at 150 V and 100 V, respectively.

All samples were first analyzed by injecting 5 µL and using the MS-only mode acquiring full-scan data in low mass range (1700 *m/z*) at a scan rate of 2 Hz and the detector in 2 GHz extended dynamic range giving a resolution (m/Δm at FWHM) of approx. 20,000 at *m/z* 322.0481. Presumably positive samples based on the two first identification criteria described in Section 2.7, were then injected once again with an injection volume of 10 µL using a targeted MS/MS mode with a list of precursors for acquiring MS/MS spectra. A collision energy of 10, 20, or 40 eV was applied to each precursor based on previous experiments to get a collision induced dissociation (CID) spectrum containing fragments and traces of the precursor. In this mode the instrument cycles between acquiring MS scans and MS/MS scans both in a rate of 6 Hz and with the detector in 4 GHz high resolution state (resolution of approx. 30,000 at *m/z* 322.0481). The computer controlling the instrument was equipped with the MassHunter Acquisition software (Acq) B.05.01 (Agilent, Santa Clara, CA, USA).

2.7 | Library spectra

CID spectra were added to the in-house library according to Broecker et al.[23] This procedure involved diluting individual 1 mg/mL stock solutions of SCs in methanol to 100 ng/mL and then 1 µL was injected on a guard column with 0.1% formic acid in water and 0.1% formic acid in acetonitrile (50:50) as mobile phase. Three CID spectra of the protonated compound using collision energies of 10, 20, and 40 eV were acquired.

The acquired CID spectra were transferred to the library file using MassHunter Qualitative software (Qual) B.07.01 and MassHunter PCDL Manager B.07.01 (Agilent). In this process the fragment masses in every spectrum were corrected to their theoretical masses. Fragments with intensities lower than 1% of the most abundant mass in each spectrum were deleted.

2.8 | Quantification and confirmation of compounds

Quantification and confirmation of the compounds was done by two injections where the first was using MS-only and the second was using targeted MS/MS. Three identification criteria (ID criteria I, II, and III) with increasing degree of confidence was used. All data files of samples, calibrators and QCs from the first injection were first processed using the MassHunter Quantitative software (Quant) B.07.01. The compounds were identified based on accurate monoisotopic mass and retention time (RT) (ID criterion I). The instrument settings in the first injection gave the widest dynamic range and 20 spectra per peak which are sufficient for quantification. Calibration curves based on peak area ratios of analyte to internal standard at each concentration level were formed using linear least square regression employing 1/x or $1/x^2$ as weighting factor. Results of the processed data presented by the software were manually reviewed and a sample was presumed positive if above the limit of quantification (LOQ) as defined in Section

2.9.1 and additionally gave a mass match score ≥ 80 in Qual software, using profile data and "Find by Formula" (ID criterion II). This score was based on accurate mass and isotopic pattern from a database of the analytes, and only the compounds with a mass error of ±15 parts per million (ppm) and a deviation of ±0.15 minutes from the RT given in the database were considered. The mass match score was calculated using the following equation:

$$\text{Mass match score} = \frac{(w_{mass} \times \text{Accuracy score}) + (w_{abundance} \times \text{Abundance score}) + (w_{spacing} \times \text{Spacing score})}{(w_{mass} + w_{abundance} + w_{spacing})} \quad (1)$$

The accuracy was weighted (w) 100, abundance was weighted 80 and isotope spacing was weighted 50.

A threshold mass match score of 80 out of 100 was chosen based on experience through method development and gave only a few presumable positive findings that were not confirmed.

In case of presumable positive findings, the MS/MS spectra acquired in a second injection were compared with a spectral library holding reference CID spectra for all the compounds in the target list obtained at 10, 20, and 40 eV. This identification was done by processing the data using the Qual software tool "Identify Compounds" and the option "Search Library." The numbers of matching and non-matching fragments and the mass accuracy of the fragments were the criteria in the identification of the compound. A score ≥ 80 out of 100 was regarded as a definite identification (ID criterion III). An example of a positive library comparison is given in Figure S1. The minimum concentration in spiked negative samples which fulfilled this most stringent criterion was defined as the limit of confirmation (LOC). This approach may result in a quantitative finding in the first assumption but the sample ending up negative after the second injection if the LOC was higher.

2.9 | Method validation

LOQ, linearity, selectivity, RT stability, carry-over, matrix effects, recovery, precision, accuracy, and stability are parameters recommended to evaluate during method validation for forensic applications.[24] All these parameters were included in the validation and the number of calibration levels, parallels and analytical runs as well as acceptance limits are described in the following paragraphs.

2.9.1 | Limit of quantification and limit of confirmation

LOQ was first evaluated for each analyte by spiking blank urine to different concentration levels (0.01–5 ng/mL). The lowest concentration level giving reproducible results when analyzed at 10 days with precision (CV) < 20% and accuracy within 80%–120% of the theoretical value was defined as LOQ.

LOC was defined as the lowest concentration identified by the library search identification criteria (ID criterion III). A serial dilution of spiked urine was first analyzed to estimate this limit. Blank urine from different individuals was then spiked at three or four concentration levels equal to and around the estimated LOC (in the range of 0.01–5 ng/mL). The concentration level where the compound was identified in all urines using criterion III was set to the LOC.

2.9.2 | Linearity

The linear range of every compound was explored by using the analyzed calibrators from the first four days of validation (all days within a week) at six calibration levels (except AB-PINACA pentanoic acid, AB-CHMINACA M1A, and RCS-4 *N*-(4-hydroxypentyl)phenol where five levels were used) in a linear least square regression employing 1/x or $1/x^2$ weighting and reported as the correlation coefficient R^2. The concentration range was defined from LOQ to highest calibration concentration. $R^2 \geq 0.990$ was regarded as accepted.

2.9.3 | Selectivity

The selectivity of the method was evaluated by spiking 10 different blank urines (creatinine concentrations 34–249 mg/dL) with a mix of 28 drugs of abuse or their corresponding metabolites commonly observed in the samples sent to the laboratory for screening for drugs of abuse. The drugs were amphetamine, methamphetamine, 3,4-methylenedioxymethamphetamine (MDMA), ephedrine, 3,4-methylenedioxyamphetamine (MDA), para-methoxy-N-methylamphetamine (PMMA), para-methoxyamphetamine (PMA), codeine, oxycodone, morphine, methadone, tramadol, O-desmethyl-tramadol, ethylmorphine, 6-monoacetylmorphine, buprenorphine, fentanyl, methadone, desmethyl-diazepam, hydroxy-alprazolam, 7-amino-nitrazepam, 7-amino-clonazepam, 7-amino-flunitrazepam, benzoylecgonine, ritalinic acid, ketamine, zolpidem, and 11-nor-9-carboxy-Δ^9 THC (THC-COOH).

2.9.4 | Retention time stability

The stability of RT and relative RT (ratio of analyte RT to internal standard RT) was monitored through an analytical sequence of minimum 14 hours at three random validation days. The deviation of RT and relative RT in QC samples through the sequence to the average RT of the calibrators in the beginning of the run was calculated. RT deviation ≤1% throughout an analytical sequence up to 14 hours was accepted.

2.9.5 | Carry-over in the LC system

The carry-over from a high concentration sample to the next was determined by injecting blank urine after a sample containing a concentration equal to its highest calibration level or at least 125 ng/mL. A carry-over <20% of LOQ was accepted.

2.9.6 | Matrix effects

To estimate the matrix effect (ME) reconstitution reagent (A) (80/20 mobile phase A/B (v/v)) and 10 extracted blank urines (B) was fortified with all compounds and analyzed to acquire the analyte signal. ME (%) was calculated as [area of B/area of A] x 100%. A value below 100% is indicative of ion suppression and a value above 100% is indicative of ion enhancement. ME values in the interval 75%–125% were regarded

as acceptable for quantification of compounds lacking a dedicated isotopically marked internal standard.

2.9.7 | Recovery

The extraction efficiency was estimated by comparing the signal in six blank urines fortified with all compounds after extraction (B) to the signal in the same samples fortified to the identical concentration level before extraction (C). Internal standards were added in the same amount to all samples after extraction. Recovery was calculated as [area of compound relative to internal standard in C/area of compound relative to internal standard in B] x 100%. Recoveries ≥75% were regarded as acceptable for quantification.

2.9.8 | Precision and accuracy

The intra-day precision was determined by analyzing 10 parallels of two concentration levels in the same sequence. The inter-day precision was calculated by analyzing one sample at two different concentration levels at 10 different days over a period of five weeks. The acceptance criterion of intra- and inter-sequence precision at both concentration levels was a CV ≤ 15%. The average value of the inter-day data was used to calculate the accuracy expressed as the deviation from theoretical/nominal value. The acceptance criterion of accuracy was values in the interval 85%–115%.

2.9.9 | Stability

The stability of the compounds was tested at different temperature conditions in spiked QC samples stored in glass tubes at one concentration level. Spiked QC samples were stored in darkness at 4°C to simulate the standard storage conditions from receiving a sample to its analysis. QC samples were analyzed after seven and 14 days. In addition QC samples were stored for three and five days at 25°C in darkness to simulate typical conditions during transport from the sampling location to the laboratory. Stored samples at 4°C and 25°C were analyzed together with freshly thawed samples and relative changes in concentration were reported. In addition the stability of extracted samples in the autosampler at 10°C was re-tested at three and seven days. An interval of three days covers the maximum time that can be experienced between first and second injection as there can be a delay between the first injection via processing and the second injection. The seven-day period was included to explore the time frame for a typical postponement due to e.g. instrument failure.

3 | RESULTS

A quantitative UHPLC–QTOF–MS screening method of 35 SC metabolites with a run time of 14 minutes was achieved. A second injection with the same run time was required for confirmation by acquiring MS/MS-spectra for library search.

3.1 | Method validation

The validation parameters were within the set criteria and requirements for the majority of analytes. However, high matrix effects and insufficient recoveries question the ability to accurately quantify 14 of the investigated analytes and therefore the method must consider being semi-quantitative for these compounds (Table 1).

3.1.1 | Chromatographic separation

Ideally the LC set-up should manage to separate all compounds with identical masses and similar MS/MS spectra. The chromatogram of calibrator 2 containing all metabolites included in the method is displayed in Figure 1. As can be observed, several compounds elute in clusters, but these co-eluting compounds are not isomers of each other and were separated based on their masses. The choice of chromatographic column, mobile phases and gradient made it possible to separate the isomeric pairs of the hydroxylated metabolites of AKB48, AM-2201, JWH-210, and UR-144. The isomers PB-22 *N*-(4-hydroxypentyl) and PB-22 *N*-(5-hydroxypentyl), though, could not be baseline separated. The isomers PB-22 *N*-(4-hydroxypentyl) and PB-22 *N*-(5-hydroxypentyl), though, could not be baseline separated. PB-22 *N*-(4-hydroxypentyl) which eluted first and is a more specific marker of PB-22 intake was kept, whereas PB-22 *N*-(5-hydroxypentyl) was excluded from the calibrators. Thus, the calibration was done based on peak height. As baseline separation was not achieved this must be regarded as semi-quantification.

3.1.2 | Limit of quantification and limit of confirmation

The lowest concentrations detected using the different ID criteria are given in the Supporting Information (Table S3). The LOQs and LOCs of the metabolites are summarized in Table 1. AB-PINACA pentanoic acid could not be confirmed by the library search at any of the levels explored. BB-22 3-carboxyindole could not be confirmed at the level of 17.5 ng/mL due to poor fragmentation and interferences in the MS/MS spectra. However, the metabolite AB-PINACA-COOH which showed an LOC of 2 ng/mL could be used as an alternative indicator for an intake of AB-PINACA, although this is also a metabolite of AMB.[25]

3.1.3 | Linearity

The LOQ and the highest calibration level for each analyte (highest limit of quantification, HLOQ) define the concentration range of the method. Correlation coefficients, LOQs and HLOQs for all compounds included in the method are given in Table 1. The correlation coefficients were above 0.990 except for RCS-4 *N*-(4-hydroxypentyl) phenol, AB-FUBINACA M3, AM-2201 *N*-(5-hydroxyindole), JWH-018 *N*-(5-hydroxypentyl), THJ-2201 *N*-pentanoic acid, JWH-210 *N*-(5-hydroxyindole), JWH-210 *N*-(5-hydroxypentyl) and JWH-210 *N*-pentanoic acid. JWH-210 *N*-5-hydroxyindole showed reduced linearity and calibration level six was excluded resulting in a less broad concentration range (1.2–72 ng/mL; ie, about 50-fold) compared to what was expected from the method optimization.

3.1.4 | Selectivity and retention time stability

Urine fortified with a mixture of 28 drugs of abuse did not give any false positive results, and the analysis identified no peaks within the

TABLE 1 Retention time (RT), limit of confirmation (LOC), limit of quantification (LOQ), highest limit of quantification (HLOQ), linearity (R^2), and precision (intra- and inter-sequence) for 35 metabolites of synthetic cannabinoids in urine. The analytes are sorted after retention time. ID refers to the numbers in Figure 1. n = number of parallels. [SEMI] = method is semi-quantitative. QC = quality control. CV = coefficient of variation

Metabolite	ID	RT min.	Can Originate From Intake Of:	LOC ng/mL	LOQ ng/mL	HLOQ ng/mL	R^2	QC Low ng/mL	QC High ng/mL	Intra-sequence CV (%) (*n* = 10)		Inter-sequence CV (%) (n = 10)	
										low[c]	high[d]	low[c]	high[d]
AB-PINACA pentanoic acid	1	3.1	AB-PINACA or 5F-AB-PINACA	– [a]	10	320	0.9927	20	200	6.4	4.0	9.5	7.3
AB-CHMINACA M1A[SEMI]	2	3.2	AB-CHMINACA	10	10	320	0.9908	20	200	3.2	2.4	6.9	5.7
RCS-4 *N*-(4-hydroxypentyl)phenol[SEMI]	3	3.5	RCS-4	10	5.0	160	0.9859	10	200	5.5	2.3	7.7	6.1
AB-FUBINACA M2[SEMI]	4	3.6	AB-FUBINACA	12	2.0	240	0.9909	20	200	5.1	4.8	6.6	7.1
5F PB-22 3-carboxyindole	5	4.1	5F-PB-22 or 5F-MDMB-PICA	5	1.0	120	0.9950	8.4	67.0	3.4	4.2	8.6	6.0
RCS-4 *N*-pentanoic acid	6	4.3	RCS-4	1.0	0.25	60	0.9940	0.5	50.0	16	4.0	10	6.1
PB-22 *N*-pentanoic acid[SEMI]	7	4.4	PB-22 or 5F-PB-22	2.5	0.25	50	0.9644	0.5	25.0	7.1	4.0	7.6	2.4
JWH-250 *N*-pentanoic acid	8	4.6	JWH-250	0.25	0.125	60	0.9936	0.5	50.0	3.2	2.1	11	10
PB-22 *N*-(4-hydroxypentyl)[SEMI]	9	4.7	PB-22	0.5	0.25	50	0.9970	1.0	50.0	3.1	4.1	5.3	4.0
JWH-073 *N*-butanoic acid	10	5.1	JWH-073 or JWH-018	0.5	0.125	60	0.9959	0.5	50.0	2.2	2.1	2.7	2.5
JWH-203 *N*-pentanoic acid	11	5.1	JWH-203	0.5	0.25	60	0.9963	0.5	50.0	2.2	2.8	6.6	2.7
PB-22 3-carboxyindole	12	5.3	PB-22 or CBL-018	12	1.0	120	0.9973	2.0	100	3.3	2.1	9.3	2.3
AB-FUBINACA M3[SEMI]	13	5.4	AB-FUBINACA, AMB-FUBINACA or EMB-FUBINACA	0.5	0.5	120	0.9836	4.3	45.0	2.5	2.5	5.0	2.1
AB-CHMINACA 3-carboxyindazole[SEMI]	14	5.4	AB-CHMINACA or AMB-CHMINACA	2.5	0.25	50	0.9957	0.5	22.5	8.6	2.4	7.3	4.5
JWH-018 *N*-pentanoic acid	15	5.4	JWH-018 or AM-2201	0.5	0.125	60	0.9970	0.5	50.0	2.6	2.2	7.1	4.1
AM-2201 *N*-(4-hydroxypentyl)[SEMI]	16	5.7	AM-2201	0.1	0.2	50	0.9962	0.5	25.0	2.5	2.1	5.6	4.6
JWH-018 *N*-(5-hydroxypentyl)[SEMI]	17	5.7	JWH-018 or AM-2201	0.25	0.25	50	0.9827	0.5	25.0	6.1	3.3	8.3	4.8
JWH-081 *N*-pentanoic acid	18	5.8	JWH-081	0.5	0.25	60	0.9951	0.5	50.0	3.1	3.1	4.8	12
AM-2201 *N*-(5-hydroxyindole)[SEMI]	19	6.0	AM-2201	0.25	0.25	60	0.9855	0.5	50.0	4.4	3.8	8.5	9.1
JWH-122 *N*-pentanoic acid	20	6.1	JWH-122 or MAM-2201	0.5	0.25	60	0.9957	0.5	50.0	3.7	3.1	10	8.6
THJ-2201 *N*-pentanoic acid[SEMI]	21	6.2	THJ-2201 or THJ-018	0.5	0.25	50	0.9867	0.5	22.5	6.7	3.2	17	4.3
BB-22 3-carboxyindole	22	6.4	BB-22, MDMB-CHMICA or ADB-CHMICA	17.5	2.0	240	0.9941	20	200	2.6	2.3	5.2	6.4
JWH-122 *N*-(5-hydroxypentyl)[SEMI]	23	6.6	JWH-122 or MAM-2201	0.5	0.25	50	0.9938	0.5	25.0	4.3	2.6	15	7.5
AB-PINACA COOH[SEMI]	24	6.7	AB-PINACA or AMB	1.0	1.0	120	0.9914	2.0	100	2.3	1.9	3.4	2.7
UR-144 *N*-pentanoic acid	25	6.8	UR-144 or XLR11	0.2	0.25	50	0.9926	0.5	25.0	3.4	1.8	9.7	2.7
JWH-210 *N*-pentanoic acid[SEMI]	26	7.2	JWH-210	0.25	0.25	30	0.9894	0.5	25.0	2.2	4.5	19	14
UR-144 *N*-(5-hydroxypentyl)	27	7.3	UR-144 or XLR11	0.1	0.1	50	0.9941	0.5	25.0	2.8	2.0	4.7	2.0
UR-144 *N*-(4-hydroxypentyl)	28	7.5	UR-144	0.1	0.1	50	0.9945	0.5	25.0	2.6	2.2	4.7	1.3
AKB48 *N*-pentanoic acid	29	7.7	AKB48 or 5F-AKB48	0.1	0.1	50	0.9980	0.5	25.0	2.9	2.4	5.8	3.9
JWH-210 *N*-(5-hydroxypentyl)[SEMI]	30	7.8	JWH-210	1.0	0.25	50	0.9814	0.5	25.0	6.3	3.8	12	13

(Continues)

TABLE 1 (Continued)

Metabolite	ID	RT min.	Can Originate From Intake Of:	LOC ng/mL	LOQ ng/mL	HLOQ ng/mL	R^2	QC Low ng/mL	QC High ng/mL	Intra-sequence CV (%) (n = 10)		Inter-sequence CV (%) (n = 10)	
										low[c]	high[d]	low[c]	high[d]
AB-CHMINACA M2	31	8.1	AB-CHMINACA or AMB-CHMINACA	1.0	1.0	50	0.9938	2.0	100	2.6	1.6	6.3	4.7
5F-AKB48 *N*-(4-hydroxypentyl)	32	8.2	5F-AKB48	0.04	0.1	120	0.9957	0.5	25.0	2.7	2.2	4.4	1.4
AKB48 *N*-(4-hydroxypentyl)	33	8.5	AKB48	0.1	0.1	25	0.9924	0.5	25.0	2.3	2.0	6.3	3.1
AKB48 *N*-(5-hydroxypentyl)	34	8.7	AKB48 or 5F-AKB48	0.1	0.1	25	0.9937	0.5	25.0	3.4	2.1	5.0	2.8
JWH-210 *N*-(5-hydroxyindole)[SEMI]	35	10.0	JWH-210	2.0	1.2	72	0.9376	2.0	– [b]	12	– [b]	17	– [b]

[a]Not determined.

[b]QC High ended up outside of the linear range and data of intra- and inter-sequence precision are therefore left out.

[c]Refers to the concentration shown in the QC Low column.

[d]Refers to the concentration shown in the QC High column.

retention time windows fulfilling the identification criteria of any of the metabolite compounds.

The acceptance criteria were met for both RT and relative RT for all analytes with the exception of RCS-4 *N*-pentanoic acid and PB-22 *N*-(4-hydroxypentyl), which in some sequences displayed a deviation up to 2%.

3.1.5 | Carry-over in LC system

No carry-over above 20% of LOQ after injecting a sample containing 125 ng/mL or the highest calibration level of AB-PINACA pentanoic acid (320 ng/mL), AB-CHMINACA M1A (320 ng/mL), RCS-4 *N*-(4-hydroxypentyl)phenol (160 ng/mL), and AB-FUBINACA M2 (240 ng/mL). This was achieved using a needle wash of eight seconds between sample draw and injection.

3.1.6 | Precision and accuracy

Precision expressed as relative standard deviation (%) and accuracy data expressed as bias (%) are given in Tables 1 and 2, respectively. The acceptance criterion of intra-sequence precision (≤ 15%) at both concentration levels was achieved for all analytes. The acceptance criterion of inter-sequence precision (≤ 15%) was achieved for all analytes except JWH-210 *N*-(5-hydroxyindole) (17%), JWH-210 *N*-pentanoic acid (19%) and THJ-2201 *N*-pentanoic acid (17%) at low concentration. The accepted accuracy of 85%–115% was achieved for all compounds except AB-FUBINACA M2 (84%), BB-22-3-carboxyindole (79%), JWH-210 *N*-pentanoic acid (131%), and JWH-210 *N*-(5-hydroxyindole) (119%) at low concentrations; AB-PINACA pentanoic acid (119%), AB-CHMINACA M1A (117%) and AM-2201 *N*-(5-hydroxyindole) (121%) at high concentrations; and AB-FUBINACA M3 at both low and high concentrations (119% and 135%, respectively). The QC high of JWH-210 *N*-(5-hydroxyindole) of 100 ng/mL was outside of the linear range and data of precision and accuracy of this level were therefore left out.

3.1.7 | Matrix effects and recovery

MEs from 57% to 262% were observed (Table 2). In general, the compounds eluting early and midway through the gradient were most influenced by the matrix. There was a relatively good agreement between MEs observed at low and high concentrations. The compounds showing the highest degree of ion suppression were AB-CHMINACA M1A (57%), PB-22 *N* pentanoic acid, PB-22 *N*-(4-hydroxypentyl) (63%) and RCS-4 *N*-(4-hydroxypentyl)phenol (74%). The compounds showing the highest degree of ion enhancement were AM-2201 *N*-(5-hydroxyindole), AB-FUBINACA-M2 and THJ-2201 *N*-pentanoic acid (220% - 262%). JWH-122 *N*-(5-hydroxypentyl, AB-PINACA COOH, AM-2201 *N*-(4-hydroxypentyl), AB-FUBINACA-M3, and AB-CHMINACA 3-carboxyindazole had somewhat less ion enhancement (133%–175%). The remaining 23 compounds were within the acceptance criterion. The level chosen for estimation of the ME at low concentrations for AB-PINACA pentanoic acid, AB-CHMINACA M1A, RCS-4 *N*-(4-hydroxypentyl)phenol, AB-FUBINACA-M2, and BB-22 3-carboxyindole gave a signal too weak to calculate an ME value.

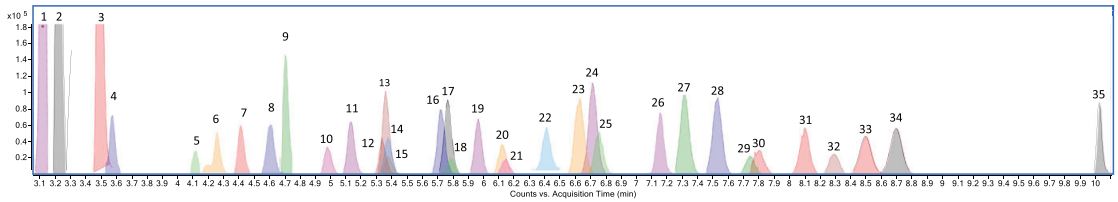

FIGURE 1 Chromatogram of calibrator 2 containing the 35 metabolites of the synthetic cannabinoids in urine. The numbers corresponds to the ID numbers shown in Table 1

TABLE 2 Accuracy, matrix effects and recovery for the 35 metabolites of synthetic cannabinoids in urine. n = number of parallels. For concentrations of QC Low and QC High, see Table 1

Metabolite	Accuracy (n = 10)		Matrix Effects (n = 10)				Recovery (n = 6)			
			QC Low		QC High		QC Low		QC High	
	QC Low %	QC High %	%	CV (%)	%	CV (%)	%	CV (%)	%	CV (%)
AB-PINACA pentanoic acid	102	119	–[a]	–[a]	123	119	98	13	105	33
AB-CHMINACA M1A	95	117	–[a]	–[a]	57	59	106	4	105	12
RCS-4 *N*-(4-hydroxypentyl)phenol	103	112	–[a]	–[a]	74	40	103	2	108	10
AB-FUBINACA M2	84	111	–[a]	–[a]	228	63	105	8	87	15
5F PB-22 3-carboxyindole	92	100	101	24	88	14	106	6	103	22
RCS-4 *N*-pentanoic acid	95	115	88	33	108	27	106	7	99	11
PB-22 *N*-pentanoic acid	96	103	64	18	63	15	106	4	103	11
JWH-250 *N*-pentanoic acid	97	108	75	12	78	10	108	7	104	7
PB-22 *N*-(4-hydroxypentyl)	96	108	62	15	72	12	101	5	98	8
JWH-073 *N*-butanoic acid	95	108	90	7	97	5	102	6	98	11
JWH-203 *N*-pentanoic acid	97	104	100	6	115	7	104	8	102	10
PB-22 3-carboxyindole	93	106	101	24	103	6	95	10	98	11
AB-FUBINACA M3	119	135	115	19	156	16	108	7	102	8
AB-CHMINACA 3-carboxyindazole	92	112	106	24	133	11	105	5	106	5
JWH-018 *N*-pentanoic acid	107	100	94	33	117	7	98	9	95	11
AM-2201 *N*-(4-hydroxypentyl)	98	98.9	146	15	175	12	99	6	97	9
JWH-018 *N*-(5-hydroxypentyl)	107	108	83	19	84	16	84	9	84	11
JWH-081 *N*-pentanoic acid	106	102	114	18	123	9	91	12	97	9
AM-2201 *N*-(5-hydroxyindole)	105	121	149	17	262	13	74	9	86	7
JWH-122 *N*-pentanoic acid	102	104	95	19	112	19	83	13	84	14
THJ-2201 *N*-pentanoic acid	101	113	195	22	220	28	96	9	96	9
BB-22 3-carboxyindole	79	109	–[a]	–[a]	114	10	84	9	93	8
JWH-122 *N*-(5-hydroxypentyl)	102	110	177	28	176	31	70	8	79	6
AB-PINACA COOH	91	113	144	27	143	23	100	9	100	7
UR-144 *N*-pentanoic acid	93	100	121	19	115	12	103	6	101	8
JWH-210 *N*-pentanoic acid	131	102	91	8	99	3	69	18	76	13
UR-144 *N*-(5-hydroxypentyl)	98	102	118	10	118	7	84	5	88	7
UR-144 *N*-(4-hydroxypentyl)	96	101	114	8	117	8	90	8	90	8
AKB48 *N*-pentanoic acid	105	107	100	6	110	3	92	9	93	11
JWH-210 *N*-(5-hydroxypentyl)	103	116	109	6	116	5	51	18	63	9
AB-CHMINACA M2	97	101	95	21	104	4	94	14	94	10
5F-AKB48 *N*-(4-hydroxypentyl)	95	104	112	6	118	5	88	9	88	8
AKB48 *N*-(4-hydroxypentyl)	93	111	102	4	109	4	76	7	79	8
AKB48 *N*-(5-hydroxypentyl)	95	110	111	5	115	8	80	9	82	9
JWH-210 *N*-(5-hydroxyindole)	119	–[b]	89	10	93	5	11	56	17	25

[a]Matrix effect was not estimated at low concentration.

[b]QC High ended up outside of the linear range and data of accuracy are therefore left out.

Recovery was above the accepted limit of 75% for all compounds except JWH-210 *N*-(5-hydroxyindole) (10%) and JWH-210 *N*-(5-hydroxypentyl) (51%) at both concentration levels (Table 2).

3.1.8 | Stability

Concentrations were considered stable when the calculated values of the stored samples were within 20% from the initial concentration measured in the sample. The QC samples stored at 4°C and 25°C were stable (data not shown), with the exception of JWH-210 N-(5-hydroxyindole) for which a decline of 25% was observed after three days of storage at 25°C. Processed samples stored at 10°C showed a decline of more than 20% after three days for JWH-018 *N*-pentanoic acid, d4-JWH-018 *N*-pentanoic acid, JWH-081 *N*-pentanoic acid, AM-2201 *N*-(5-hydroxyindole), JWH-122 *N*-pentanoic acid, BB-22 3-carboxyindole, JWH-122 *N*-(5-hydroxypentyl), JWH-210 *N*-(5-hydroxyindole), JWH-210 *N*-(5-hydroxypentyl), and JWH-210 *N*-pentanoic acid (data not shown).

3.2 | Results of authentic samples

One or more metabolites were quantified and confirmed in 21 of the total of 1000 samples and in two additional samples metabolites were quantified and identified with ID criterion II, giving a frequency of positive findings of 2.3%. A total of seven different metabolites were confirmed and two identified with ID criterion II. Additionally two metabolites were subsequently identified based on new reference substances. A summary of the findings, with suggestions of which drug(s) that have been ingested in each case, is given in Table 3. JWH-018 *N*-pentanoic acid, JWH-018 *N*-(5-hydroxypentyl), and JWH-073 *N*-pentanoic acid were the most frequently confirmed metabolites. JWH-018 *N*-pentanoic acid was confirmed in 13 samples and quantified in a range from 0.5 to 10 ng/mL. JWH-018 *N*-(5-hydroxypentyl) was confirmed in seven samples and quantified from 0.25 to 8.7 ng/mL. JWH-073 *N*-pentanoic acid was confirmed in seven samples and quantified in a range from 0.5 to 12 ng/mL. AKB-48 *N*-pentanoic acid was confirmed in six samples and quantified in a range from 0.28 to 14 ng/mL. AB-FUBINACA M3 was confirmed in six samples and quantified in a range from 1.4 to 2300 ng/mL. 5F-PB-22 3-carboxyindole was identified, but not confirmed, in three samples at a concentration range from 2.5 to 8.9 ng/mL. BB-22 3-carboxyindole was identified, but not confirmed, in one sample at a concentration of 12 ng/mL. In one sample metabolites from three different drugs were confirmed. Metabolites that may originate from more than one drug was confirmed in 17 of 23 samples.

4 | DISCUSSION

4.1 | Method validation

A screening method capable for quantification and confirmation of a variety of SC metabolites at concentrations relevant for clinical and toxicological investigations has been developed. Quantitative screening results are essential when a recent intake needs to be distinguished from residual drug excretion caused by a former intake and repeated samples are available from the same individual.[14] Moreover, the access of quantitative methods is crucial in order to carry out pharmacokinetic studies (ie, to estimate half-lives, peak concentrations, and detection times in urine). The validation of this method demonstrates a satisfactory recovery and selectivity, linearity, precision and accuracy within accepted limits for a majority of the investigated metabolites. No carry-over following injection of high concentration samples was observed with the selected needle wash settings.

However, some limitations need to be acknowledged. Especially early eluting polar compounds suffer from more pronounced MEs, higher LOQs and LOCs, and less precise quantification. Due to poor quality of MS/MS spectra acquired for a few analytes, relatively high concentrations were needed to achieve acceptable library-search scores, with correspondingly high LOCs. Co-eluting isomeric species suppressing or contaminating the MS/MS spectra by introducing additional fragment masses or poor ionization and fragmentation of the precursor can cause these problems. Generally, the LOC is expected to be higher than the LOQ. For AM-2201 *N*-(4-hydroxypentyl), 5F-AKB48 *N*- (4-hydroxypentyl), and UR-144 *N*-(5-hydroxypentyl), however, the opposite was observed. This was due to MS/MS spectra acquired at concentrations lower than LOQ meeting the threshold scores of ID criterion III. Nevertheless, this had no practical impact as levels below LOQ were not confirmed with a second injection and library search.

There are limited data available on the expected concentrations of the different metabolites in urine after recreational use, but a relatively broad range of concentration levels, from under one and up to hundreds of ng/mL, has been reported.[5,7,26] The majority of the analytes have an LOC at 1 ng/mL or below which will be sufficient to confirm them at their presumable levels in urine. The window of detection will obviously be narrower if the LOC is higher. LOC of AB-PINACA pentanoic acid, RCS-4 *N*-(4-hydroxypentyl)phenol, RCS-4-*N*-pentanoic acid, AB-FUBINACA M2, PB-22 3-carboxyindole, and BB-22 3-carboxyindole was up to 50 times higher compared to LOQs presented using LC–MS/MS based methods.[4,6,7,26,27] The majority of these elute early (RTs < 4 minutes) and are more prone to ME as they co-elute with matrix components. Higher LOC values than LOQ values were expected as the LOC is based on a more stringent identification criterion. The LOQ is in most methods based on the signal-to-noise ratio of the quantifier transition together with accuracy of the concentration measurement. In the presented method, the instrument is both acquiring MS and MS/MS which compromise the sensitivity. Other compounds like AKB48 *N*-(4-hydroxypentyl), AKB48 *N*-(5-hydroxypentyl), AKB48 *N*-pentanoic acid, AM-2201 *N*-(4-hydroxypentyl), JWH-018 *N*-(5-hydroxypentyl), JWH-203 *N*-pentanoic acid, JWH-018 *N*-pentanoic acid, JWH-210 *N*-pentanoic acid, JWH-250 *N*-pentanoic acid, UR-144 *N*-5-hydroxypentyl, UR-144 *N*-pentanoic acid, and UR-144 *N*-(4-hydroxypentyl) had an LOC at the same level or even below the LOQ achieved in methods with a comparable panel of analytes based on LC–MS/MS.[4,5,7,28-30]

With the exception of AB-FUBINACA M3, the HLOQs in this method are sufficiently high to encompass the relevant levels in the positive patient samples as well as previous published levels of SCs in urine, without further dilution. In some studies it has been shown that

TABLE 3 List of samples with one or more metabolites above limit of confirmation (LOC)

Sample Number	Metabolite I	Conc. (ng/mL)	Metabolite II	Conc. (ng/mL)	Metabolite III	Conc. (ng/mL)	Metabolite IV	Conc. (ng/mL)	Consistent with Intake of
1	JWH-018 *N*-pentanoic acid	< LOC[a]	JWH-018 *N*-(5-hydroxypentyl)	0.48	JWH-073 *N*-butanoic acid	< LOC[a]	JWH-018 *N*-(4-hydroxypentyl)[b]	–[e]	JWH-018 in a mix with JWH-073 or alone
2	5F-PB-22 3-carboxyindole	4.8							5F-PB-22 or 5F-MDMB-PICA
3	AB-FUBINACA M3	2300	AKB-48 *N*-pentanoic acid	14	AKB-48-hydroxy met.[c]	29[d]	5F-AKB-48-hydroxy met.[b]	–[e]	AB-FUBINACA (or AMB-FUBINACA or EMB-FUBINACA) and 5F-AKB-48
4	AB-FUBINACA M3 BB-22 3-carboxyindole	1400 12	AKB-48 *N*-pentanoic acid	6	AKB-48-hydroxy met.[c]	13[d]	5F-AKB-48-hydroxy met.[b]	–[e]	AB-FUBINACA (or AMB-FUBI9NACA or EMB-FUBINACA), 5F-AKB-48 and BB-22 (or MDMB-CHMICA or ADB-CHMICA)
5	AB-FUBINACA M3	5.2	AKB-48 *N*-pentanoic acid	1	AKB-48 *N*-(5-hydroxypentyl)	0.48	5F-AKB-48-hydroxy met.[b]	–[e]	AB-FUBINACA (or AMB-FUBINACA or EMB-FUBINACA) and 5F-AKB-48
6	AKB-48 *N*-pentanoic acid	1.3	AKB-48 *N*-(5-hydroxypentyl)	0.88	5F-AKB-48-hydroxy met.[b]	–[e]			5F-AKB-48
7	AB-FUBINACA M3	340							AB-FUBINACA or AMB-FUBINACA or EMB-FUBINACA
8	AB-FUBINACA M3	800	AKB-48 *N*-pentanoic acid	0.68	AKB-48-hydroxy met.[c]	18.6[d]	5F-AKB-48-hydroxy met.[b]	–[e]	AB-FUBINACA (or AMB-FUBINACA or EMB-FUBINACA) and 5F-AKB-48
9	5F-PB-22 3-carboxyindole	8.9							5F-PB-22 or 5F-MDMB-PICA
10	AB-FUBINACA M3	1.35	AKB-48 *N*-pentanoic acid	0.28					AB-FUBINACA (or AMB-FUBINACA or EMB-FUBINACA) together with 5F-AKB-48 or AKB-48
11	5F-PB-22 3-carboxyindole	4.9							5F-PB-22 or 5F-MDMB-PICA
12	JWH-018 *N*-pentanoic acid	0.78	JWH-018 *N*-(5-hydroxypentyl)	< LOC	JWH-073 *N*-butanoic acid	0.82	JWH-018 *N*-(4-hydroxypentyl)[b]	–[e]	JWH-018 in a mix with JWH-073 or alone
13	JWH-018 *N*-pentanoic acid	< LOC[a]	JWH-018 *N*-(5-hydroxypentyl)	8.7	JWH-073 *N*-butanoic acid	< LOC[a]	JWH-018 *N*-(4-hydroxypentyl)[b]	–[e]	JWH-018 in a mix with JWH-073 or alone
14	JWH-018 *N*-pentanoic acid	1.6	JWH-018 *N*-(5-hydroxypentyl)	1.6	JWH-073 *N*-butanoic acid	2.2	JWH-018 *N*-(4-hydroxypentyl)[b]	–[e]	JWH-018 in a mix with JWH-073 or alone
15	JWH-018 *N*-pentanoic acid	3.5	JWH-018 *N*-(5-hydroxypentyl)	0.87	JWH-073 *N*-butanoic acid	2.7	JWH-018 *N*-(4-hydroxypentyl)[b]	–[e]	JWH-018 in a mix with JWH-073 or alone
16	JWH-018 *N*-pentanoic acid	10	JWH-018 *N*-(5-hydroxypentyl)	2.3	JWH-073 *N*-butanoic acid	12	JWH-018 *N*-(4-hydroxypentyl)[b]	–[e]	JWH-018 in a mix with JWH-073 or alone
17	JWH-018 *N*-pentanoic acid	< LOC[a]	JWH-018 *N*-(5-hydroxypentyl)	2.0	JWH-073 *N*-butanoic acid	< LOC[a]	JWH-018 *N*-(4-hydroxypentyl)[b]	–[e]	JWH-018 in a mix with JWH-073 or alone
18	JWH-018 *N*-pentanoic acid	< LOC	JWH-018 *N*-(5-hydroxypentyl)	0.32	JWH-073 *N*-butanoic acid	0.57	JWH-018 *N*-(4-hydroxypentyl)[b]	–[e]	JWH-018 in a mix with JWH-073 or alone
19	JWH-018 *N*-pentanoic acid	0.50	JWH-018 *N*-(5-hydroxypentyl)	0.58	JWH-073 *N*-butanoic acid	0.67	JWH-018 *N*-(4-hydroxypentyl)[b]	–[e]	JWH-018 in a mix with JWH-073 or alone

(Continues)

TABLE 3 (Continued)

Sample Number	Metabolite I	Conc. (ng/mL)	Metabolite II	Conc. (ng/mL)	Metabolite III	Conc. (ng/mL)	Metabolite IV	Conc. (ng/mL)	Consistent with Intake of
20	JWH-018 *N*-pentanoic acid	< LOC[a]	JWH-018 *N*-(5-hydroxypentyl)	0.28	JWH-073 *N*-butanoic acid	< LOC[a]	JWH-018 *N*-(4-hydroxypentyl)[b]	–[e]	JWH-018 in a mix with JWH-073 or alone
21	JWH-018 *N*-pentanoic acid	< LOC[a]	JWH-018 *N*-(5-hydroxypentyl)	0.46	JWH-073 *N*-butanoic acid	< LOC[a]	JWH-018 *N*-(4-hydroxypentyl)[b]	–[e]	JWH-018 in a mix with JWH-073 or alone
22	JWH-018 *N*-pentanoic acid	< LOC[a]	JWH-018 *N*-(5-hydroxypentyl)	0.42	JWH-073 *N*-butanoic acid	0.52	JWH-018 *N*-(4-hydroxypentyl)[b]	–[e]	JWH-018 in a mix with JWH-073 or alone
23	JWH-018 *N*-(5-hydroxypentyl)	0.39	JWH-018 *N*-(4-hydroxypentyl)[b]	–[e]					JWH-018

[a]Analyte detected but in a concentration below the LOC.
[b]Based on subsequent identification with additional reference substances.
[c]Hydroxylated on the adamantyl ring.
[d]Based on AKB-48 *N*-(5-hydroxypentyl) calibration.
[e]Not quantified.

the ingestion of JWH-018, JWH-122, JWH-210, AM-2201, UR-144, and AB-PINACA can result in high metabolite concentrations (approximately 200 to above 2000 ng/mL),[5,7,26,31] which are above the upper calibration limits of the method, but such high levels were not observed in the authentic samples in this study. Of the 23 positive samples analyzed, only four samples had levels above the linear range and therefore had to be diluted to achieve a precise quantification. These samples were diluted 1:20 with blank urine and then re-analyzed. The method showed good selectivity indicating that other commonly abused compounds should have no influence on the quantification and confirmation of SCs. RTs were proven to be very stable within a worklist of up to 14 hours and can be used as an important ID criterion. The deviation of up to 2% seen for RCS-4 *N*-pentanoic acid and PB-22 *N*-(4-hydroxypentyl) is within the RT window used in ID criteria and will not compromise the detection and quantification.

The majority of compounds showed MEs and recoveries within the acceptance criteria. A general sample preparation, which was chosen here, can be used for extraction of analytes with a broad spectrum of physico-chemical properties, but a high ME and thereby unfavorable influence on the analytical quality was observed for some compounds. Choosing a sample preparation method that removes matrix more effectively may most likely decrease the MEs but also potentially reduce the recoveries of many of the analytes. The measured MEs outside the accepted range indicate that both ion suppression and ion enhancement occur. Quantifications with corresponding internal standards for all analytes would potentially compensate for the MEs. However, in a screening method this is not easily achieved and a compromise on the analytical quality for certain analytes must be accepted. Moreover, a tendency toward lower recovery for the compounds eluting late indicates that these compounds also are adsorbed strongly on the SPE sorbent. This must be taken in to account when introducing new compounds to the screening method. As a consequence of high MEs, low recoveries and the absence of dedicated isotopically labeled internal standards, the method must be regarded as semi-quantitative for the following analytes: AB-CHMINACA M1A, AB-CHMINACA 3-carboxyindazole, AB-FUBINACA-M2, AB-FUBINACA-M3, AB-PINACA COOH, AM-2201 *N*-(4-hydroxypentyl), AM-2201 *N*-(5-hydroxyindole), JWH-122 *N*-(5-hydroxypentyl), JWH-210 *N*-(5-hydroxyindole), JWH-210 *N*-(5-hydroxypentyl), JWH-210 *N*-pentanoic acid, PB-22 *N*-pentanoic acid, PB-22 *N*-(4-hydroxypentyl), RCS-4 *N*-(4-hydroxypentyl)phenol, and THJ-2201 *N*-pentanoic acid.

Our stability results of processed samples stored at 72 hours and 4°C are not in agreement with those previously reported by Scheidweiler et al, who did not reveal any degradation of the metabolites under investigation after 24 hours in room temperature.[9] Previous studies of the stability and storage of naturally occurring cannabinoids in urine have proven loss of these types of compounds under different conditions.[32-35] In our method, the use of glass materials and the temperature of 10°C can possibly result in a reduction of analyte due to degradation or adherence to the glass surface. Injections should therefore be done directly after processing the urine samples. If samples are injected three or more days after being processed, the response of JWH-018 *N*-pentanoic acid, JWH-081 *N*-pentanoic acid, AM-2201 *N*-(5-hydroxyindole), JWH-122 *N*-pentanoic acid, BB-22 3-carboxyindole, JWH-122 *N*-(5-

hydroxypentyl), JWH-210 *N*-(5-hydroxyindole), JWH-210 *N*-(5-hydroxypentyl), and JWH-210 *N*-pentanoic acid will be lower than freshly prepared samples. This degradation can compromise the quantitative quality of the method.

4.2 | Authentic samples

In the 1000 authentic samples analyzed, a total of 10 different metabolites were confirmed or identified with ID criterion II. The majority of the chosen metabolites in the method can be produced by more than one drug (Table 1) which means that a definite identification of the ingested substance(s) is difficult. However, such a list of substances will probably never cover all possibilities as new derivatives with minor chemical modifications will continue to be synthesized. JWH-018 *N*- pentanoic acid, JWH-018 *N*-(5-hydroxypentyl) and JWH-073 *N*-pentanoic acid can be a result of consumption of both JWH-018 and AM-2201. JWH-018 *N*-(4-hydroxypentyl) is formed after JWH-018 consumption but small amounts of JWH-018 can be produced when smoking AM-2201 which may result in trace levels of JWH-018 *N*-(4-hydroxypentyl).[17,18]

Retrospectively, a reference standard of JWH-018 *N*-(4-hydroxypentyl) was analyzed with the method and acceptable chromatographical separation from the 5-OH isomer was achieved. When samples positive for JWH-018 *N*-(5-hydroxypentyl) were re-investigated also JWH-018 *N*-(4-hydroxypentyl) was confirmed by RT and MS/MS spectrum. JWH-018 *N*-(4-hydroxypentyl) was not quantified but the peak areas were similar to those of JWH-018 *N*-(5-hydroxypentyl) in the same sample. The peak areas in the positive samples show that the two metabolites were formed in similar amounts, indicating that JWH-018 and not AM-2201 was the drug of origin. The concentrations of JWH-018 *N*-pentanoic acid and JWH-073 *N*-pentanoic acid in these samples analyzed by LC–MS/MS have previously been published by our group.[6] In that study, elimination half-lives of these compounds were determined and detection times established based on the LOQs of that method.[6] The relatively high LOCs of JWH-073 *N*-pentanoic acid and JWH-018 *N*-pentanoic acid in the present study as compared to the LOQ of the LC–MS/MS method, which was 0.1 ng/mL, will result in detection times of days instead of weeks.

The pentanoic acid metabolite of AKB48 was detected in six samples. The specific metabolite of 5F-AKB48 hydroxylated at the pentyl chain (5F-AKB48-*N*-(4-hydroxypentyl)) was not detected in any of the samples suggesting that our findings originated from AKB48 and not the 5-fluoro analogue. However, the seizure statistics from KRIPOS indicate that the use of 5F-AKB48 was more frequent than AKB48 at the time of sample collection. Previous studies have showed that both AKB48 and 5F-AKB48 are metabolized to AKB-48 *N*-pentanoic acid and AKB48-*N*-(5-hydroxypentyl).[21,36] Our initial findings could therefore not unambiguously determine which compounds were taken by these individuals.

A retrospective search for the general formula of hydroxylated 5F-AKB48 ($C_{23}H_{30}FN_3O_2$) revealed a peak three minutes earlier than 5F-AKB48-*N*-(4-hydroxypentyl) in five out of the six positive samples. By acquiring CID spectra of this compound the fragmentation pattern could be compared with the literature[21,36] and reveal the structure (Figure 2). The detection of the fragments *m/z* 151.1117 and 133.1012 corresponding to a hydroxylated adamantyl cation $[C_{10}H_{15}O]^+$ and water loss, and not the *m/z* 135.1168 which dominate the spectra when fragmenting the metabolite hydroxylated at the pentyl chain, strongly suggested that the metabolite was hydroxylated at the adamantyl group. Sample #10 had the lowest concentration of AKB48 *N*-pentanoic acid indicating that the absence of a detected hydroxylated metabolite was sensitivity related. Three synthesized metabolites of 5F-AKB48 hydroxylated at the adamantyl group (hydroxy-group in position 3 and both axial and equatorial orientation in position 4) kindly donated by the Department of Forensic Genetics

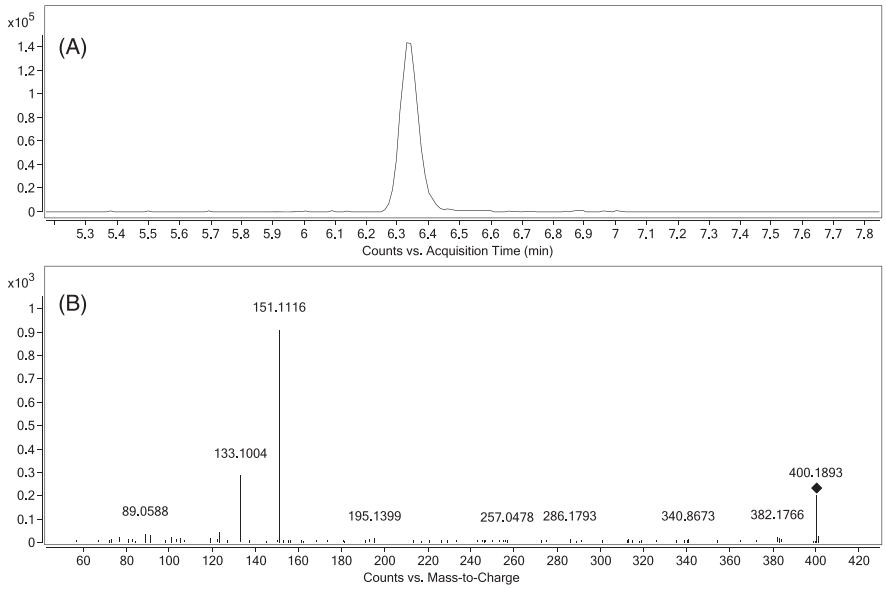

FIGURE 2 A, extracted ion chromatogram of $[C_{23}H_{30}FN_3O_2 + H]^+$. B, a CID-spectrum of the precursor at collision energy of 20 eV

and Forensic Toxicology, National Board of Forensic Medicine (Linköping, Sweden) were analyzed. Chromatographic separation was achieved and RT and fragmentation pattern of the equatorial positioned structure was congruent with the peak detected in the samples. The position of the hydroxyl group on the adamantyl group influenced the fragmentation pattern significantly. The hydroxyl group at position 3 resulted in the proton to seek the carboxamide giving the dominant *m/z* 250.1085 and 233.1350. In position 4 the hydroxyl group is closer to the cleavage which can explain the formation of the dominating adamantyl cation (*m/z* 151.1117 and 133.1012). Chromatographic separation and fragmentation of the three synthesized metabolites are given in Figure 3 and NMR spectra are presented in the Supporting Information (Figure S2).

The detected AKB48-OH metabolite in samples # 3, 4 and 8 eluted slightly earlier than AKB48-*N*-(5-hydroxypentyl), but baseline separation was not achieved. The CID spectra of the precursor ($C_{23}H_{31}N_3O_2$, mono-hydroxylated metabolite of AKB48) at this RT showed a fragmentation pattern typical of the AKB48 metabolite hydroxylated at the adamantyl group while the CID spectra produced at the RT of AKB48-*N*-(5-hydroxypentyl) confirmed the presence of this metabolite as well (Figure 4). Concentration estimation of the metabolite in these samples was based on the calibration curve of AKB48-*N*-(5-hydroxypentyl). The hydroxylated metabolite in samples #5 and #6 was confirmed to be AKB48-*N*-(5-hydroxypentyl), indicating individual differences in the metabolic pathways. The original choice of AKB48 and 5F-AKB48 metabolites was not sufficient for deciding the specific consumption of these drugs. The method allowed a retrospective investigation of metabolites outside of the original panel, which gave us the possibility to confirm the drug of origin to be 5F-AKB48. The absence of AKB48-*N*-(4-hydroxypentyl) in any of the samples supports the theory that AKB48 was not the drug of origin in any of the cases. Sample #10 was the only sample of these where distinguishing between intake of AKB48 or 5F-AKB48 was not possible.

The AB-FUBINACA M3 metabolite was semi-quantified in six samples with a concentration range of 1.35 to 2300 ng/mL. The samples with a concentration above the linear range were diluted 1:20 with blank urine and re-analyzed. A carry-over at this high concentration was not tested during validation, but no carry-over was observed in the samples injected after the samples containing AB-FUBINACA M3. AB-FUBINACA M3 is formed by oxidation of the primary amide producing a carboxylic acid, while M2 is formed by oxidation at the oxobutane moiety. M3 has, in contrast to M2, previously been demonstrated to be one of top three markers of AB-FUBINACA.[20,37] Having

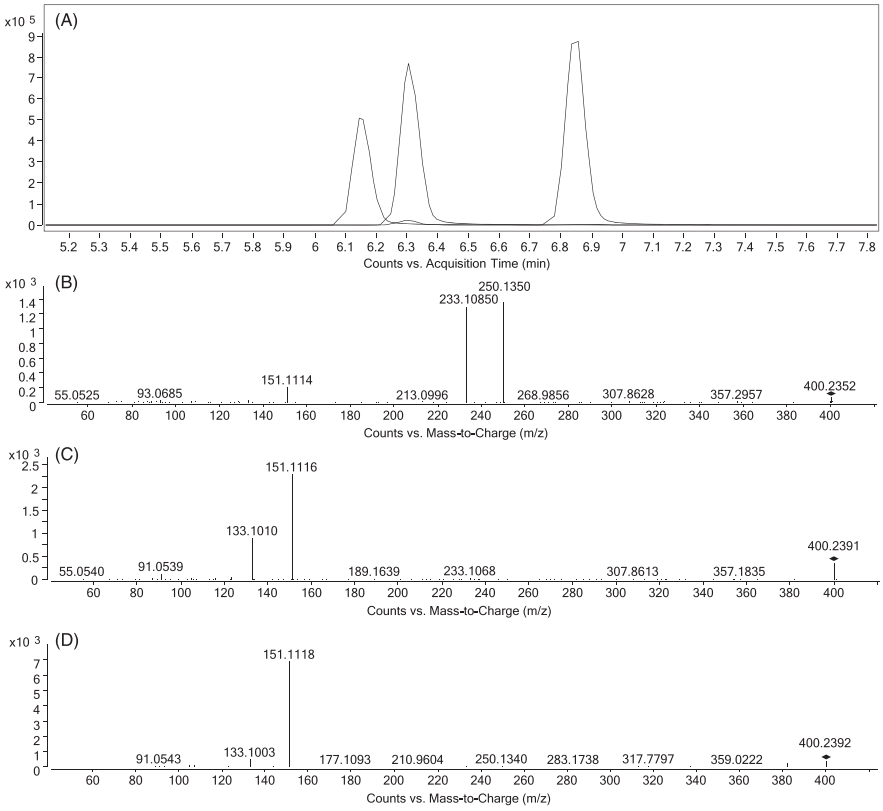

FIGURE 3 A, extracted ion chromatogram of the protonated synthesized metabolites of 5F-AKB48 hydroxylated at different positions at the adamantyl group. B, CID-spectrum of the first eluting compound with hydroxyl-group in position 3. C, CID-spectrum of second eluting compound with hydroxy-group with equatorial orientation in position 4. D, CID-spectrum of third eluting compound with hydroxy-group with axial orientation in position 4. All CIDs with a collision energy of 20 eV

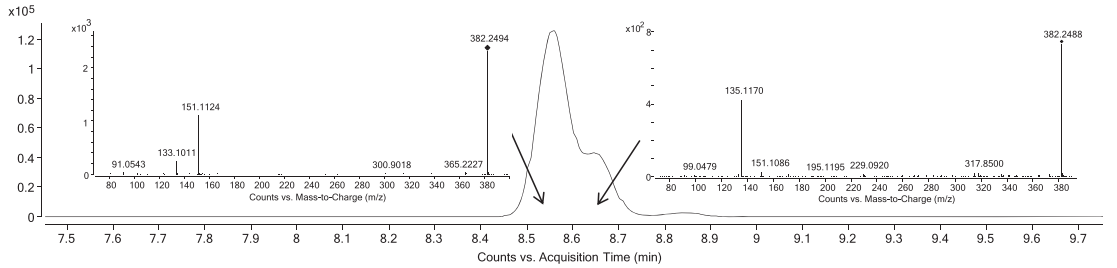

FIGURE 4 Extracted ion chromatogram of hydroxylated AKB48 $[C_{23}H_{31}N_3O_2 + H]^+$ and a CID-spectrum acquired of the precursor from the beginning of the peak and a CID-spectrum from the shoulder of the peak. Both CID-spectrum with a collision energy of 10 eV

AB-FUBINACA M2 as an analyte in the panel and not detecting it is an additional proof to the studies cited above of M2 being a unsuitable marker. AB-FUBINACA itself was not included in the method, but a retrospective search for the formula of this compound returned a positive finding in samples #3, 4, and 8, which were also the samples with the highest concentrations of AB-FUBINACA M3. The more non-polar mother substance was not detected in samples #5, 7, and 10 demonstrating both the extensive metabolism of this compound and the increased detection time when choosing the more polar metabolites as markers. This method is to the best of the authors' knowledge the first published comprehensive screening method containing AB-FUBINACA M3. The results show that including this marker is essential to be able to detect AB-FUBINACA. It must be emphasized, though, that the methyl ester analogue AMB-FUBINACA (also known as MMB-FUBINACA)[19] and the ethyl ester analogue EMB-FUBINACA also can result in AB-FUBINACA M3.

In five of the six samples containing AB-FUBINACA M3 at least one metabolite of 5F-AKB48 was also detected. This can be a result of concomitant intake of either AB-FUBINACA, AMB-FUBINACA or EMB-FUBINACA and 5F-AKB48 from two different products, but it can also be caused by intake of a product containing both drugs either sold as a mix or the one being a contamination of the other. Information from KRIPOS shows that in only one out of 11 AB-FUBINACA seizures 5F-AKB48 was detected in the same product. In two out of 11 seizures of AB-FUBINACA a seizure of 5F-AKB48 was made in the same case. As our samples were anonymized before analysis we could not determine if some of them were from the same individual(s) or from the same geographical area. A corresponding situation was seen with JWH-073, which was always detected when any of the metabolites of JWH-018 were present. A demethylation of JWH-018 to JWH-073 and further oxidation to JWH-073-*N* pentanoic acid has previously been hypothesized and cannot be ruled out.[8]

5F-PB-22 3-carboxyindole could not be confirmed with spectral library in the two samples where a concentration below the LOC (< 5 ng/mL) was observed. The second injection, however, provided MS spectra that strongly indicated the presence of the compound at a concentration > 2.5 ng/mL even though the concentration was too low to be confirmed with ID criteria III. Neither 5F-PB-22 3-carboxyindole nor BB-22 3-carboxyindole are specific markers of 5F-PB or BB-22 intake, respectively. 5F-PB-22 3-carboxyindole can origin from 5F-MDMB-PICA[38] and a biotransformation of MDMB-CHMICA to BB-22 3-carboxyindole can take place.[39] Other specific markers were not available as certified reference materials. In the case of BB-22, the absence of specific metabolites for MDMB-CHMICA and AMB-CHMICA in biological samples must be documented to prove intake of this substance.[40]

In statistics provided by KRIPOS of seizures in Norway in 2014, 5F-AKB48 was at the top with 43 seizures followed by 5F-PB-22, BB-22, AB-FUBINACA and AM-2201 with 15, 15, 11, and 10 seizures respectively. JWH-210, PB-22, UR-144, AKB48, JWH-018, JWH-073, AB-CHMINACA, JWH-122, and JWH-081 were reported in five or fewer seizures. With the present method, metabolites of 5F-AKB48 were found in six samples. In addition we found metabolites of five other SCs or their closely related analogs.

The introduction of new SCs to the global market puts the laboratories in a challenging position. Covering all existing and new SCs in the analytical repertoire is a labor-intensive task, but knowledge of the current situation in a nation and the neighboring countries is a valuable tool to design relevant methods. The statistics of seized drugs of abuse in Norway in recent years show that a couple of new drugs have appeared on the marked. At the same time those dominating in 2014 are still occurring, but at a much lower frequency. This requires a frequent revision of the analytes covered by the method and potentially an addition of new compounds if standards for relevant metabolites become available. With a generic sample preparation and the analytical methodology presented here the addition of new analytes is relatively straight forward with a limited number of validation experiments depending on whether the analyte is added for qualitative or quantitative purposes. Qualitative validation should include experiments to determine LOC, selectivity, retention time, carry-over, and stability of the new compound. For quantitative purposes additional experiments to determine LOQ, ME, recovery, precision, accuracy and linearity should be conducted.

5 | CONCLUSIONS

A UHPLC–QTOF–MS method was developed and validated for quantification and confirmation of 35 metabolites of SCs. The method was based on two injections where the first facilitated the identification and quantification based on full spectra MS data and the second acquired MS/MS data for confirmation. The method showed acceptable performance for its purpose. The sensitivity expressed as LOC was sufficient

to confirm the analytes at their presumable levels in urine with a few exceptions which primarily were caused by matrix effects, low recoveries or interference of MS/MS spectra used for confirmation. As a consequence of matrix effects, low recoveries and linearities below the acceptance criteria, in combination with absence of dedicated isotopically labeled internal standards, the method must be regarded as semi-quantitative for the following analytes: AB-CHMINACA M1A, AB-CHMINACA 3-carboxyindazole, AB-FUBINACA-M2, AB-FUBINACA-M3, AB-PINACA COOH, AM-2201 *N*-(4-hydroxypentyl), AM-2201 *N*-(5-hydroxyindole), JWH-018 *N*-pentanoic acid, JWH-122 *N*-(5-hydroxypentyl), JWH-210 *N*-(5-hydroxyindole), JWH-210 *N*-(5-hydroxypentyl), JWH-210 *N*-pentanoic acid, PB-22 *N*-pentanoic acid, PB-22 *N*-(4-hydroxypentyl), RCS-4 *N*-(4-hydroxypentyl)phenol, and THJ-2201 *N*-pentanoic acid. Presence of AB-PINACA pentanoic acid could not be confirmed by MS/MS-spectra.

Relatively generic method settings were chosen to cover a broad range of analytes. This is an advantage if the panel is to be expanded and updated as new SCs are introduced to the marked, but can also result in compromised analytical performance as were demonstrated by those analytes not meeting the defined validation criteria. The validated method was applied to 1000 authentic samples from subjects undergoing drug treatment programs. Interpretation of the analytical results revealed the need for the method to contain specific urine markers if the exact compounds have to be decided. This is a challenge as the availability of commercially synthesized metabolites is limited and the constant release of structurally similar compounds which are biotransformed to metabolites identical to analytes already present in the method. As shown by the presented method, retrospectively processing previously analyzed samples based on new information can detect additional important metabolites that later can be confirmed and included in the method. The presented method is an approach to the analytical challenges that the evolving drug market brings. The targets in the method have to be adjusted according to the drugs used and the current legislation.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

How to cite this article: Gundersen POM, Spigset O, Josefsson M. Screening, quantification, and confirmation of synthetic cannabinoid metabolites in urine by UHPLC–QTOF–MS. *Drug Test Anal.* 2018;1–17. https://doi.org/10.1002/dta.2464

Supporting information – Paper I

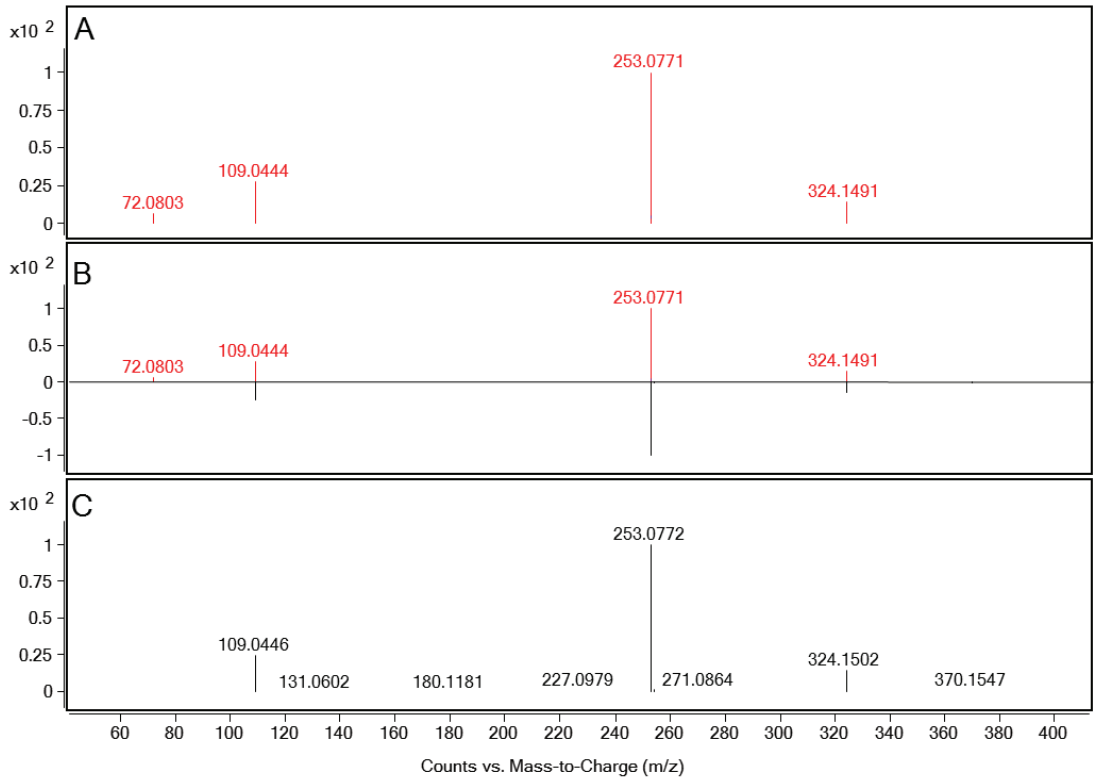


Figure S1: Acquired CID of AB-FUBINACA M3 in positive sample (A) and the library CID of AB-FUBINACA M3 (C). (B) is a comparison of the two.

Supporting material Figure S2-A1 to S2-C3: 1H, 13C and 19F-NMR spectra were recorded on a Varian Mercury 300 MHz instrument at 25 °C in CDCl3, MeOH-d4 or acetone-d6.

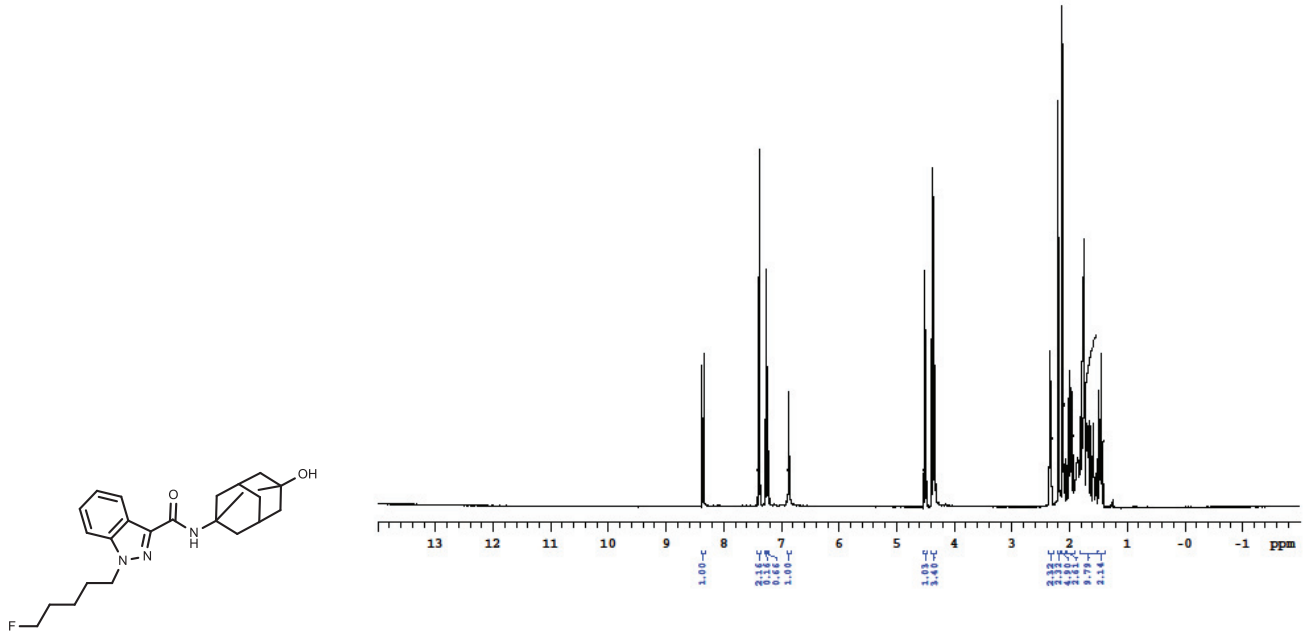


Figure S2-A1: Structure and ^{1}H-NMR Spectrum of 5F-AKB48 with hydroxy-group in position 3, Solvent: $CDCl_3$

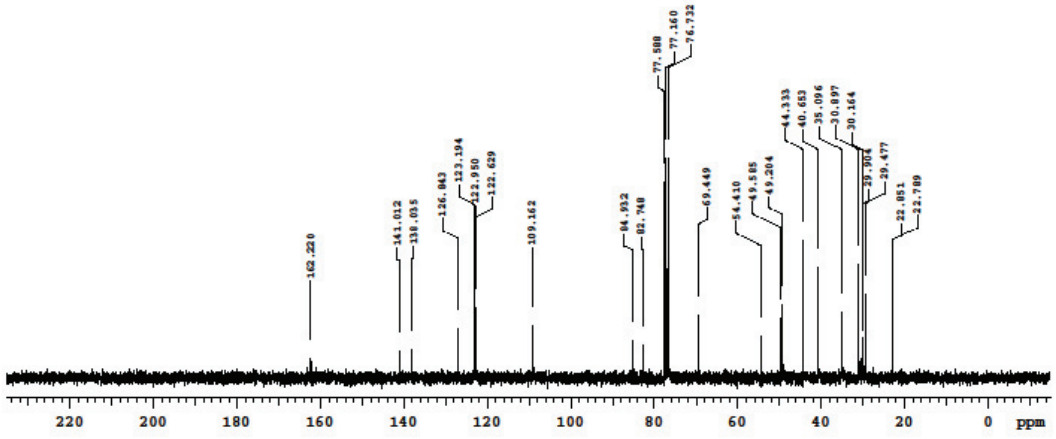


Figure S2-A2: ^{13}C-NMR Spectrum of 5F-AKB48 with hydroxy-group in position 3, Solvent: Solvent: $CDCl_3$

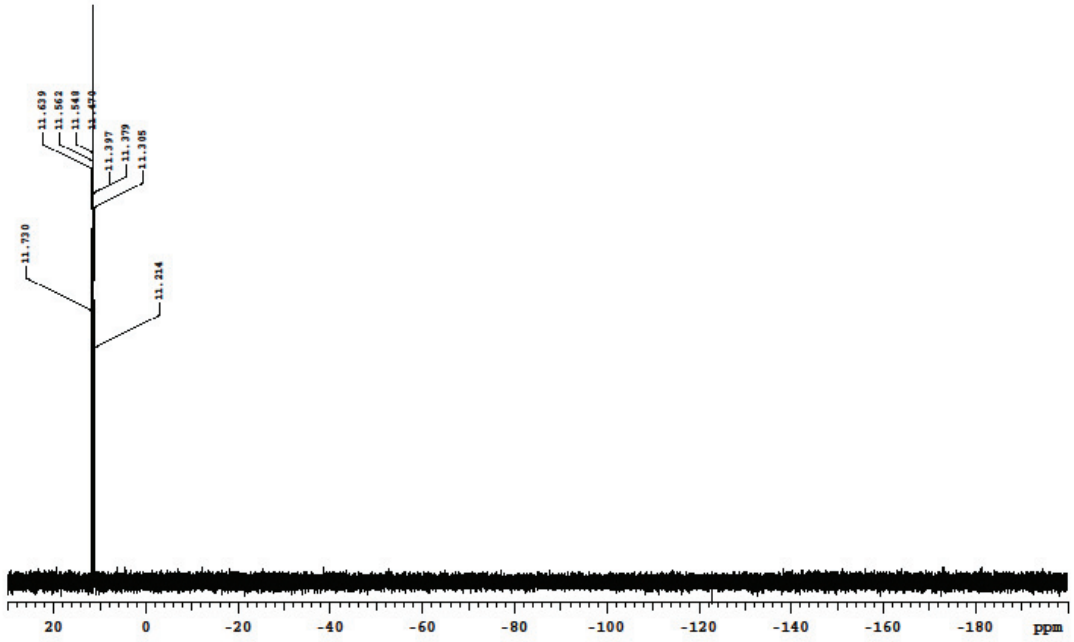


Figure S2-A3: ^{19}F-NMR Spectrum of 5F-AKB48 with hydroxy-group in position 3, Solvent: Solvent: $CDCl_3$

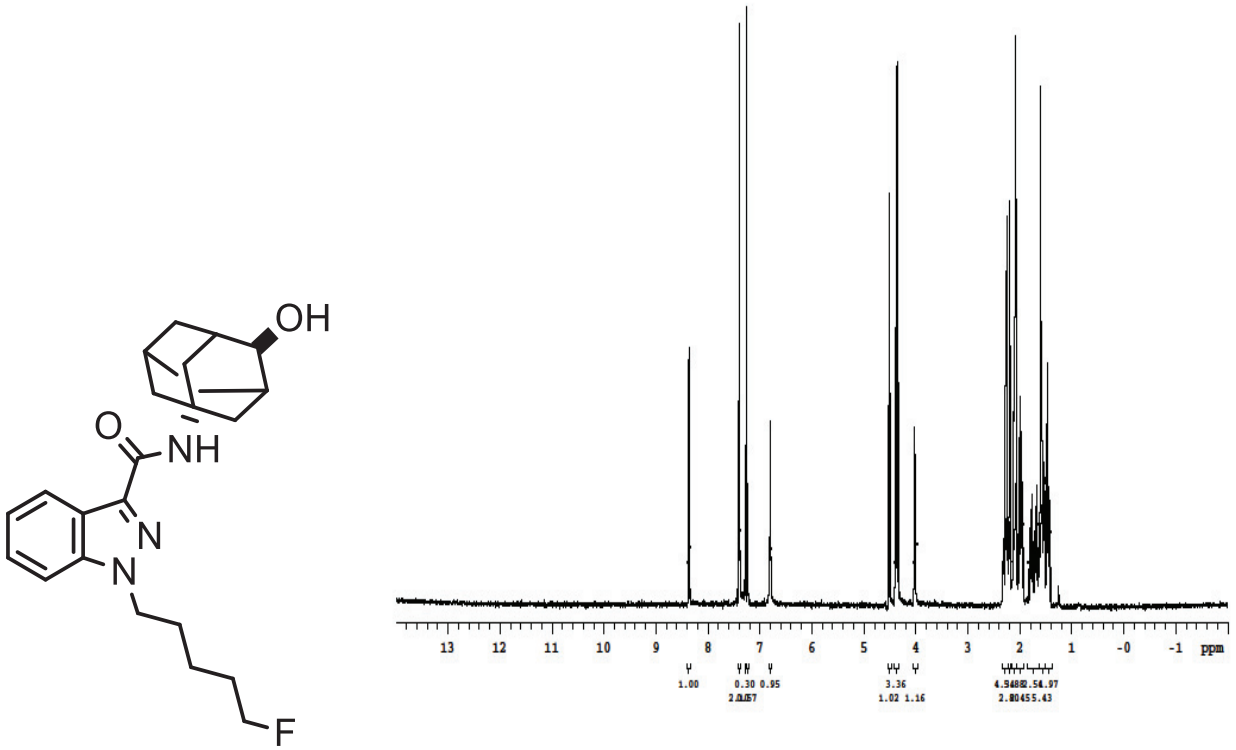


Figure S2-B1: Structure and ^{1}H-NMR Spectrum of 5F-AKB48 with hydroxy-group in position 4 with equatorial orientation, Solvent: $CDCl_3$

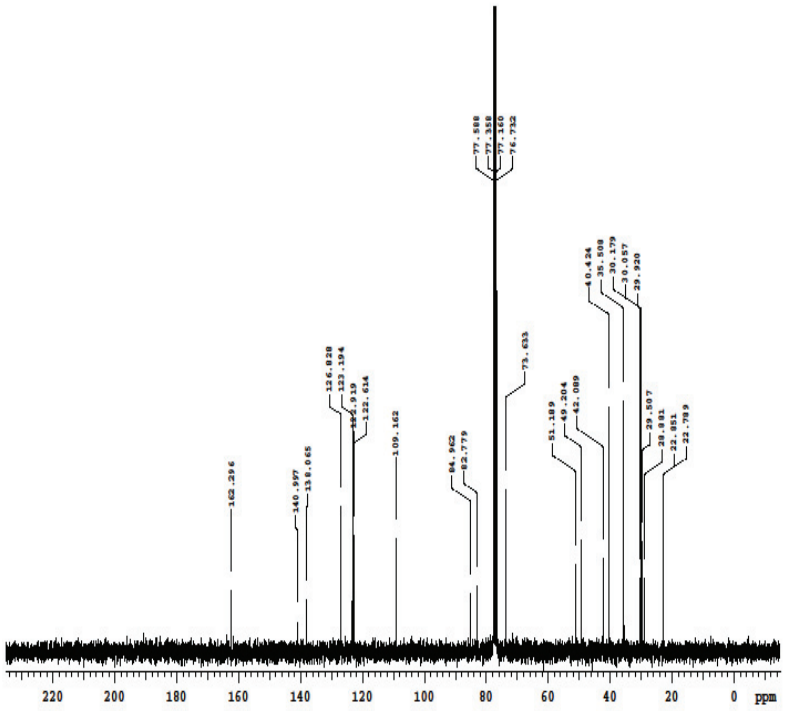


Figure S2-B2: ^{13}C-NMR Spectrum of 5F-AKB48 with hydroxy-group in position 4 with equatorial orientation, Solvent: Solvent: $CDCl_3$

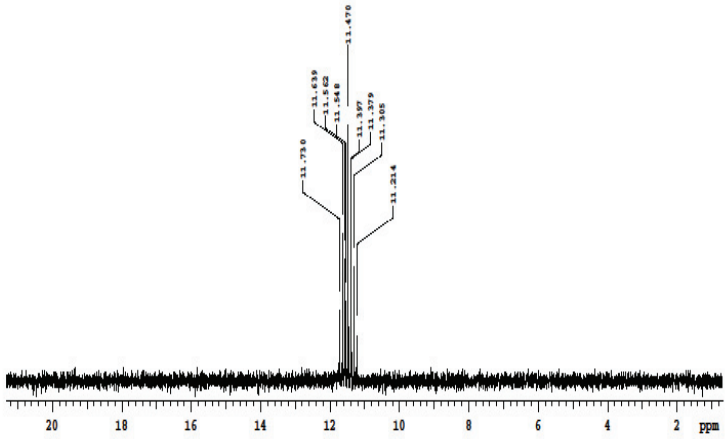


Figure S2-B3: ^{19}F-NMR Spectrum of 5F-AKB48 with hydroxy-group in position 4 with equatorial orientation, Solvent: Solvent: $CDCl_3$

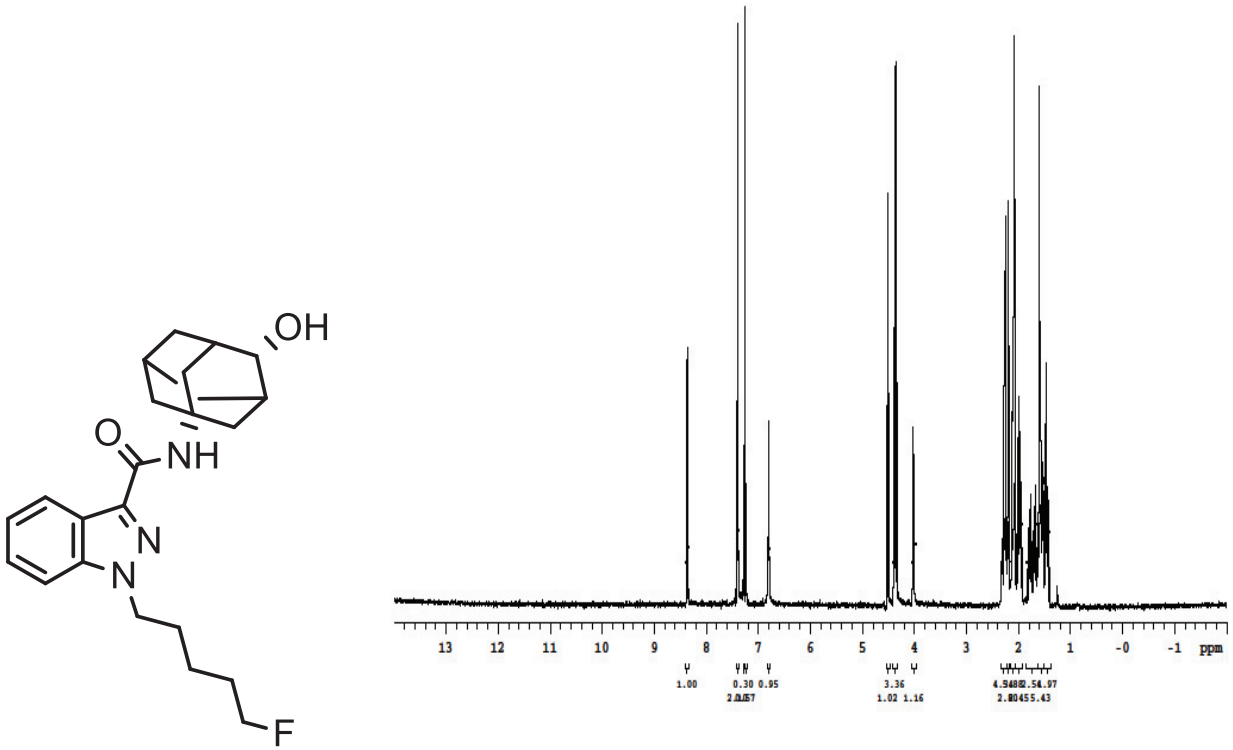


Figure S2-C1: Structure and ^{1}H-NMR Spectrum of 5F-AKB48 with hydroxy-group in position 4 with axial orientation, Solvent: $CDCl_3$

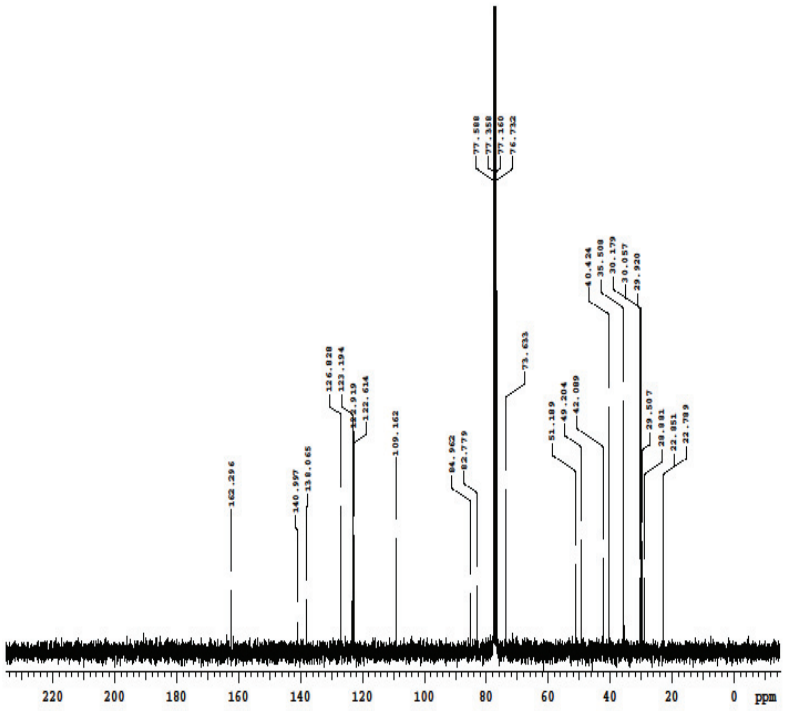


Figure S2-C2: ^{13}C-NMR Spectrum of 5F-AKB48 with hydroxy-group in position 4 with axial orientation, Solvent: Solvent: $CDCl_3$

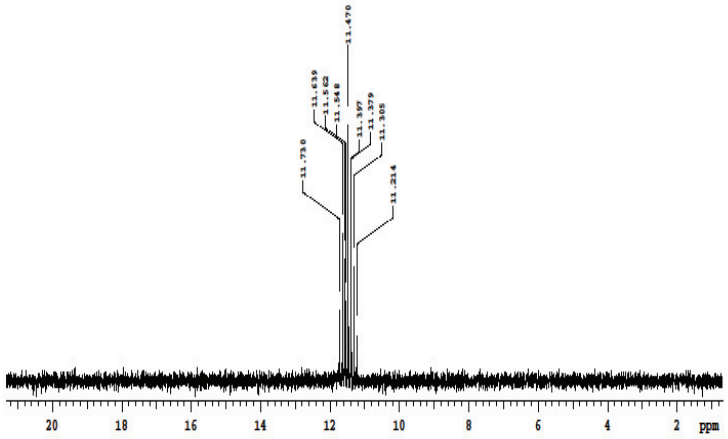
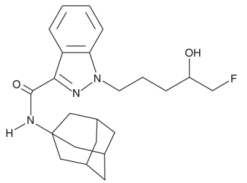
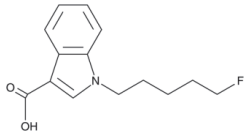
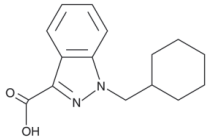
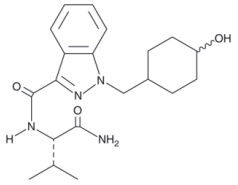
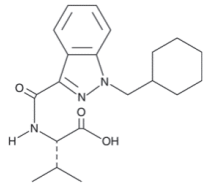
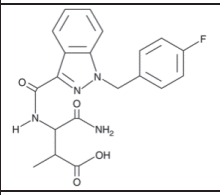
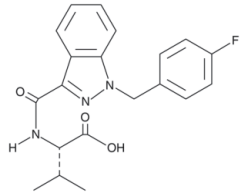
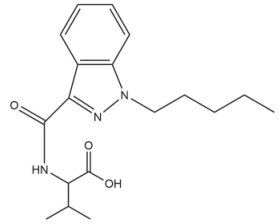
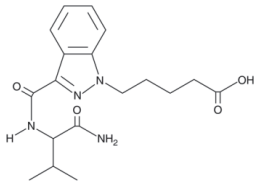
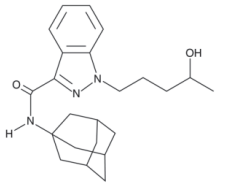
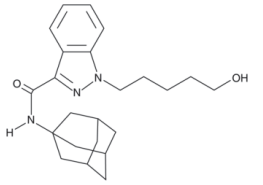
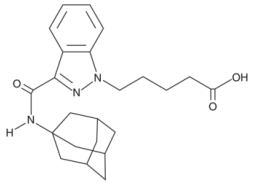
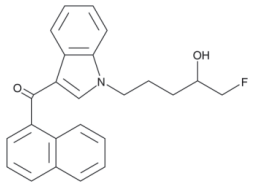


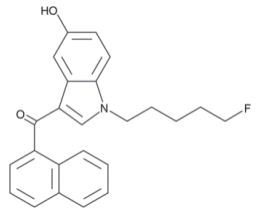
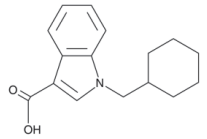
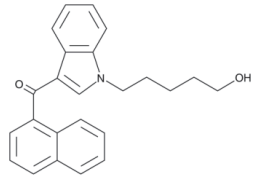
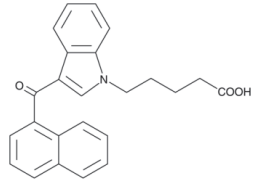
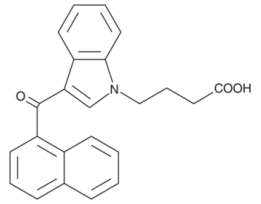
Figure S2-C3: ^{19}F-NMR Spectrum of 5F-AKB48 with hydroxy-group in position 4 with axial orientation, Solvent: Solvent: $CDCl_3$

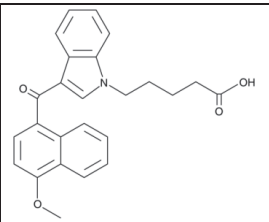
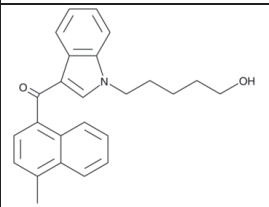
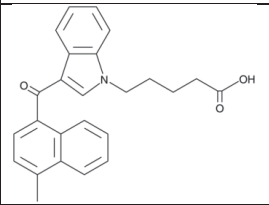
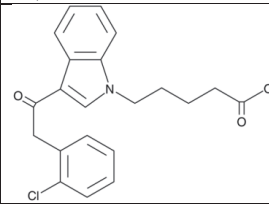
Table S1: The name, formula, accurate mass, CAS-number, IUPAC Name and structure of 35 metabolites of synthetic cannabinoids and three deuterium labeled internal standards in the method

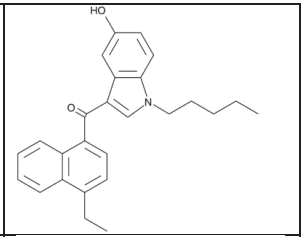
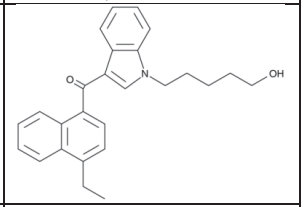
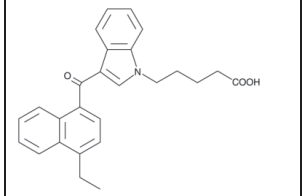
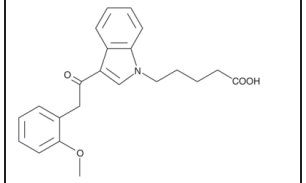
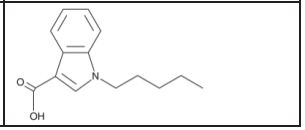
Name	Formula	Mass	CAS	IUPAC Name	Structure
5F-AKB48 *N*-(4-hydroxypentyl)	$C_{23}H_{30}FN_3O_2$	399.23221	1843184-42-8	N-[(3s,5s,7s)-Adamantan-1-yl]-1-(5-fluoro-4-hydroxypentyl)-1H-indazole-3-carboxamide	
5F-PB-22 3-carboxyindole	$C_{14}H_{16}FNO_2$	249.11651	1432794-98-3	1-(5-Fluoropentyl)-1H-indole-3-carboxylic acid	
AB-CHMINACA 3 carboxyindazole	$C_{15}H_{18}N_2O_2$	258.13683	1271630-11-5	1-(Cyclohexylmethyl)-1H-indazole-3-carboxylic acid	
AB-CHMINACA M1A[2]	$C_{20}H_{28}N_4O_3$	372.21614	NA[1]	N-[(2S)-1-Amino-3-methyl-1-oxo-2-butanyl]-1-[(4-hydroxycyclohexyl)methyl]-1H-indazole-3-carboxamide	

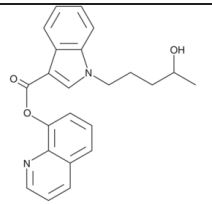
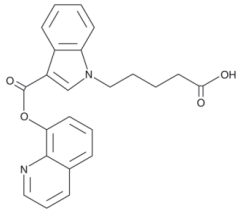
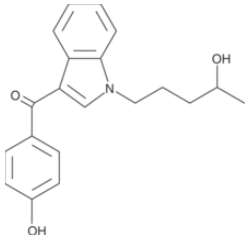
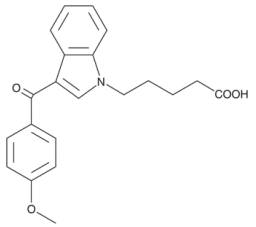
AB-CHMINACA M2[3]	$C_{20}H_{27}N_3O_3$	357.20524	1185887-51-7	N-[[1-(cyclohexylmethyl)-1H-indazol-3-yl]carbonyl]-L-valine	
AB-FUBINACA M2[4]	$C_{20}H_{19}FN_4O_4$	398.13903	NA	N2-{[1-(4-Fluorobenzyl)-1H-indazol-3-yl]carbonyl}-3-methyl-α-asparagine	
AB-FUBINACA M3[3]	$C_{20}H_{20}FN_3O_3$	369.14887	1877243-60-1	(2S)-2-({1-[(4-Fluorophenyl)methyl]indazole-3-carbonyl}amino)-3-methylbutanoic acid	
AB-PINACA COOH	$C_{18}H_{25}N_3O_3$	331.18959	NA	(2S)-3-methyl-2-[(1-pentyl-1H-indazole-3-carbonyl)amino]butanoic acid	

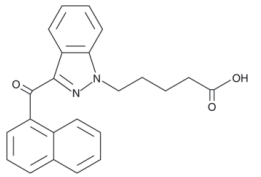
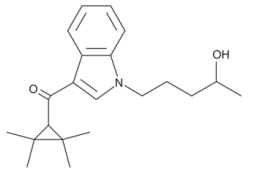
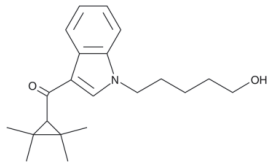
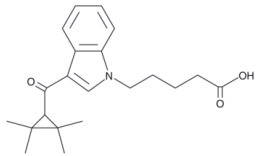
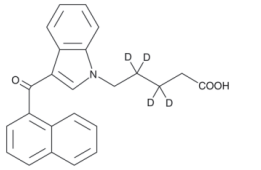
AB-PINACA pentanoic acid	$C_{18}H_{24}N_4O_4$	360.17976	1879029-93-2	5-{3-[(1-Amino-3-methyl-1-oxo-2-butanyl)carbamoyl]-1H-indazol-1-yl}pentanoic acid	
AKB48 N-(4-hydroxypentyl)	$C_{23}H_{31}N_3O_2$	381.24163	1843184-41-7	N-[(3s,5s,7s)-Adamantan-1-yl]-1-(4-hydroxypentyl)-1H-indazole-3-carboxamide	
AKB48 *N*-(5-hydroxypentyl)	$C_{23}H_{31}N_3O_2$	381.24163	1778734-77-2	N-[(3s,5s,7s)-Adamantan-1-yl]-1-(5-hydroxypentyl)-1H-indazole-3-carboxamide	
AKB48 *N*-pentanoic acid	$C_{23}H_{29}N_3O_3$	395.22089	1630022-94-4	5-{3-[(3s,5s,7s)-Adamantan-1-ylcarbamoyl]-1H-indazol-1-yl}pentanoic acid	
AM-2201 *N*-(4-hydroxypentyl)	$C_{24}H_{22}FNO_2$	375.16346	1427521-34-3	[1-(5-Fluoro-4-hydroxypentyl)-1H-indol-3-yl](1-naphthyl)methanone	

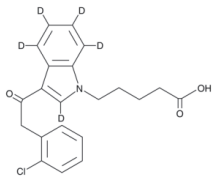
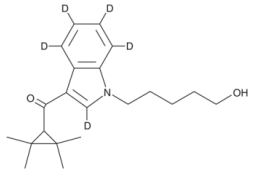
AM-2201 *N*-(5-hydroxyindole)	$C_{24}H_{22}FNO_2$	375.16346	NA	[1-(5-Fluoropentyl)-5-hydroxy-1H-indol-3-yl](1-naphthyl)methanone	
BB-22 3-carboxyindole	$C_{16}H_{19}NO_2$	257.14158	858515-71-6	1-(Cyclohexylmethyl)-1H-indole-3-carboxylic acid	
JWH-018 *N*-(5-hydroxypentyl)	$C_{24}H_{23}NO_2$	357.17288	335161-21-2	[1-(5-Hydroxypentyl)-1H-indol-3-yl](1-naphthyl)methanone	
JWH-018 *N*-pentanoic acid	$C_{24}H_{21}NO_3$	371.15214	1254475-87-0	5-[3-(1-Naphthoyl)-1H-indol-1-yl]pentanoic acid	
JWH-073 *N*-butanoic acid	$C_{23}H_{19}NO_3$	357.13649	1307803-52-6	4-[3-(1-Naphthoyl)-1H-indol-1-yl]butanoic acid	

JWH-081 *N*-pentanoic acid	$C_{25}H_{23}NO_4$	401.16271	1537889-08-9	5-[3-(4-Methoxy-1-naphthoyl)-1H-indol-1-yl]pentanoic acid	
JWH-122 *N*-(5-hydroxypentyl)	$C_{25}H_{25}NO_2$	371.18853	1379604-68-8	[1-(5-Hydroxypentyl)-1H-indol-3-yl](4-methyl-1-naphthyl)methanone	
JWH-122 *N*-pentanoic acid	$C_{25}H_{23}NO_3$	385.16779	1537889-09-0	5-[3-(4-Methyl-1-naphthoyl)-1H-indol-1-yl]pentanoic acid	
JWH-203 *N*-pentanoic acid	$C_{21}H_{20}ClNO_3$	369.11317	1449675-70-0	5-{3-[(2-Chlorophenyl)acetyl]-1H-indol-1-yl}pentanoic acid	

JWH-210 *N*-(5-hydroxyindol)	$C_{26}H_{27}NO_2$	385.20418	1427325-81-2	(4-Ethyl-1-naphthyl)(5-hydroxy-1-pentyl-1H-indol-3-yl)methanone	
JWH-210 *N*-(5-hydroxypentyl)	$C_{26}H_{27}NO_2$	385.20418	1427521-40-1	(4-Ethyl-1-naphthyl)[1-(5-hydroxypentyl)-1H-indol-3-yl]methanone	
JWH-210 *N*-pentanoic acid	$C_{26}H_{25}NO_3$	399.18344	1427521-36-5	5-[3-(4-Ethyl-1-naphthoyl)-1H-indol-1-yl]pentanoic acid	
JWH-250 *N*-pentanoic acid	$C_{22}H_{23}NO_4$	365.16271	1379604-65-5	5-{3-[(2-Methoxyphenyl)acetyl]-1H-indol-1-yl}pentanoic acid	
PB-22 3-carboxyindole	$C_{14}H_{17}NO_2$	231.12593	727421-73-0	1-Pentyl-1H-indole-3-carboxylic acid	

PB-22 *N*-(4-hydroxypentyl)	$C_{23}H_{22}N_2O_3$	374.16304	NA	8-Quinolinyl 1-(4-hydroxypentyl)-1H-indole-3-carboxylate	
PB-22 *N*-pentanoic acid	$C_{23}H_{20}N_2O_4$	388.14231	NA	5-{3-[(8-Quinolinyloxy)carbonyl]-1H-indol-1-yl}pentanoic acid	
RCS-4 *N*-(4-hydroxypentyl)phenol	$C_{20}H_{21}NO_3$	323.15214	1379604-66-6	[1-(4-Hydroxypentyl)-1H-indol-3-yl](4-hydroxyphenyl)methanone	
RCS-4 *N*-pentanoic acid	$C_{21}H_{21}NO_4$	351.14706	1427521-39-8	5-[3-(4-Methoxybenzoyl)-1H-indol-1-yl]pentanoic acid	

THJ-2201 *N*-pentanoic acid	$C_{23}H_{20}N_2O_3$	372.14739	1850409-18-5	5-[3-(naphthalene-1-carbonyl)-1H-indazol-1-yl]pentanoic acid	
UR-144 *N*-(4-hydroxypentyl)	$C_{21}H_{29}NO_2$	327.21983	1537889-04-5	[1-(4-Hydroxypentyl)-1H-indol-3-yl](2,2,3,3-tetramethylcyclopropyl)methanone	
UR-144 *N*-(5-hydroxypentyl)	$C_{21}H_{29}NO_2$	327.21983	895155-95-0	[1-(5-Hydroxypentyl)-1H-indol-3-yl](2,2,3,3-tetramethylcyclopropyl)methanone	
UR-144 *N*-pentanoic acid	$C_{21}H_{27}NO_3$	341.19909	1451369-33-7	5-{3-[(2,2,3,3-Tetramethylcyclopropyl)carbonyl]-1H-indol-1-yl}pentanoic acid	
d4-JWH-018 *N*-pentanoic acid	$C_{24}H_{17}D_4NO_3$	375.17725	1320363-49-2	5-[3-(1-Naphthoyl)-1H-indol-1-yl](3,3,4,4-2H4)pentanoic acid	

d4-JWH-250 *N*-pentanoic acid	$C_{22}H_{19}D_4NO_4$	369.18782	NA	4-{3-[(2-methoxyphenyl)acetyl](2H5)-1H-indol-1-yl}butanoic acid	
d5-UR-144 *N*-(5-hydroxypentyl)	$C_{21}H_{24}D_5NO_2$	332.25121	NA	[1-(5-Hydroxypentyl)(2H5)-1H-indol-3-yl](2,2,3,3-tetramethylcyclopropyl)methanone	

[1]Not available [2]Hydroxylated at cyclohexyl [3]Hydroxylation of primary amide [4]Carboxylation of the oxobutane moiety

Table S2: Concentration levels of the 35 metabolites in the six calibration levels and two quality control levels distributed in five groups of working solutions.

Metabolite	Level 1 [ng/ml]	Level 2 [ng/ml]	Level 3 [ng/ml]	Level 4 [ng/ml]	Level 5 [ng/ml]	Level 6 [ng/ml]	QC low [ng/ml]	QC high [ng/ml]
5F PB-22 3-carboxyindole[4]	1.00	4.00	20.0	40.0	60.0	120	0.500	67.0
5F-AKB48 *N*-(4-hydroxypentyl)[1]	0.100	0.400	1.00	5.00	10.0	25.0	0.500	25.0
AB-CHMINACA 3-carboxyindazole[2]	0.250	1.00	2.50	10.0	20.0	50.0	0.500	22.5
AB-CHMINACA M1A[5]	10.0	40.0	80.0	160	320	-	20.0	200
AB-CHMINACA M2[4]	1.00	4.00	20.0	40.0	60.0	120	2.00	100
AB-FUBINACA M2[4]	2.00	8.00	40.0	80.0	120	240	20.0	200
AB-FUBINACA M3[4]	1.00	4.00	20.0	40.0	60.0	120	2.00	45.0
AB-PINACA COOH[4]	10.0	40.0	80.0	160	320	-	2.00	100
AB-PINACA pentanoic acid[5]	1.00	4.00	20.0	40.0	60.0	120	20.0	200
AKB48 *N*-(4-hydroxypentyl)[1]	0.100	0.400	1.00	5.00	10.0	25.0	0.500	25.0
AKB48 *N*-(5-hydroxypentyl)[1]	0.100	0.400	1.00	5.00	10.0	25.0	0.500	25.0
AKB48 *N*-pentanoic acid[1]	0.200	0.800	2.00	10.0	20.0	50.0	0.500	25.0
AM-2201 *N*-(4-hydroxypentyl)[1]	0.200	0.800	2.00	10.0	20.0	50.0	0.500	25.0
AM-2201 *N*-(5-hydroxyindole)[3]	0.250	1.00	2.50	12.5	25.0	60.0	0.500	50.0
BB-22 3-carboxyindole[4]	2.00	8.00	40.0	80.0	120	240	2.00	200
JWH-018 *N*-(5-hydroxypentyl)[2]	0.250	1.00	2.50	10.0	20.0	50.0	0.500	50.0
JWH-018 *N*-pentanoic acid[3]	0.250	1.00	2.50	12.5	25.0	60.0	0.500	22.5
JWH-073 *N*-butanoic acid[3]	0.250	1.00	2.50	12.5	25.0	60.0	0.500	50.0
JWH-081 *N*-pentanoic acid[3]	0.250	1.00	2.50	12.5	25.0	60.0	0.500	50.0
JWH-122 *N*-(5-hydroxypentyl)[2]	0.250	1.00	2.50	10.0	20.0	50.0	0.500	25.0
JWH-122 *N*-pentanoic acid[3]	0.250	1.00	2.50	12.5	25.0	60.0	0.500	50.0
JWH-203 *N*-pentanoic acid[3]	0.250	1.00	2.50	12.5	25.0	60.0	0.500	50.0
JWH-210 *N*-(5-hydroxyindole)[4]	1.20	4.80	24.0	48.0	72.0	144	2.00	25.0
JWH-210 *N*-(5-hydroxypentyl)[2]	0.250	1.00	2.50	10.0	20.0	50.0	0.500	50.0
JWH-210 *N*-pentanoic acid[3]	0.250	1.00	2.50	6.25	12.5	30.0	0.500	25.0
JWH-250 *N*-pentanoic acid[3]	0.250	1.00	2.50	12.5	25.0	60.0	0.500	50.0
PB-22 3-carboxyindole[4]	1.00	4.00	20.0	40.0	60.0	120	2.00	100
PB-22 *N*-(4-hydroxypentyl)[2]	0.250	1.00	2.50	10.0	20.0	50.0	0.500	22.5
PB-22 *N*-pentanoic acid[1]	0.250	1.00	2.50	10.0	20.0	50.0	0.500	25.0
RCS-4 N-(4-hydroxypentyl)phenol[5]	5.00	20.0	40.0	80.0	160	-	10.0	200
RCS-4-*N*-pentanoic acid[3]	0.250	1.00	2.50	12.5	25.0	60.0	0.500	50.0
THJ-2201-*N*-pentanoic acid[2]	0.250	1.00	2.50	10.0	20.0	50.0	0.500	22.5
UR-144 *N*-(4-hydroxypentyl)[1]	0.200	0.80	2.00	10.0	20.0	50.0	0.500	25.0
UR-144 *N*-(5-hydroxypentyl)[1]	0.200	0.80	2.00	10.0	20.0	50.0	0.500	25.0
UR-144 *N*-pentanoic acid[1]	0.250	1.00	2.50	10.0	20.0	50.0	0.500	25.0

[1]working solution 1
[2]working solution 2
[3]working solution 3
[4]working solution 4
[5]working solution 5

Table S3: The 35 metabolites of synthetic cannabinoids in urine and the lowest concentration detected using the different ID criteria where (+) or (-) means detected/not detected at the correspondent concentration. Criteria I means identified by mass accuracy and RT using MassHunter Quantitative, criteria II means identified using Find by formula algorithm with a minimum score of 80 and criteria III means confirmed by MS/MS library spectra both using MassHunter Qualitative.

	Conc	ID Criteria		
Metabolite	[ng/ml]	I	II	III
5F-AKB-48 *N*-(4-hydroxypentyl)	0.01 0.04	- +	- +	- +
5F PB-22 3-carboxyindole	1 2.5 5	- +	- (4 GHz)[1]	- - +
AB-CHMINACA 3-carboxyindazole	0.5 1 2.5	+	- - +	- - +
AB-CHMINACA M1A	2 5 10	- - +	- - +	- - +
AB-CHMINACA M2	0.4 1	+	- +	- +
AB-FUBINACA M2	1 2 12	- +	- - +	- - +
AB-FUBINACA M3	0.5 1 2.5	+	- (4 GHz) +	+
AB-PINACA-carboxylic acid	1 2 12	+	 (4 GHz) +	 +
AB-PINACA pentanoic acid	2 5 10	- - +	- - (4 GHz)	- - -
AKB48 *N*-(4-hydroxypentyl)	0.01 0.04 0.1	- - +	- - +	- - +
AKB48 *N*-(5-hydroxypentyl)	0.01 0.04 0.1	- - +	- - +	- - +
AKB48 *N*-pentanoic acid	0.01 0.04 0.1	- - +	- - +	- - +
AM-2201 *N*-(4-hydroxypentyl)	0.1 0.2 1	+	- +	+
AM-2201 *N*-(5-hydroxyindole)	0.1 0.25 0.5	+	- - +	- +
BB-22 3-carboxyindole	2 5 12 17.5	+	- - +	- - - +
JWH-018 *N*-(5-hydroxypentyl)	0.25 0.5 1	+	(4 GHz) - +	+
JWH-018 *N*-pentanoic acid	0.1 0.25 0.5	- +	- - -	- - +
JWH-073 *N*-butanoic acid	0.1 0.25 0.5	- +	- (4 GHz) +	- - +

JWH-081 *N*-pentanoic acid	0.1 0.25 0.5	- +	- (4 GHz) +	- - +
JWH-122 *N*-(5-hydroxypentyl)	0.25 0.5	+	- -	- +
JWH-122 *N*-pentanoic acid	0.25 0.5 5	+	- - -	- +
JWH-203 *N*-pentanoic acid	0.25 1 2.5	+	- - (4 GHz)	- - +
JWH-210 *N*-(5-hydroxyindol)	1 2	+	- +	- +
JWH-210 *N*-(5-hydroxypentyl)	0.25 1 2.5	+	- +	- - +
JWH-210 *N*-pentanoic acid	0.1 0.25 0.5	- +	- (4 GHz) +	- - +
JWH-250 *N*-pentanoic acid	0.1 0.25 0.5	+	- - +	- +
PB-22 3-carboxyindole	1 5 12	+	(4 GHz) +	- - +
PB-22 *N*-(4-hydroxypentyl)	0.1 0.25 0.5	+	- +	- - +
PB-22 *N*-pentanoic acid	0.2 1 2.5	- +	- - +	- - +
RCS-4 N-(4-hydroxypentyl)phenol	1 5 10	+	- - (4 GHz)	- - +
RCS-4-*N*-pentanoic acid	0.25 0.5 1 2.5	+	- (4 GHz) - +	- - +
THJ-2201-*N*-pentanoic acid	0.25 0.5 1	+	- - +	- +
UR-144 *N*-(5-hydroxypentyl)	0.04 0.1 0.2	- +	- +	- +
UR-144 *N*-(4-hydroxypentyl)	0.01 0.04 0.1	- - +	- - +	- - +
UR-144 *N*-pentanoic acid	0.1 0.2 1	- +	- - +	- +

[1]Metabolite identified at this concentration using 4 GHz data from second injection.

Paper II

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Article

Metabolite Profiling of Ortho-, Meta- and Para-Fluorofentanyl by Hepatocytes and High-Resolution Mass Spectrometry

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Abstract

New psychoactive substances are emerging on the illegal drug market. Synthetic opioids including fentanyl analogues are of special concern due to their high potency. This indicates the possibility of low drug concentrations in vivo and calls for sensitive analytical methods and identification of the most appropriate analytical targets. In this study the in vitro metabolism of ortho-, meta- and para-fluorofentanyl, three fluorinated derivatives of fentanyl, has been investigated using human hepatocytes and compared to the results from an authentic human urine sample. Based on knowledge on the metabolism of similar fentanyl analogues *N*-dealkylation and hydroxylation was hypothesized to be the most central pathways. The three fluorofentanyl isomers were incubated with pooled human hepatocytes at 1, 3 and 5 h. Liquid chromatography quadrupole time of flight mass spectrometry operating in data-dependent mode was used to analyse the hepatocyte samples, as well as the hydrolysed and non-hydrolysed authentic urine sample. Data were analysed by a targeted approach with a database of potential metabolites. The major metabolite formed in vitro was the *N*-dealkylation product norfluorofentanyl. In addition various hydroxylated metabolites, a *N*-oxide, dihydrodiol metabolites and a hydroxymethoxy metabolite were found. In total, 14 different metabolites were identified for each fluorofentanyl isomer. In the authentic urine sample, three metabolites were detected in addition to the ortho-fluorofentanyl parent compound, with hydroxymethoxy metabolite having the highest abundance followed by norfluorofentanyl and a metabolite hydroxylated on the ethylphenyl ring. This in vitro study showed that the metabolic pattern for ortho-, meta-, and para-fluorofentanyl was close to those previously reported for other fentanyl analogues. We suggest that the hydroxymethoxy metabolite and the metabolite hydroxylated on the ethylphenyl ring should be the metabolites primarily investigated in further studies to determine the most appropriate marker for intake of fluorofentanyl derivatives in urine drug screening for human subjects.

Key words: Fluorofentanyl, metabolism, human hepatocytes, high-resolution mass spectrometry

Introduction

Numerous structural analogs of the opioid fentanyl have entered the illegal drug market in recent decades. These synthetic opioids were originally designed with the purpose of becoming medications used in humans or animals. Some, e.g., remifentanil and alfentanil, are medications approved for human use, while others solely appear as illicit drugs after being produced at clandestine laboratories. New analogs created from existing compounds by substitution with halogens or other functional groups are also adding to the increasing number of potential drugs of abuse. The positional isomers ortho-, meta- and para-fluorofentanyl (o-, m- and p-fluorofentanyl), also named 2-, 3- and 4-fluorofentanyl, respectively, are derivatives of fentanyl with a fluorine atom located at the *N*-phenyl moiety. Para-fluorofentanyl (*N*-(4-fluorophenyl)-*N*-[1-(2-phenylethyl)-4-piperidinyl] propanamide) is classified as a narcotic under the United Nation's Single Convention on Narcotic Drugs (1). The ortho- and meta-derivatives (*N*-(2-fluorophenyl)-*N*-[1-(2-phenylethyl)-4-piperidinyl] propanamide and *N*-(3-fluorophenyl)-*N*-[1-(2-phenylethyl)-4-piperidinyl] propanamide, respectively) were first reported to the EU early warning system of the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) in 2016 and are now controlled substances in many European countries as well as in the USA (2, 3).

There have been several reported seizures of these compounds in Europe since 2016, and a case report of a death after intake of ortho-fluorofentanyl has been reported (4). Little research has been performed on the pharmacokinetics and toxicity of these substances. However, in a study on the interaction with cloned human opioid receptors, para-fluorofentanyl was found to be a more potent agonist than fentanyl (5). The high potency indicates the possibility of low drug concentrations *in vivo* and calls for sensitive analytical methods using carefully selected analytical targets to detect a possible intake of these drugs.

In general, metabolites of illicit drugs are formed by complex enzymatic processes. The best way to study these is to use authentic human urine samples. However, due to the limited access to such samples from subjects with a known or suspected drug intake as well as uncertainties related to which drug(s) has been ingested, *in vitro* model systems of drug metabolism have emerged as an important tool. The metabolic pattern of fentanyl and some other fentanyl derivatives has previously been investigated both *in vitro* and in biological samples (6–14) but no studies of metabolism have been performed on ortho-, meta- or para-fluorofentanyl. Based on the knowledge of the metabolic pattern of previously studied fentanyl derivatives, the *N*-dealkylated metabolite and hydroxylated metabolites were expected to be major metabolites.

High resolution mass spectrometry (HR-MS) by liquid chromatography quadrupole time of flight mass spectrometry (LC-QTOF-MS) is a powerful tool for screening for and identification of previous known or unknown compounds like metabolites in metabolite profiling (15). LC-QTOF-MS can be operated in a data-dependent MS/MS mode (Auto-MS/MS), switching between acquiring full spectra MS and MS/MS spectra of ions fulfilling the requirements set in the method. The high-resolution MS data files can be processed in the search for compounds likely to be metabolites of ortho-, meta- and para-fluorofentanyl and the corresponding MS/MS spectra can be used to elucidate their structures.

The primary aim of this study was to investigate the metabolism of ortho-, meta- and para-fluorofentanyl using human hepatocytes and elucidate the structure of the metabolites using HR-MS. Identifying the major metabolites is important as they may serve as analytical targets for urinary drug screening. A secondary aim was to investigate whether the exact position of the fluorine atom had any influence on the degree of formation of the various metabolites.

Material and Methods

Chemicals and reagents

Ortho-, meta- and para-fluorofentanyl were purchased from Cayman Chemicals (Ann Arbor, MI, USA). LC-MS grade acetonitrile, formic acid and methanol were purchased from Fisher Scientific (Gothenburg, Sweden). Ammonium formate was obtained from Sigma–Aldrich (Stockholm, Sweden) and 99.5% ethanol from Kemetyl (Haninge, Sweden). Divide; Cryo-preserved human hepatocytes (LiverPool™, 10-donor-pool, Lot nr. RBR) and InVitro Gro HT medium were from BioreclamationIVT (Baltimore, MD, USA). Williams medium E (without L-glutamine and phenol red), L-glutamine 200 mM and Hepes 1 M buffer solution from Gibco® by life technologies™ were purchased from Thermo (Stockholm, Sweden). MilliQ Gradient 10 production unit from Millipore (Billerica, MA, USA) was used to produce high-purity water. *β*-Glucuronidase/arylsulfatase stock solution (Helix promatia), with activities of 4.5 and 14 U/ml respectively, was purchased from Roche Diagnostics (Mannheim, Germany).

Preparation of drug solutions and positive control

Stock solutions were diluted in William medium E to form individual drug solutions of 10 µM ortho-, meta- or para-fluorofentanyl with a maximum organic content of 0.2%. A positive control containing a mix of the cytochrome P-450 (CYP) substrates caffeine (CYP1A2), bupropion (CYP2B6), diclofenac (CYP2C9), omeprazole (CYP2C19), dextromethorphane (CYP2D6), chlorzoxazone (CYP2E1) and midazolam (CYP3A4) was diluted in William medium E to a concentration of 10 µM. Internal standards (IS) were diluted in acetonitrile to a final concentration of 300 ng/mL amphetamine-d8 and phenobarbital-d5, 100 ng/mL diazepam-d5 and 50 ng/mL mianserin-d3.

Incubation with human hepatocytes

The incubation of human hepatocytes with the fluorofentanyl isomers was performed in accordance to the protocol used by Åstrand *et al*. (13). Cryopreserved pooled human hepatocytes were thawed at 37°C and poured into HT medium. After centrifugation (60 g for 5 min at room temperature), the supernatant was removed and the cells were re-suspended in Williams E medium. The cells were centrifuged (60 g for 5 min at room temperature) again, the supernatant was removed and the cells were re-suspended in Williams E medium making a final volume of 2 mL. The concentration of viable cells was evaluated with Trypan blue (0.4% v/v) exclusion dye method. Cells were diluted to 2.0 x 10^6 cells/mL.

Each fluorofentanyl isomer at a concentration of 5 µM was incubated with 10^5 cells (100 µL total volume) in 96-well plates in duplicate. The incubations were stopped after 1, 3 and 5 h by adding 100 µL ice cold acetonitrile (including IS) to each well. A negative control (only cells) and a degradation control (only drug) were incubated for 5 h and positive controls were incubated for 0 and 5 h. The controls incubated for 0 h were prepared by adding acetonitrile to the drug immediately before adding the cells. The plates were vortexed and left at −20°C for a minimum of 10 min before centrifugation (1100 g for 15 min at 4°C). Finally 100 µL of the extracts were transferred to an injection plate.

Preparation of an authentic human urine sample

A urine sample from a patient intoxicated with ortho-fluorofentanyl was used for metabolite profiling and comparison with the *in vitro* results. Clinical information about the case has been presented elsewhere (4). The sample was analyzed with and without enzymatic hydrolysis. For the hydrolyzed sample preparation, 100 µL urine was incubated with 10 µL β-glucuronidase stock solution at 40°C for 1 h in a water bath and diluted with 300 µL of mobile phase mixture (A/B, 50:50; see later). No pH adjustment was done. The nonhydrolyzed sample was diluted with 310 µL mobile phase mixture. Finally, both samples were filtered using a 13 mm syringe filter with 0.45 µm polytetrafluoroethylene membrane (VWR, Radnor, PA, USA) before injection.

LC-QTOF analysis

The chromatographic separation of the hepatocyte samples (1 µL injection volume) and the diluted authentic urine sample (5 µL injection volume) was performed by an Agilent 1290 Infinity system equipped with an Acquity HSS T3 column (150 mm x 2.1 mm, 1.8 µm) from Waters (Milford, MA, USA). Separation was achieved using a mobile phase consisting of 0.05% formic acid in 10 mM ammonium formate (A) and 0.05% formic acid in acetonitrile (B). A linear gradient with a flow of 0.50 mL/min starting at 1% B increasing to 40% in 13 min and continuing to 95% in the next 2 min was employed. After a 3.1-min hold at 95% B the column was re-equilibrated for 2.9 min at 1% B, giving a total cycle time of 21 min. Autosampler and column temperatures were set to 7 and 60°C, respectively.

The MS analysis was performed using a 6550 QTOF-MS (Agilent, Santa Clara, CA, USA) with electrospray ionization and iFunnel interface. Positive ionization was used with a fragmentor voltage of 380 V, VCap at 3500 V, gas temperature and flow at 150°C and 18 L/min, respectively, nebulizer pressure at 50 psig and sheath gas temperature at 380°C. Data were acquired in data-dependent Auto MS/MS mode. MS spectra and MS/MS spectra were acquired in the mass range of 100–950 m/z at a rate of 5 Hz and 50–950 m/z at 10 Hz, respectively. The detector was operated in 2 GHz extended dynamic range giving a resolution (m/Δm at FWHM) of approx. 20,000 at m/z 322.0481. Precursor selection was based on abundance, and an intensity threshold of 5000 counts was applied. After one spectrum from a precursor was acquired, this specific precursor was excluded in 0.03 min. Lock masses 121.0509 and 922.0098 were applied for automated mass correction in all spectra.

The data files were processed by the Agilent MassHunter Qualitative Analysis (B.07.00), using the algorithm Find by Formula. This is a targeted approach with a database with formulas of possible metabolites including mono-, di- and trihydroxylations, carbonylation, dihydrodiol formation, methylation, carboxylation, defluorination, *N*-dealkylations (including loss of the fluorophenyl group), amide hydrolysis, glucuronidation and combinations of these. Only compounds with a mass error within 15 ppm and a peak area above 10,000 were regarded as potential metabolites. In addition, the fragment ions in the MS/MS spectrum associated with the precursor ion of the compound were evaluated. Potential metabolites detected in the negative control, 0 h samples or in the degradation controls were excluded.

Results and Discussion

Fragmentation pattern of the parent compounds

For each fluorofentanyl analog, 14 potential metabolites were detected in the *in vitro* experiment (Table I). The elucidation of MS/MS spectra from metabolites was based on the fragmentation of the parent compound. The MS/MS spectra of the parent compounds ortho-, meta- and para-fluorofentanyl ($C_{22}H_{27}FN_2O$) with a precursor molecule ion of m/z 355.2180 were identical, containing the fragment ions of m/z 84.0808, 105.0702, 134.0965, 150.0710, 188.1438, 234.1291 and 299.1912 (Figure 1 shows the MS/MS spectrum of the isomer ortho-fluorofentanyl). The fragment ion 84.0808 corresponds to an unchanged piperidine ring and the base peak 105.0702 results from the cleavage between the piperidine ring and the phenetyl moiety. The fragment ion 134.0965 corresponds to the phenethyl moiety and parts of the piperidine ring. The fragment ion 150.0710 corresponds to degradation of the piperidine ring. The fragment ion 188.1438 corresponds to the complete phenethylpiperidine structure. The fragment ion 234.1291 corresponds to cleavage of the piperidine ring. Lastly, a fragment ion 299.1912, corresponding to the elimination of the amide alkyl chain, was detected (Figure 1).

Identification of the metabolites

Ortho- meta- and para-fluorofentanyl produced metabolites by the same principal metabolic pathways, but with certain differences in retention times (RTs) and absolute chromatographic peak areas (Table I). For simplicity, the metabolites are presented in the text as they originate from one of the parents. The compounds eluted from 4.75 to 12.81 min, and all had a mass error of less than 4.52 ppm. Table I lists all metabolites and parent compounds with their RTs, specific biotransformation, formulas, masses of protonated molecular ions, mass errors, peak areas and diagnostic ions. When the molecular position for the specific biotransformation step could be proposed, the metabolite was given a name in correspondence with a letter- and number-based system given in Figure 1. The metabolites were named using O (ortho), M (meta) or P (para) and numbered 1–14 corresponding to RT order. The MS/MS spectra and proposed fragmentation of the metabolites are shown in the supplementary information (Figure S1). The chromatographic separation of the metabolites of ortho-fluorofentanyl formed *in vitro* is given in Figure 2A. Major metabolites were formed by *N*-dealkylation, hydroxylations at the alkyl chain, ethylphenyl ring and/or piperidine ring and methylation. Detector saturation for the most abundant metabolite (O2/M2/P2) made estimation of relative abundance of the major metabolites impossible.

N-dealkylation at the piperidine ring resulting in the loss of the phenethyl moiety and forming norfluorofentanyl (O2/M2/P2) was observed to be the main metabolite *in vitro*. The MS/MS spectra showed one abundant fragment ion at m/z 84.0815, indicating that the piperidine ring was intact in these metabolites. The enzyme CYP3A4 has previously been shown to be responsible for the *N*-dealkylation of fentanyl (16). The metabolite was detected *in vitro* already in the 0 h samples (Table I), but not in the degradation control. This indicates that norfluorofentanyl is rapidly formed. A compound (O1/M1/P1) corresponding to hydroxylation (addition of 15.9949 u, i.e., +O) of the *N*-dealkylated metabolite was also detected. The fragment ion m/z 84.0805 was also dominating these spectra suggesting hydroxylation at the amide alkyl chain or *N*-phenyl ring.

Four different hydroxylated metabolites (addition of 15.9949 u, i.e., +O, when compared to the mass of the parent) with the protonated molecular ion [$C_{22}H_{27}FN_2O$ +H] and m/z 371.2131 were detected eluting from 9.31 to 12.81 min. The most abundant of these metabolites was O12/M12/P12. Unfortunately, an exact structure cannot be elucidated for this metabolite with MS/MS data only.

Table I. Ortho-, Meta- and Para-Fluorofentanyl with Proposed Biotransformation, Retention Time (RT), Formula, Accurate Mass of Protonated Compound (m/z), Mass Error of Protonated Compound, Peak Areas in Hepatocyte Samples and in Authentic Urine Sample and Diagnostic Ions

ID	Biotransformation	RT[1] (min)	Formula	m/z[1]	Mass error[1] (ppm)	Peak area in hepatocyte samples at various time points (two parallels at each time point, marked #1 and #2, respectively)								Peak area in urine sample		Diagnostic ions (m/z)
						0 h #1	0 h #2	1 h #1	1 h #2	3 h #1	3 h #2	5 h #1	5 h #2	Hyd[2]	Nonhyd[3]	
O1	*N*-Dealkylation +	4.76	$C_{14}H_{19}FN_2O_2$	267.1489	−4.52	ND	ND	9.2E+04	1.0E+05	1.5E+05	1.5E+05	1.4E+05	1.6E+05	ND	ND	84.0815
P1	monohydroxylation at the	4.86		267.1500	−1.84	ND	ND	2.8E+04	2.8E+04	4.3E+04	4.2E+04	4.7E+04	5.0E+04			
M1	amide group	4.75		267.1502	−1.43	ND	ND	8.6E+04	7.8E+04	1.1E+05	1.1E+05	1.5E+05	1.4E+05			
O2	*N*-Dealkylation	7.55	$C_{14}H_{19}FN_2O$	251.1559	0.96	3.0E+05	3.0E+05	Sat	Sat	Sat	Sat	Sat	Sat	4.4E+04	5.5E+04	84.0805
P2		7.62		251.1556	−0.27	2.3E+05	2.4E+05	Sat	Sat	Sat	Sat	Sat	Sat			
M2		7.57		251.1555	−0.50	2.1E+05	2.4E+05	Sat	Sat	Sat	Sat	Sat	Sat			
O3	Dihydrodiol: At the *N*-alkyl	7.98	$C_{22}H_{29}FN_2O_3$	389.2230	−1.41	ND	ND	3.1E+04	3.1E+04	1.0E+05	9.3E+04	9.9E+04	1.1E+05	ND	ND	164.0864,
P3	chain	8.02		389.2237	0.14	ND	ND	6.9E+04	7.3E+04	2.4E+05	2.2E+05	3.0E+05	2.9E+05			207.1297
M3		7.98		389.2230	−1.40	ND	ND	3.6E+04	3.1E+04	9.0E+04	8.3E+04	1.0E+05	9.1E+04			
O4	Dihydroxylation: at the	8.22	$C_{22}H_{27}FN_2O_3$	387.2071	−1.99	ND	ND	ND	ND	ND	ND	4.5E+04	4.4E+04	ND	ND	NDI
P4	*N*-alkyl chain, piperidine ring	8.35		387.2069	−2.68	ND	ND	ND	ND	ND	ND	2.4E+04	2.5E+04			
M4	and/or the amide chain	8.29		387.2072	−1.51	ND	ND	ND	ND	ND	ND	5.1E+04	4.7E+04			
O5	Dihydrodiol: at the *N*-alkyl	8.39	$C_{22}H_{29}FN_2O_3$	389.2250	−2.74	ND	ND	ND	ND	ND	ND	1.6E+04	1.8E+04	ND	ND	164.0875
P5	chain	8.44		389.2226	−2.17	ND	ND	ND	ND	ND	ND	4.6E+04	3.7E+04			
M5		8.41		389.2224	−3.13	ND	ND	ND	ND	ND	ND	1.9E+04	1.5E+04			
O6	Dihydroxylation: at the	9.10	$C_{22}H_{27}FN_2O_3$	387.2071	−2.25	ND	ND	ND	ND	ND	ND	1.8E+04	1.9E+04	ND	ND	NDI
P6	*N*-alkyl chain, piperidine ring	9.10		387.2069	−1.81	ND	ND	ND	ND	ND	ND	7.6E+03	8.3E+03			
M6	and/or the amide chain	9.09		387.2068	−1.94	ND	ND	ND	ND	ND	ND	7.5E+03	7.2E+03			
O7	Monohydroxylation: at the	9.31	$C_{22}H_{27}FN_2O_2$	371.2127	−0.60	ND	ND	1.9E+06	1.9E+06	2.3E+06	2.1E+06	1.9E+06	2.1E+06	ND	ND	84.0803, 105.0690,
P7	amide alkyl chain	9.40		371.2129	−0.41	ND	ND	5.5E+05	4.5E+05	6.6E+05	6.4E+05	6.6E+05	6.6E+05			134.0960, 150.0697,
M7		9.35		371.2128	−0.59	9.3E+04	9.3E+04	1.7E+06	1.3E+06	1.4E+06	1.4E+06	1.7E+06	1.5E+06			188.1434, 299.1908
O8	Monohydroxylation: at	9.81	$C_{22}H_{27}FN_2O_2$	371.2131	0.0	ND	ND	6.2E+05	6.7E+05	7.3E+05	6.5E+05	4.9E+05	6.4E+05	1.2E+04	4.1E+03	84.0808, 121.0646,
P8	phenethyl moiety	9.78		371.2128	−0.22	ND	ND	9.8E+05	8.3E+05	1.2E+06	1.2E+06	1.3E+06	1.2E+06			150.0688, 204.1376
M8		9.77		371.2131	−0.07	ND	ND	7.1E+05	5.0E+05	5.4E+05	5.4E+04	5.4E+05	4.8E+05			
O9	Methylation +	10.11	$C_{23}H_{29}FN_2O_3$	401.2257	4.47	ND	ND	5.7E+04	5.9E+04	6.6E+04	6.0E+04	ND	5.6E+04	6.0E+04	6.0E+04	119.0487, 151.0749,
P9	dihydroxylation: at the	10.08		401.2242	1.28	ND	ND	1.4E+05	1.2E+05	1.6E+05	1.6E+05	1.2E+05	1.3E+05			164.0867, 234.1503
M9	phenethyl moiety	10.08		401.2227	−0.61	ND	ND	4.8E+04	3.3E+04	ND	3.4E+04	3.5E+04	ND			
O10	Dihydroxylation: At the phenethyl moiety and	10.17	$C_{22}H_{27}FN_2O_3$	387.2080	0.07	ND	ND	ND	ND	ND	ND	5.6E+04	5.5E+04	ND	ND	121.0643, 164,0863
P10	2-position of piperidine ring	10.21		387.2071	−2.51	ND	ND	ND	ND	ND	ND	1.1E+04	1.2E+04			
M10	and/or amide group	10.20		387.2066	−2.94	ND	ND	ND	ND	ND	ND	1.1E+04	1.1E+04			

(Continued)

Table I. Continued

ID	Biotransformation	RT[1] (min)	Formula	m/z[1]	Mass error[1] (ppm)	Peak area in hepatocyte samples at various time points (two parallels at each time point, marked #1 and #2, respectively)								Peak area in urine sample		Diagnostic ions (m/z)
						0 h #1	0 h #2	1 h #1	1 h #2	3 h #1	3 h #2	5 h #1	5 h #2	Hyd[2]	Nonhyd[3]	
O11	Dihydroxylation at piperidine ring and amide group or	10.28	$C_{22}H_{27}FN_2O_3$	387.2077	−2.25	ND	ND	ND	ND	ND	ND	4.7E+04	6.5E+04	ND	ND	105.0690, 164.0871, 186.1276
P11	*N*-oxide + hydroxylation at	10.64		387.2077	−0.91	ND	ND	ND	ND	ND	ND	6.8E+04	6.8E+04			
M11	the amide group	10.52		387.2070	−2.08	ND	ND	ND	ND	ND	ND	6.6E+04	6.2E+04			
O12	Monohydroxylation: at	10.54	$C_{22}H_{27}FN_2O_2$	371.2128	−0.79	ND	ND	3.8E+06	3.8E+06	4.2E+06	3.7E+06	3.2E+06	3.6E+06	ND	ND	105.0695, 150.0710,
P12	phenethyl linker or piperidine	10.53		371.2129	−0.12	ND	ND	4.3E+06	3.4E+06	4.6E+06	4.5E+06	4.4E+06	4.5E+06			160.0864, 186.1277,
M12	ring	10.55		371.2130	−0.28	ND	ND	5.6E+06	3.8E+06	4.2E+06	4.4E+06	5.1E+06	4.4E+06			204.1380, 353.2020
O	Parent compound (fluorofentanyl)	11.56	$C_{22}H_{27}FN_2O$	355.2180	0.03	Sat	Sat	Sat	Sat	Sat	Sat	Sat	Sat	4.3E+04	1.2E+04	84.0808, 105.0702, 134.0965, 150.0710,
P		11.51		355.2178	−0.57	Sat	Sat	Sat	Sat	Sat	Sat	Sat	Sat			188.1438, 234.1291,
M		11.55		355.2179	−0.25	Sat	Sat	Sat	Sat	Sat	Sat	Sat	Sat			299.1912
O13	Dihydroxylation at phenethyl moiety and amide group or	11.84	$C_{22}H_{27}FN_2O_3$	387.2080	−0.23	ND	ND	1.6E+05	1.7E+05	2.9E+05	2.7E+05	2.5E+05	2.9E+05	ND	ND	164.0867, 207.1279
P13	*N*-oxide + hydroxylation at	12.02		387.2068	−3.18	ND	ND	1.7E+04	1.8E+04	2.5E+04	2.3E+04	2.1E+04	2.5E+04			
M13	the amide group	11.99		387.2073	−1.84	ND	ND	3.0E+04	2.5E+04	3.8E+04	3.5E+04	4.0E+04	3.6E+04			
O14	*N*-oxide: at the piperidine	12.66	$C_{22}H_{27}FN_2O_2$	371.2129	0.00	ND	ND	9.9E+05	1.0E+06	1.6E+06	1.5E+06	1.4E+06	1.6E+06	ND	ND	105.0714, 150.0700,
P14	ring	12.81		371.2130	0.01	ND	ND	3.5E+05	3.3E+05	5.3E+05	5.2E+05	5.8E+05	5.8E+05			164.0868
M14		12.79		371.2129	−0.79	ND	ND	ND	3.4E+05	5.2E+05	5.2E+05	6.3E+05	5.8E+05			

[1]Measured at 5 h and sample #2 (except for metabolite 2 measured at 1 h).
[2]Hydrolyzed.
[3]Not hydrolyzed.
Abbreviations: ID = identification (O = ortho, M = meta, P = para; the number that follows represents a consecutive numbering of the metabolites according to their RTs); ND = not detected; NDI = no diagnostic ions found; Sat = detector saturated.

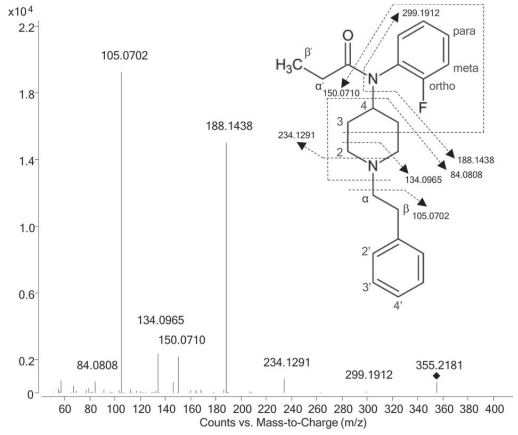

Figure 1. MS/MS spectrum of ortho-fluorofentanyl, suggested explanation of fragmentation and a positional system for indicating the position of substituents.

The addition of an oxygen to fragment ion m/z 188.1438 forming m/z 204.1380 (with water loss to m/z 186.1277) and the fragment ions m/z 150.0710 and 164.0864 indicate hydroxylation in the 2-position. The fragment ion m/z 353.2020 can correspond to an elimination of H_2O from the metabolite which favors an interpretation towards aliphatic hydroxylation, and the fragment ion m/z 105.0695 may have been formed from a *β*-hydroxy-metabolite. Therefore we propose that O12/M12/P12 is a monohydroxy metabolite with the hydroxy group at either the piperidine ring or at the ethyl linker.

The second most abundant monohydroxylated metabolite was O7/M7/P7. Fragment ion m/z 188.1434 corresponds to an intact phenethylpiperidine structure and together with m/z 299.1908 (Figure 1) it indicates hydroxylation on the amide alkyl chain. Traces of this metabolite were detected in the 0 h sample of meta-fluorofentanyl. The Monohydroxylated metabolite O8/M8/P8 was hydroxylated at the phenethyl moiety as indicated by the fragment ions m/z 121.0646 (mass of phenethyl moiety +O). Hydroxylation at the phenyl ring (position 2′, 3′ or 4′) or a *β*-hydroxy at the *N*-alkyl chain is most probable as α-hydroxy-metabolites are not known to exist (intermediate to the *N*-dealkylation pathway). The last monohydroxylated metabolite O14/M14/P14 elutes after the parent drug (Table I) which is unexpected for a more polar compound. These late eluting metabolites have been described previously for *N*-oxide metabolites of fentanyl analogs (7, 10) and in an *in vitro* study of the metabolism of nicotine (17). Based on its RT and mass we propose O14/M14/P14 to be an *N*-oxide (Figure 3). Also, fragment ions m/z 105.0700 and m/z 164.0868 indicate an unchanged phenethyl moiety and *N*-phenyl ring, which is consistent with an *N*-oxide, as shown with similar compounds in previous studies (7, 13).

Five di-hydroxylated metabolites (addition of 2x15.9949 u, i.e., $+O_2$, when compared to the mass of the parent) with the protonated molecular ion $[C_{22}H_{27}FN_2O_3 + H]$ and m/z 387.2078 were detected per parent *in vitro*. O13/M13/P13 was already detected in the 1 h samples while the other four were only present in the 5 h samples. No di-hydroxylated metabolites were detected in the authentic urine sample. The most abundant O13/M13/P13 with fragment ion at m/z 164.0867 and lack of m/z 188.1434 indicates an unchanged *N*-phenyl ring and that hydroxylation has occurred at the amide alkyl chain, phenethyl moiety or at the 2-position of the piperidine ring. Fragment ion m/z 207.1279 can be formed by loss of the amide alkyl chain and the phenethyl moiety (with one carbon left; $C_{12}H_{16}FN_2$), see supplementary information (Figure S1). This opens up for a second interpretation that includes a monohydroxylated *N*-oxide or a water loss from an *N*-oxide with dihydrodiol. O11/M11/P11 shows a fragment of m/z 186.1276, which indicates that the first hydroxylation is at position 2 at the piperidine ring (after water loss similar to O12/M12/P12) and the fragments with m/z 105.0700 and 164.0868 suggesting an intact *N*-phenyl ring and phenethyl moiety and therefore the second hydroxylation at the amide alkyl chain. The presence of a fragment ion at m/z 207.1247 means that the first oxidation can be an *N*-oxide (as shown for O13/M13/P13). The third di-hydroxylated metabolite O10/M10/P10 was detected and the fragment ion m/z 121.0643 indicates hydroxylation on the phenethyl moiety. The location of the second hydroxyl group could not be determined by the MS/MS spectrum but 164.0863 indicates position 2 at the piperidine ring. The MS/MS spectrum of O4/M4/P4 and O6/M6/P6 did not contain any diagnostic ions which could help interpret the position of the hydroxyl groups.

Two compounds corresponding to dihydrodiol metabolites were detected, O3/M3/P3 and O5/M5/P5. Theoretically a dihydrodiol can be located on the ethylphenyl ring or the *N*-phenyl ring. The common fragment ion of m/z 164.0864/164.0875 suggests that the *N*-phenyl ring is unchanged in both compounds and that the ethylphenyl ring is the target of the biotransformation. Watanabe *et al*. suggested in a study of the metabolism of 4-fluoro-isobutyrylfentanyl that a water loss from one of the dihydrodiol hydroxyl groups leads to re-aromatization and that this is a favorable reaction that explains the absence of the expected fragment ion m/z 139.0754 but the presence of fragment ions m/z 121.0640/121.0633 (7). The exact position of the dihydrodiol hydroxyl groups on the ethylphenyl ring could not be determined from the fragmentation pattern. Finally a compound corresponding to a hydroxylated and methoxylated metabolite with the protonated molecular ion $[C_{23}H_{29}FN_2O_3 + H]$ was detected, O9/M9/P9. The fragment ion at m/z 151.0749 suggests the presence of hydroxyl and a methoxy group at the phenethyl moiety (4′-hydroxy-3′-methoxy-fluorofentanyl). The presence of m/z 119.0487 is caused by the loss of methanol.

Metabolites detected in an authentic urine sample

Three of the metabolites found *in vitro* were also detected in the authentic ortho-fluorofentanyl urine sample. These were norfluorofentanyl (O2), 2′, 3′ or 4′-hydroxy-fluorofentanyl (O8) and either 4′-hydroxy-3′-methoxy-fluorofentanyl or 3′-hydroxy-4′-methoxy-fluorofentanyl (O9). The chromatographic separation of the metabolites of ortho-fluorofentanyl in the authentic urine sample is shown in Figure 2B. To enable comparisons between MS/MS spectra of metabolites detected in both urine and *in vitro* samples these spectra are shown as supplementary information (Figure S2). In urine, O9 had the highest abundance, in contrast to the *in vitro* experiment where this metabolite was of low abundance compared to the other metabolites. Norfluorofentanyl (O2) and 2′, 3′ or 4′-hydroxy-fluorofentanyl (O8) was the second and third most abundant metabolite in the authentic urine sample.

Differences between metabolites of ortho-, meta- and para-fluorofentanyl

When comparing the RTs and chromatographic peak areas of the corresponding metabolites from the three fluorofentanyl isomers,

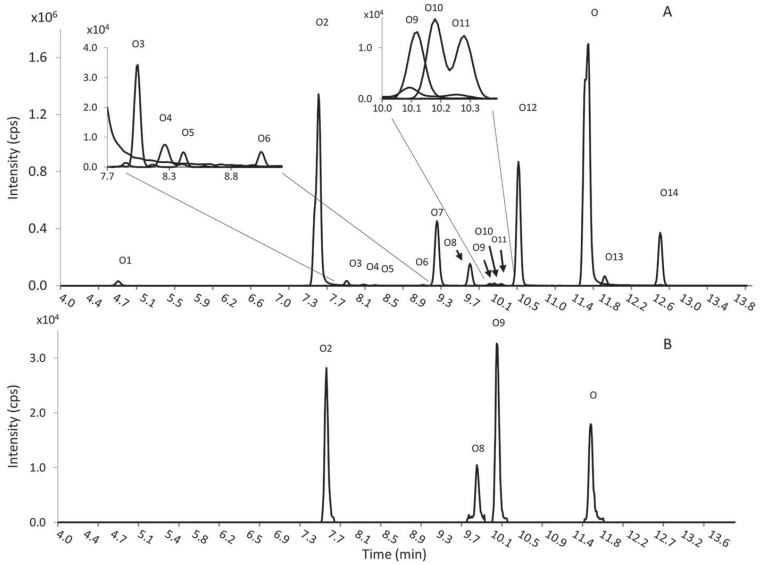

Figure 2. Extracted ion chromatogram (EIC) of ortho-fluorofentanyl and metabolites from 5 h incubation with human hepatocytes (A) and the hydrolyzed authentic urine sample (B). Letter and numbers on top of the peaks correspond to those used in Table I; O represents ortho-fluorofentanyl and O1-O14 represents its metabolites. Magnified EICs of the minor metabolites O3, O4, O5, O6, O9, O10 and O11 are also displayed.

there were some notable differences. The three parent compounds eluted within 0.06 min from each other with para-fluorofentanyl eluting first (Table I). Such a small difference could be a result of instrumental instability, but the two IS (amphetamine-d8 and midazolam-d3) showed excellent RT stability (within 0.01 min) between the samples, indicating that the difference between the parent compounds is real. The retention order varied from metabolite to metabolite (Table I), and the RT difference varied from 0.02 min (O12/M12/P12) to 0.37 min (O11/M11/P11). The position of the fluorine atom obviously affects the RT more for some metabolites than for other. There were also differences in peak areas between isomers, but without any clear pattern. Metabolites from all three parent compounds were formed by the same principle pathways even though the difference in peak areas for some of the metabolites was considerable; e.g., for O13/M13/P13 it was 10-fold. As relative peak areas between metabolites probably vary between individuals, they can most likely not be used as an identification parameter.

Metabolic pathways

The general metabolic pattern for the three fluorofentanyl isomers could be suggested as shown for ortho-fluorofentanyl in Figure 3. Metabolites were formed through *N*-dealkylation and/or oxidations. In addition, a pathway of hydroxylation and methylation via dihydrodiol forming O9/M9/P9 could be proposed. The same pathway was shown by. Watanabe *et al*., which suggested that the dihydrodiol compound can be metabolized by the enzyme cathechol-O-methyltransferase to a metabolite containing a hydroxyl group and methylation of a second hydroxyl group giving O9/M9/P9, possibly through an intermediate catechol compound (7). However, such a dihydroxylated precursor of O9/M9/P9 could not be detected among the metabolites. The main *in vitro* metabolites norfluorofentanyl (O2/P2/M2) and hydroxyl fluorofentanyl (O7/P7/M7, O8/P8/M8, O12/P12/M12 and O14/P14/M14) were consistent with findings in previous studies on fentanyl and some other analogs (6–8, 10). Glucuronidated metabolites were not observed. This is consistent with previous studies performing *in vitro* experiments of fentanyl analogs with hepatocytes. Watanabe *et al*. detected only one glucuronidated metabolite from the fentanyl analogs acetylfentanyl, acrylfentanyl, furanylfentanyl and 4-fluoro-isobutyrylfentanyl. Carboxylated metabolites have been detected in previous studies of fentanyl analogs, e.g., 2,2,3,3-tetramethyl-cyclopropylfentanyl (13) and crotonylfentanyl (14). No metabolites of this type were detected for fluorofentanyl. The amide hydrolysis product, fluoro-4-anilino-*N*-phenylpiperidine, was detected in the degradation control, 0 h sample and with a declining peak area throughout the experiment. This finding indicates that the compound is not formed *in vitro* which is in contrast to other studies of similar fentanyl analogs where amide hydrolysis is a significant metabolic pathway (7–9). The relative low number of metabolites detected in the authentic urine sample compared to the *in vitro* study can at least partly be attributed to the low drug concentration in the urine sample and the simple dilute-and-shoot sample preparation.

Norfluorofentanyl (O2/P2/M2) may be a suitable marker of drug intake as it was the most abundant metabolite found *in vitro* and was also detected in the authentic urine sample. However, norfluorofentanyl might not be specific enough to be used as a single analytical target. Future fentanyl analogs could potentially produce this metabolite as well, and a more specific marker will be necessary to unambiguously identify drug exposure. O8/P8/M8 includes the whole structure of its parent drug and might therefore be a better candidate. Unfortunately, even though abundant *in vitro*, only traces were detected in the authentic urine sample. O9/P9/M9 is another specific marker and according to the results of the authentic urine sample likely to be in relative high abundance.

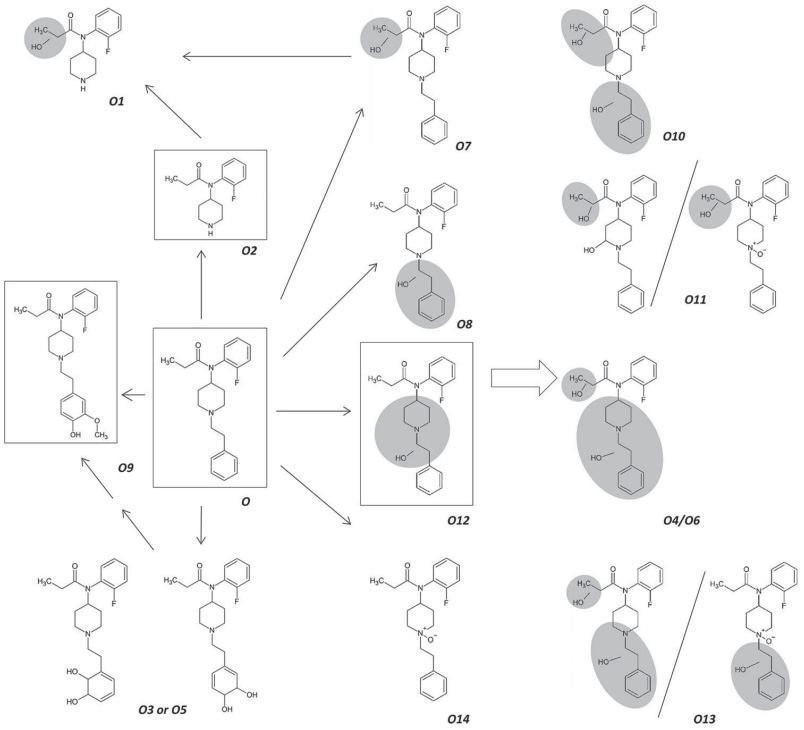

Figure 3. Metabolic pathway of ortho-fluorofentanyl (O). Compounds in frames were detected both in the *in vitro* study and in the authentic urine sample. The others were detected in the *in vitro* study, only. Highlighted parts of the molecules indicate possible positions of hydroxylation. The numbering of the metabolites corresponds to those used in Table I.

The elimination half-lives of ortho- meta- and para-fluorofentanyl are not studied, but it is reasonable to believe that they would be similar to fentanyl itself, and thus to be somewhere between 3 and 12 hours (18). Detection times in urine might be extended by using the O9/P9/M9 and/or O8/P8/M8 as markers, but this must be confirmed by analyses of several positive samples.

Strengths and weaknesses

Human hepatocytes were chosen over human liver microsomes in this *in vitro* model, as they are living cells and contain all endogenous enzymes, cofactors, drug transporters and drug-binding proteins to mimic human drug metabolism. However, due to extrahepatic drug metabolism and transport as well as inter-individual differences, the in vitro results may differ from those obtained *in vivo*. Even though only one authentic urine sample was available, the results indicate that there are differences both in the range of metabolites and the number detected. However, just having one authentic urine sample available is clearly a weakness of this study and a definite recommendation on the most appropriate marker to choose when analyzing human urine cannot be given.

Compounds corresponding to the hydroxylated metabolites of diclofenac, omeprazole and midazolam were detected in the positive control samples incubated for 5 h, showing that the cells were functional. No glucuronidated metabolites were detected in the *in vitro* study or in the authentic sample, which may be due to a very limited formation of these and/or lack of detection capacity with the analytical instrument used. The similar peak areas observed for the same metabolite in the hydrolyzed and nonhydrolyzed authentic urine sample indicate that glucuronidation is not taking place, but again, caution should be exercised in the interpretations as only one sample was available.

The use of LC-QTOF-MS or other comparable HR-MS instrumentation is a well suited approach for acquiring identification data from *in vitro* experiments, as accurate masses and MS/MS spectra can be used to tentatively elucidate the structures. However, complete determination of the structures is not possible without synthesizing and characterizing (by e.g., nuclear magnetic resonance spectroscopy and chromatography) a range of possible candidates and comparing these with those detected *in vitro*.

Conclusions

Fourteen metabolites for each of the three parent compounds ortho- meta- and parafluorofentanyl were formed after incubation with hepatocytes and detected by LC-QTOF-MS analysis. There were no principal differences in which metabolites were formed by the three positional isomers. The detected metabolites were in accordance with the expectations based on *in vitro* data from other similar fentanyl analogs and included norfluorofentanyl, an *N*-oxide at the piperidine ring, hydroxylated and methylated metabolites. The most abundant metabolite *in vitro* was norfluorofentanyl which was also detected in the authentic urine sample together with a monohydroxylated

metabolite and a both hydroxylated and methylated metabolite. The current approach, using hepatocytes and HR-MS, is a relatively cost-effective and straight-forward tool to generate information on the metabolism and identify potential targets for metabolite of illicit drug detection in urine samples, thereby being able to determine both the specific substances ingested and increasing the time window for detection after intake.

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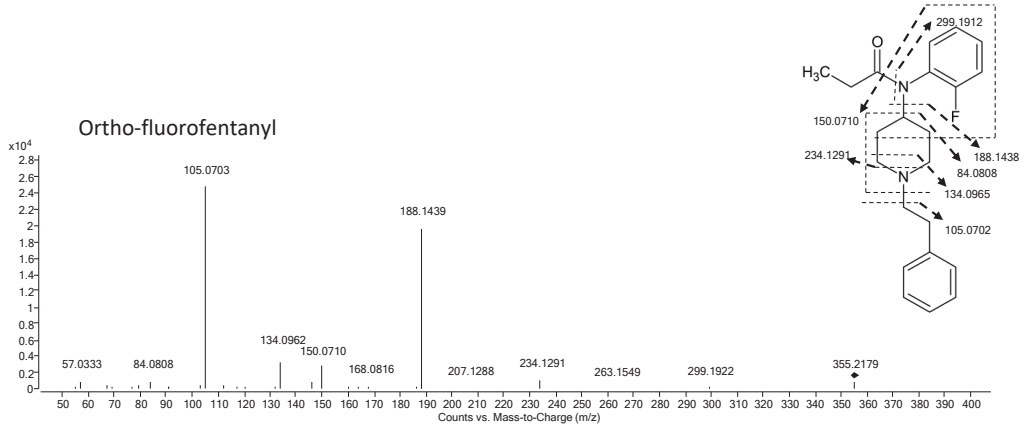
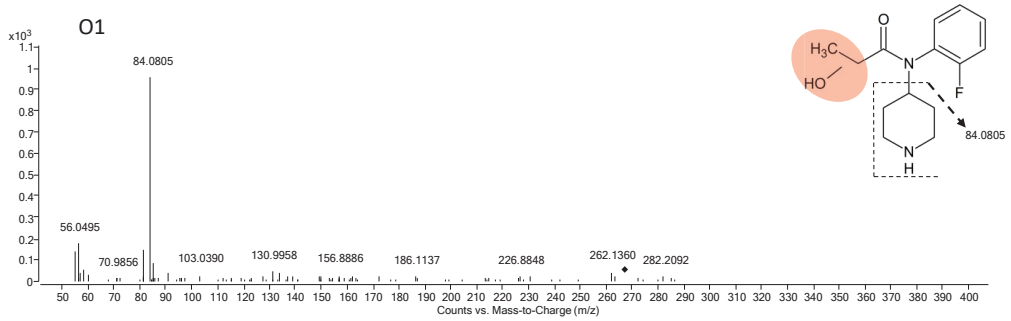
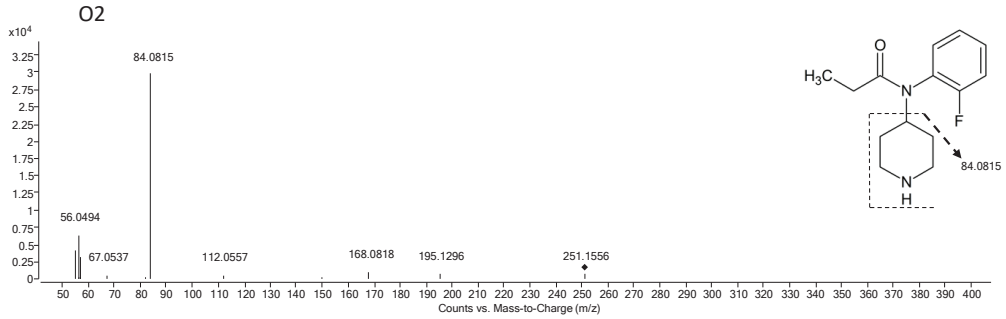
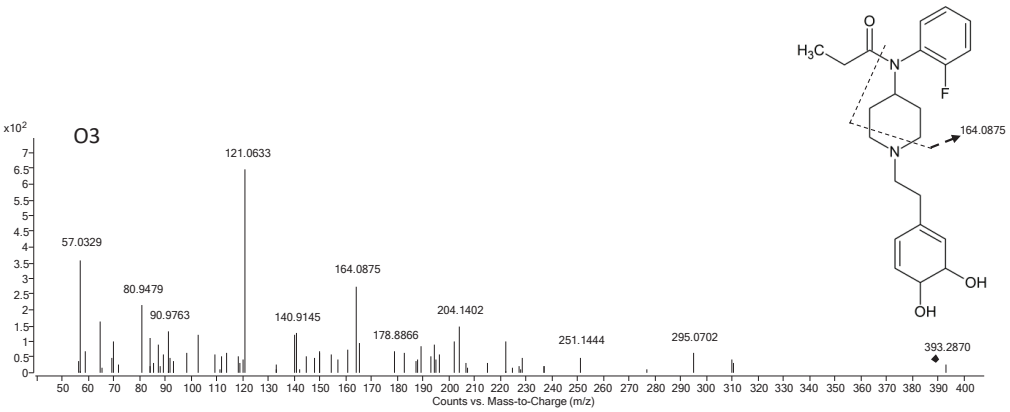
Supplementary information – Paper II

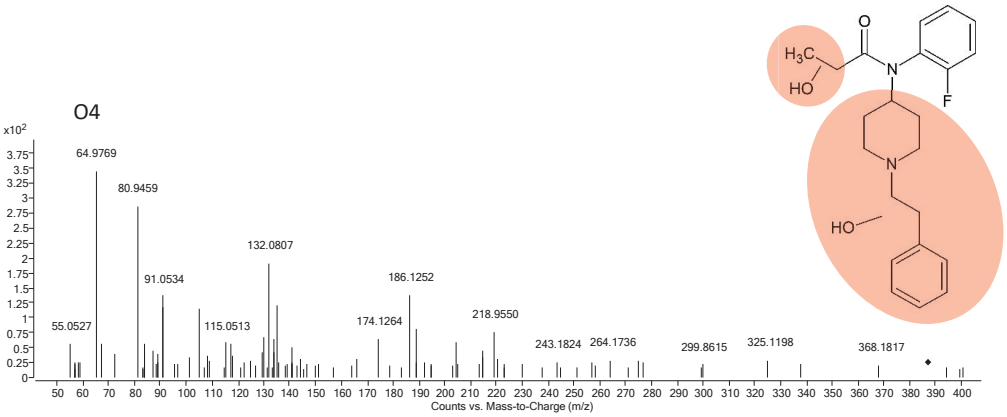
Figure S1: Ortho-fluorofentanyl and its 14 metabolites O1-O14 identified in the in vitro study, with their MS/MS spectra and purposed structures. Highlighted parts of the molecules indicate possible positions of hydroxylation.








O3
x10²
121.0633
57.0329
164.0875
80.9479
90.9763
140.9145
204.1402
178.8866
251.1444
295.0702
393.2870
Counts vs. Mass-to-Charge (m/z)
O
H3C
N
F
164.0875
OH
OH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x10²
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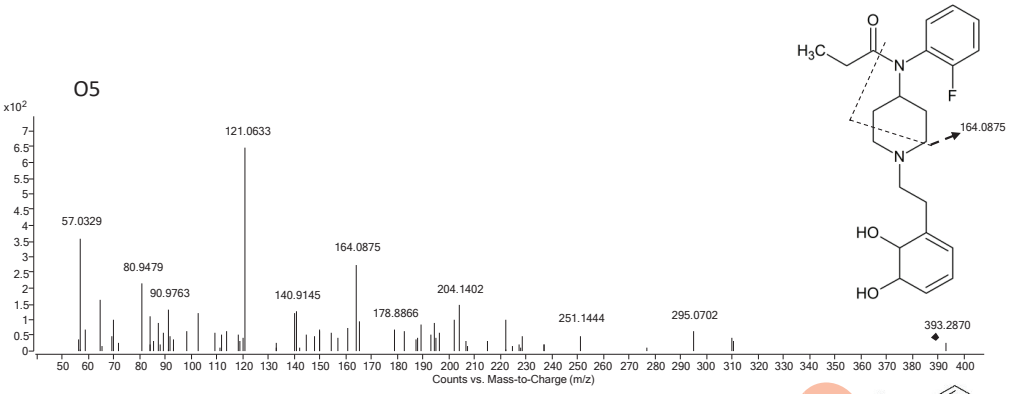
132.0807
91.0534
186.1252
55.0527
115.0513
174.1264
218.9550
243.1824
264.1736
299.8615
325.1198
368.1817
Counts vs. Mass-to-Charge (m/z)
O
H3C
HO
N
F
HO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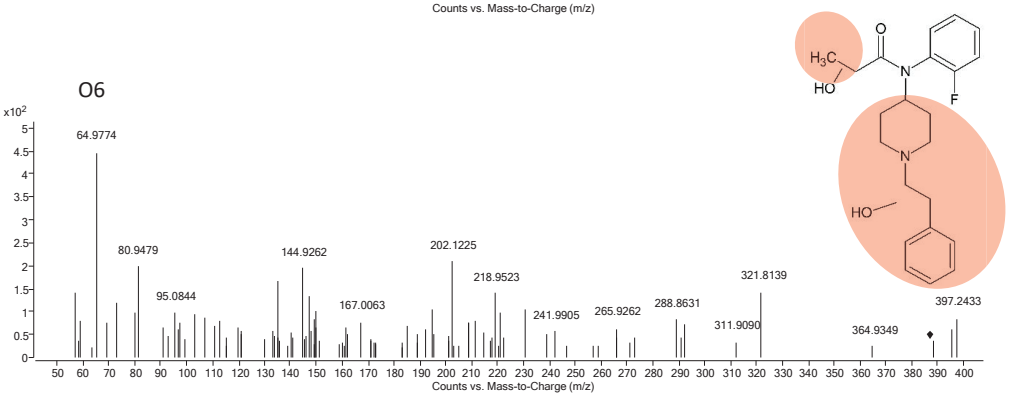
57.0329
164.0875
80.9479
90.9763
140.9145
204.1402
178.8866
251.1444
295.0702
393.2870
Counts vs. Mass-to-Charge (m/z)
O
H3C
N
F
164.0875
HO
HO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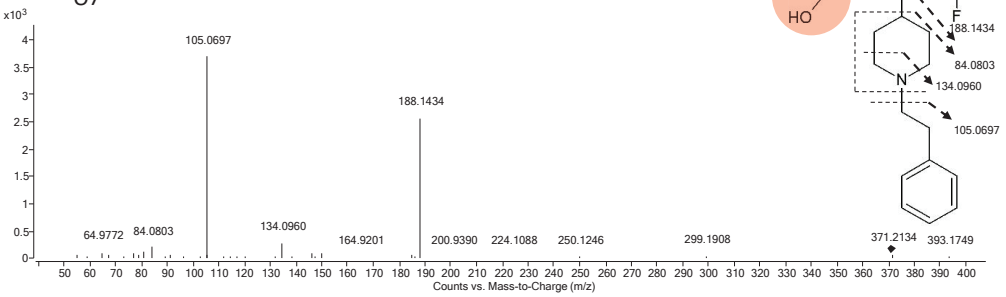
202.1225
218.9523
95.0844
167.0063
241.9905
265.9262
288.8631
321.8139
311.9090
364.9349
397.2433
Counts vs. Mass-to-Charge (m/z)
O
H3C
HO
N
F
HO

O7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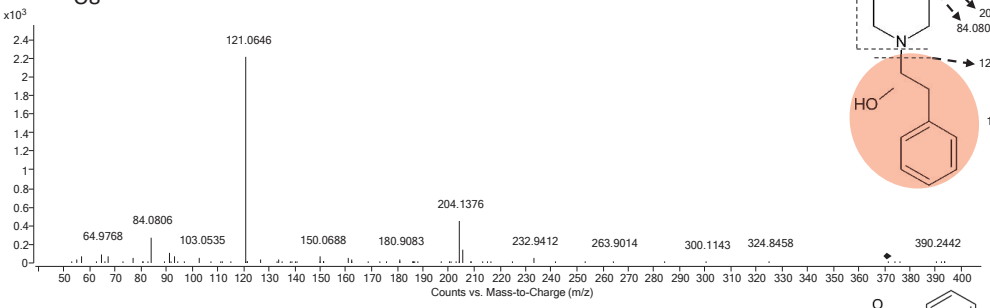
188.1434
64.9772
84.0803
134.0960
164.9201
200.9390
224.1088
250.1246
299.1908
371.2134
393.1749
Counts vs. Mass-to-Charge (m/z)

299.1908
188.1434
84.0803
134.0960
105.0697

O8


x10^3
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204.1376
84.0806
64.9768
103.0535
150.0688
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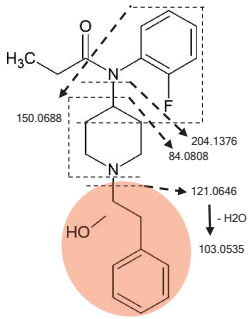
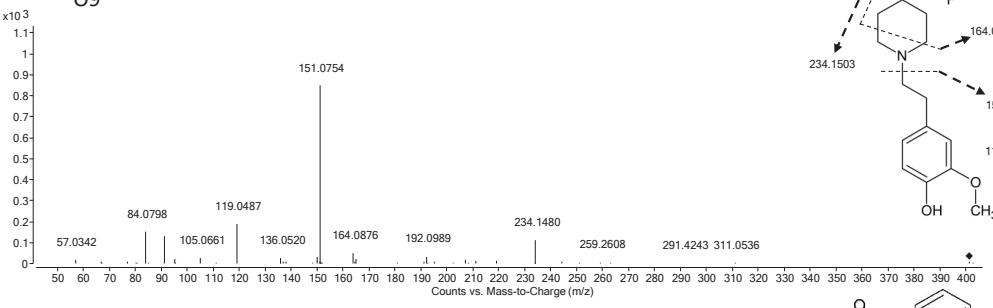
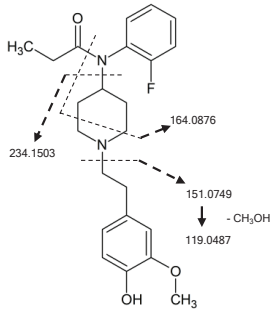
232.9412
263.9014
300.1143
324.8458
390.2442
Counts vs. Mass-to-Charge (m/z)

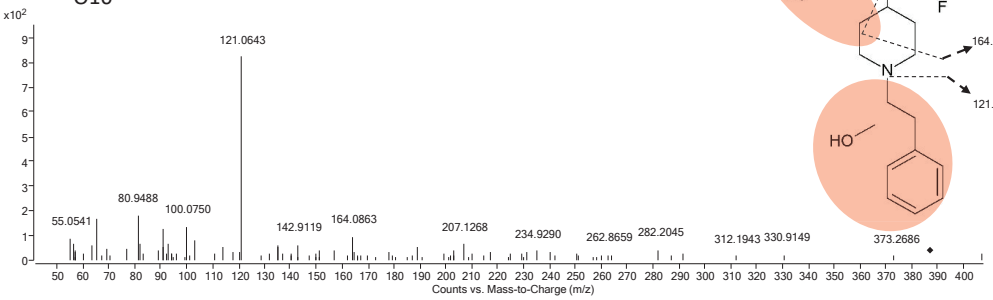

150.0688
204.1376
84.0808
121.0646
- H2O
103.0535

O9


x10 3
151.0754
84.0798
119.0487
57.0342
105.0661
136.0520
164.0876
192.0989
234.1480
259.2608
291.4243
311.0536
Counts vs. Mass-to-Charge (m/z)

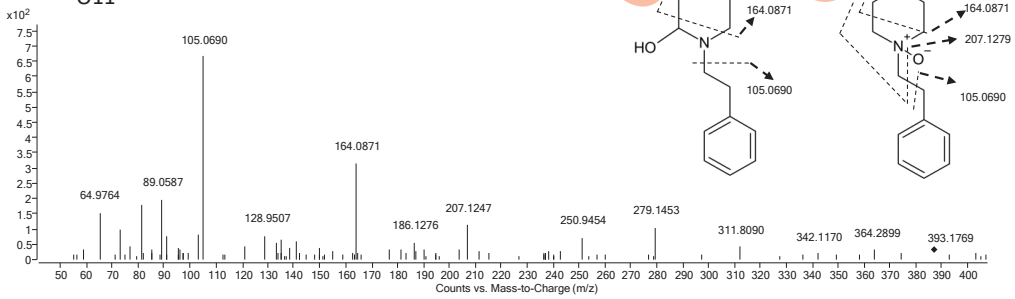

164.0876
234.1503
151.0749
- CH3OH
119.0487

O10

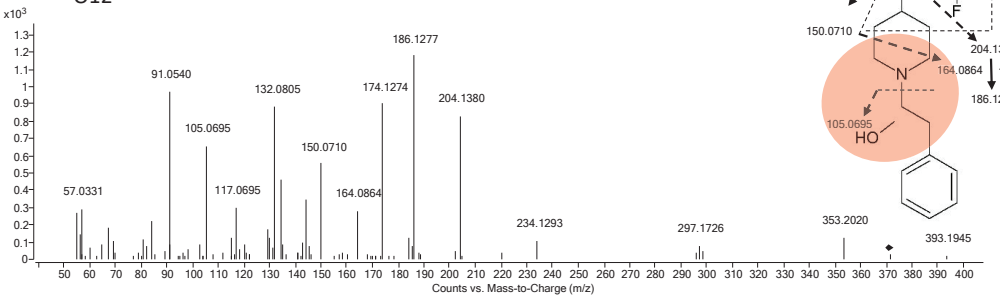

x10^2
121.0643
80.9488
100.0750
55.0541
142.9119
164.0863
207.1268
234.9290
262.8659
282.2045
312.1943
330.9149
373.2686
Counts vs. Mass-to-Charge (m/z)

164.0863
121.0643

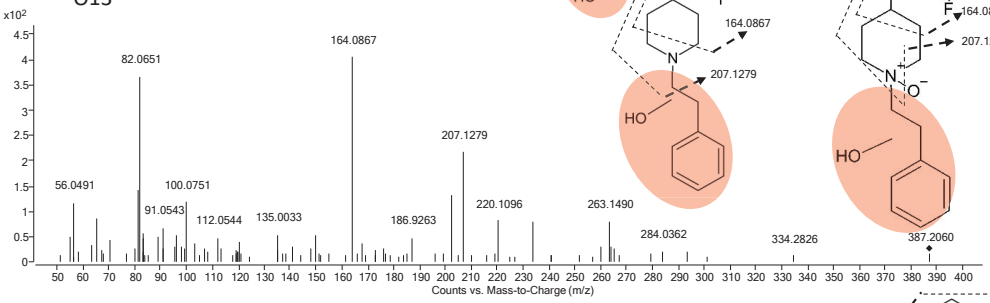
O11



O12



O13



O14

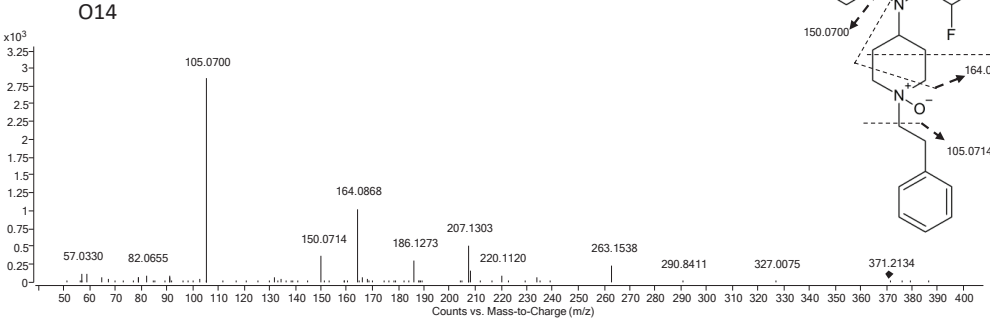
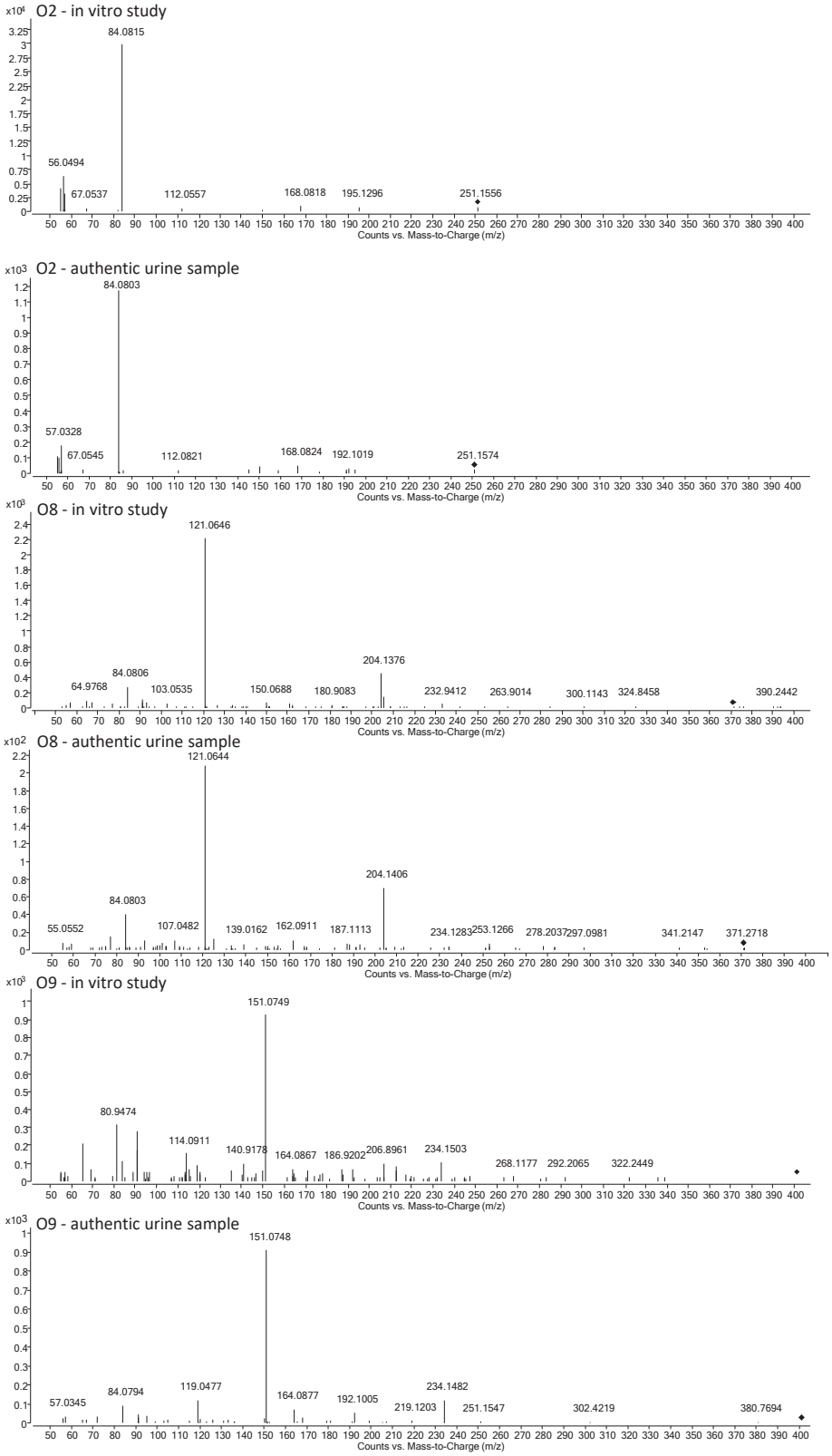


Figure S2: MS/MS spectra of the three metabolites O2, O8 and O9 which were detected in the in vitro study (upper panels) as well as in the authentic urine sample (lower panels).



Paper III

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Retrospective screening of synthetic cannabinoids, synthetic opioids and designer benzodiazepines in data files from forensic post mortem samples analysed by UHPLC-QTOF-MS from 2014 to 2018

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ABSTRACT

The introduction of new psychoactive substances (NPS) on the illicit drug market has led to major challenges for the analytical laboratories. Keeping screening methods up to date with all relevant drugs is hard to achieve and the risk of missing important findings in biological samples is a matter of concern. Aiming for an extended retrospective data analysis, diagnostic fragment ions from synthetic cannabinoids (n = 251), synthetic opioids (n = 88) and designer benzodiazepines (n = 26) not included in our original analytical method were obtained from the crowdsourced database HighResNPS.com and converted to a personalized library in a format compatible with the analytical instrumentation. Data files from the analysis of 1314 forensic post mortem samples with an Agilent 6540 ultra high pressure liquid chromatography quadrupole time-of-flight mass spectrometry (UHPLC-QTOF-MS) performed in our laboratory from January 2014 to December 2018 were retrieved and retrospectively processed with the new personalized library. Potentially positive findings were grouped in two: The most confident findings contained MS/MS data for library match (category 1) whereas the less confident findings lacked such data (category 2). Five new category 1 findings were identified: Flubromazepam in two data files from 2015 and 2016, respectively, phenibut (4-amino-3-phenylbutyric acid) in one data file from 2015, fluorofentanyl in one data file from 2016 and cyclopropylfentanyl in one data file from 2018. Retention time matches with reference standards further strengthened these findings. A list of 35 presumably positive category 2 findings was generated. Of these, only one finding of phenibut was considered plausible after checking retention times and signal-to-noise ratios. This study shows that new compounds can be detected retrospectively in data files from QTOF-MS using an updated library containing diagnostic fragment ions. Automatic screening procedures can be useful, but a manual re-evaluation of positive findings will always be necessary.

1. Introduction

In recent years, there has been a continuously increasing number of new psychoactive substances (NPS) appearing on the European illicit drug market [1]. The diversity and high number of new compounds pose challenges for clinical and toxicological laboratories who strive to keep their drug screening methods updated. Synthetic cannabinoids, i.e. compounds acting as cannabinoid receptor agonists and produced as alternatives to Δ-9-tetrahydrocannabinol (THC) represent the largest and most structurally diverse group [2]. New synthetic opioids, and in particular the fentanyl analogues, have been of mounting concern because of their formidable toxic potential [3–6]. Designer benzodiazepines is another group in focus due to the high prevalence of use, at least in our country [7], compared to other groups of NPS.

To develop, establish and maintain a screening method capable of detecting all drugs relevant at any given time is a major challenge. The use of high resolution mass spectrometry (HR-MS)

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e.g. quadrupole time of flight mass spectrometry (QTOF-MS) instrumentation has proven to be an applicable tool when searching for drugs of abuse in biological samples [8–13]. The detection of unknown compounds is time consuming and hardly feasible on a routine basis with a large number of samples. Consequently, the method must encompass a screening or targeted approach, based on an extensive and comprehensive database containing multiple types of data for identification. Such data can be retention times (RTs) and fragmentation data from collision-induced dissociation (CID), in silico or other theoretical evaluations, in addition to the molecular formula of the substance. The database can be created and maintained "in-house" by the laboratory. This requires access to a high number of well-defined reference compounds. Procurement of reference standards is costly, particularly if a database should be up to date with as many new and relevant compounds as possible. Databases are also commercially available from suppliers of MS instruments (e.g. the Forensic Toxicology Personal Compound Database and Library from Agilent), but users are dependent on the frequency of new releases and/or additions being up to date. There are also examples of commercial operators offering free databases (e.g. the mzCloud from ThermoFisher). Another opportunity is crowdsourced databases with information submitted by global HR-MS users. One such example is HighResNPS.com [14]. When performing CID on a certain compound, different instrument configurations tend to generate the same diagnostic fragment ions even though the relative abundance may vary. Thus, fragment data acquired on one instrument can then be used as identification across platforms [14–16]. In principle, the same is true for a crowdsourced database with diagnostic fragments acquired by instruments from different manufacturers, providing that the added fragment masses are converted to theoretical values.

In contrast to analytical methods based on single ion monitoring or multiple reaction monitoring, HR-MS full-spectrum data remain available and permit the identification of non-target compounds and retrospective analysis, also called post-target analysis. For data from HR-MS instrumentation with fragmentation capabilities, e.g. QTOF-MS or linear ion trap Orbitrap, fragmentation data are also available. In principle, all compounds are available for investigation at a certain level, but the data available are limited by sample extraction recovery, chromatographic selectivity and the degree of ionization and fragmentation. Depending on which acquisition mode is used, the QTOF-MS data also contain fragment ions originating from the molecular ions generated in the ion source. Based on new knowledge, post-targeted analysis of data can generate new findings in a specific toxicological or clinical sample and ultimately change the conclusion in a particular case. A retrospective study is also important as an internal quality check for the laboratory to assess whether the screening repertoire used is comprehensive and relevant. In addition, new trends in drug abuse can be identified, as exemplified in the study by Kriikku et al. where the toxic lifespan of U-47700 was explored [17].

The number of studies applying such a retrospective approach in a forensic or clinical toxicology setting are limited. Noble et al. processed 2339 forensic samples retrospectively with a targeted screening method to detect 50 4-anilidopiperidine-related fentanyl analogues [18]. In another case study U-47700, diclazepam and flubromazepam were detected in retrospect [19]. Mollerup et al. applied a post-targeted approach when developing a screening method for valproate using positive ionisation mode [20]. Retrospective analysis of urine samples has been used to detect metabolites of pesticides [21]. Post-targeted analysis of data has also been used for detection of drugs and pesticides in non-human matrices including sewage water, surface water and food [22–26].

Since December 2013, our laboratory has utilized a workflow based on ultra-high performance liquid chromatography (UHPLC) coupled to a 6540 QTOF-MS from Agilent (Santa Clara, CA, USA) for therapeutic drugs and drugs of abuse in post mortem blood samples. The same UHPLC and MS method has been applied from 2014 to the present. A commercial database supplied with entries added manually after analysing reference materials has been used for identification. However, in order to detect a new or previously unknown drug in a biological sample, additional information connected to the case or sample (e.g. a seizure) has to be available. In our experience, such information is rarely available, and this may increase the risk of missing detection of NPS. The consistency of the screening method enables retrospective analysis so that new compounds can be found. The use of HighResNPS for identifying compounds in samples analysed on Agilent QTOF-MS has previously been shown, but only files from data independent acquisition (DIA) could be investigated with this approach [14]. Our method was based on data dependent acquisition (DDA) which, as opposed to DIA, involves acquiring of MS/MS spectra after selection of precursor ions isolated by the quadrupole. A thorough explanation of the differences between DIA and DDA can be found e.g. in the papers of Sundström et al. [27] and Broecker et al. [8]. To be able to use HighResNPS, diagnostic fragment information from the database had to be converted to spectra in the format accepted by the Agilent MassHunter Qualitative searching tool. In Agilent terminology, a library is the sum of compounds in a database containing MS/MS spectra and these databases and libraries are called Personal Compound Database and Library (PCDL).

The aim of this study was to re-process data files of forensic post mortem samples acquired from January 2014 to December 2018 in a PCDL-facilitated search for NPS belonging to the subgroups synthetic cannabinoids, synthetic opioids and designer benzodiazepines.

2. Materials and method

2.1. Chemicals and reagents

Reference substances used in the experiments to calculate recoveries and matrix effects and explore instrument sensitivities were purchased as solid material or stock solutions from either of the following sources: Cayman Chemicals (Ann Arbor, MI, USA), Chiron AS (Trondheim, Norway), Sigma Aldrich (St. Louis, MO, USA) and Lipomed (Arlesheim, Switzerland). Individual stock solutions in the range from 0.2 to 1.0 mg/mL were prepared and combined into working solutions which were spiked into blood. For confirmation of tentative findings, reference substances of tilidine, phenibut (4-amino-3-phenylbutyric acid) and JWH-167 were purchased from Sigma Aldrich, Chiron AS and Cayman Chemicals respectively. LC–MS quality acetonitrile, methanol, LiChrosolve® water and ARISTAR® formic acid were all purchased from VWR Chemicals (Oslo, Norway). Ammonium acetate of LC–MS grade was from Sigma Aldrich (St. Louis, MO, USA). A solution of the internal reference standards codeine-d3, morphine-d3, benzoylecgonine-d3 and griseofulvin was prepared by diluting stock solutions in 20% methanol (v/v) in water to a final concentration of 200 ng/mL. D3-codeine, d3-morphine and d3-benzoylecgonine were from Lipomed whereas griseofulvin was from Janssen Chimica (Geel, Belgium).

2.2. Validation of original screening method

2.2.1. Instrument sensitivity and limit of identification

The same UHPLC-QTOF-MS instrumental method, sample preparation and internal reference standard concentration were used for all the samples throughout the period. The peak area results and RTs of the internal reference standards in one data file

per batch were extracted in order to illustrate the variation in response over time. Limit of identification (LOI) was evaluated for a selection of synthetic cannabinoids (MDMB-CHMICA, AB-CHMINACA, BB-22, JWH-018, PB-22 and THJ-018), synthetic opioids (fentanyl, remifentanil, cyclopropylfentanyl, para-fluorofentanyl, furanylfentanyl, acetylfentanyl) and designer benzodiazepines (deschloroetizolam, diclazepam, etizolam, flubromazepam, flubromazolam, pyrazolam and meclonazepam). Blood samples were spiked at 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, 10 and 20 ng/mL, and prepared in triplicates with the same method as described for the post mortem samples. LOI was defined as the minimum concentration where the compound was identified and at least one MS/MS spectrum was acquired for library search in all three parallels (see Section 2.5 for details on identification).

2.2.2. Recovery and matrix effects

Recoveries (REs) and matrix effects (MEs) were calculated for the same compounds as used in the LOI experiment. Subsamples of pooled whole blood were spiked after (B) or before (C) extraction to a final concentration of 0.1 μg/mL. The peak areas in neat standard solution of the same concentration (A), sample B and C were used to calculate RE and ME (Eqs. (1) and (2)). An ME below 100% indicates ion suppression whereas a value above 100% indicates ion enhancement.

$$RE\ (\%) = \frac{C}{B} \times 100 \quad (1)$$

$$ME\ (\%) = \frac{B}{A} \times 100 \quad (2)$$

2.3. Original analysis of the blood samples

Data files included in this study were from the analyses of post mortem blood samples from forensic autopsies sent to our laboratory in the period from January 2014 to December 2018. In a limited number of cases where blood was not available, spleen tissue was used. Samples from a total of 1314 cases were analysed in this period. Permission to re-process the data files (in this context meaning opening the data file and run the algorithm with the new PCDL) was given by the Regional Committee of Medical and Health Research Ethics in Mid Norway (approval No. 2018/2157). The data files were anonymized and the analyst had no information about the original findings when doing the re-processing. A second person compared the new findings with the analytical report originally attached to the relevant cases. According to the permission granted from the ethics committee, re-analysis of the sample specimens as such could not be performed. The samples were originally processed with the commercially available *Forensic Toxicology Personal Compound Database and Library* from Agilent (Santa Clara, CA, USA) with more than 3000 compounds containing MS/MS spectra complemented with between 250 and 300 compounds with RTs.

2.3.1. Sample preparation

Each blood sample was thawed at room temperature and 200 mg was weighed into a micro tube and 50 μL solution of internal reference standard and 800 μL ice-cold acetonitrile were added. The tube was then mixed on a vortex mixer for 30 s and centrifuged at 7000 *g* for 10 min. before 500 μL of the supernatant was transferred to a 96-well plate, evaporated to dryness and reconstituted in 50 μL of 30% acetonitrile (v/v) in 0.03 mg/mL ammonium formate. In the cases where only spleen was available, sample preparation was adjusted according to the condition of the tissue. If a blood-like material could be obtained from the spleen, it was handled as a blood sample. In the other cases a subsample of tissue material was homogenized with an equal volume of H_2O, and 200 mg of this material were processed like a blood sample. The samples were prepared in weekly batches by the same procedure throughout the period.

2.3.2. Instrumentation

Instrumental analysis was performed using a 6540 QTOF-MS (Agilent, Santa Clara, CA, USA) with electrospray ionization (ESI) coupled with a 1290 Infinity UHPLC system from Agilent equipped with an Acquity HSS T3 column (100 mm × 2.1 mm, 1.8 μm) from Waters (Milford, MA, USA). An injection volume of 2 μL was used. Separation was achieved using a mobile phase consisting of 0.05% formic acid in 10 mM ammonium formate (A) and 0.05% formic acid in acetonitrile (B). A gradient with a flow of 0.50 ml/min starting at 5% B increasing to 50% in 10 min. and continuing to 100% over the next 6 min. was used. After a 4-minute hold at 100% B the column was re-equilibrated for 2 min. at 5% B, giving a total cycle time of 22 min. Autosampler and column temperatures were set to 10 °C and 50 °C, respectively.

Positive ESI was used and with fragmentor voltage at 120 V, capillary voltage at 3500 V, gas temp at 320 °C, gas flow at 8 L/min, nebulizer pressure at 40 psig and sheath gas temperature at 380 °C. Data was acquired in data dependent Auto MS/MS mode. MS spectra and MS/MS spectra were both acquired in the mass range of 50–1000 *m/z* at a rate of 6 Hz. The detector operated in 2 GHz extended dynamic range giving a resolution (m/Δm at FWHM) of approx. 20,000 at *m/z* 322.0481. Precursor selection was based on abundance and an intensity threshold of 1000 counts was applied. After one spectrum from a precursor was acquired, this specific precursor was excluded for 0.03 min. Precursors were fragmented in the collision cell using an electron voltage according to Eq. (3):

$$Collision\ energy\ (eV) = 4 + (0.06 \times m/z\ of\ precursor) \quad (3)$$

The computer controlling the instrument was equipped with the MassHunter Acquisition software (Acq) B.05.01 (Agilent, Santa Clara, CA, USA). The acquired data files consisted of MS1 (full spectrum MS-only) of all ionized compounds and MS/MS spectra of the precursors selected for fragmentation. The *m/z* masses of 121.0509 and 922.0098 were applied for automated mass correction in all MS spectra. A daily performance sample of amphetamine (0.74 ng/mL), diazepam (0.35 ng/mL), 7-amino-flunitrazepam (0.35 ng/mL), morphine (0.35 ng/mL) and Δ9-tetrahydrocannabinol (0.5 ng/mL) in MeOH was injected at the beginning of every analytical run to monitor important instrument parameters. Samples were not analysed if large deviations in RTs (more than 0.2 min.), mass accuracies (more than 5 ppm) or peak areas from the historical averages were observed for the compounds in the daily performance sample.

2.4. Creating a new PCDL

HighResNPS (highresnps.com) is a free, online, spreadsheet-format, crowdsourced HR-MS database for NPS-screening initiated and managed by a group of researchers at Section of Forensic Chemistry at the University of Copenhagen [14]. Several contributors worldwide submit fragmentation data when new drugs (reference standards or seizures etc.) are detected and analysed by a HR-MS instrument. Also, diagnostic ions derived from theoretical dissociations of the molecules are supplied. From this HighResNPS database (total number of entries in May 2019 was 1782 including duplicates, and 1304 contained at least one diagnostic fragment ion), 374 unique compounds with minimum one diagnostic fragment primarily belonging to the drug classes synthetic cannabinoids, synthetic opioids or designer benzodiazepines were selected. NPS already present in the screening method

Table 1
Number of new compounds included in the HighResNPS subset Personal Compound Database and Library (PCDL) grouped according to drug class and source of diagnostic fragment ions.

	Synthetic cannabinoids	Synthetic opioids	Designer benzodiazepines	Total
Library spectra based on diagnostic ions from standards	126	47	22	195
Library spectra based on diagnostic ions from theoretical evaluation	116	40	0	156
Library spectra based on diagnostic ions from seizures	4	2	4	10
Library spectra based on diagnostic ions from RESPONSE project[a] (seizures or test purchase on-line)	13	–	–	13
Total number of unique compounds (database entries)	259	89	26	374

[a] A European project named Response to challenges in forensic drug analysis. https://www.policija.si/apps/nfl_response_web/seznam.php.

implemented in 2014 were filtered out. Based on this selection a PCDL was developed. For this purpose each compound was added as an individual database entry. Then the software tool "Spectrum Generator" created by Broeckers Solutions (Berlin, Germany) was used to convert the text-based information of diagnostic ions from the HighResNPS database into the Agilent "cef" file format which allows an import of library spectra for each PCDL entry. Table 1 shows the resulting HighResNPS subset PCDL content. By this approach the diagnostic fragment ions were stored as a library spectrum. Relative abundance of the ions was not taken into account even though this would be possible by the software "Spectrum Generator". The collision energy of the library spectra was chosen by the software as 20 eV just to have any value in the PCDL. An example of the library entry of flubromazepam is shown in Fig. 1. A complete list of the 374 unique compounds is given in the supplementary material (Table S1).

2.5. Data processing

Of the 1314 data files available, batches of approx. 250 were re-processed using MassHunter DA Reprocessor software B.09.00 (Agilent, Santa Barbara, CA, USA). The re-processing was relatively fast, approximately 1 min. per sample, when using a computer equipped with a 2.67 GHz processor and 8 GB of RAM. This process was running in the background allowing re-processed data files to be opened and evaluated in batches of 50–80 simultaneously in MassHunter Qualitative Analysis Software (version 10.0) (Agilent, Santa Clara, CA, USA).

The qualitative method used in the re-processing was based on the algorithm "Find by formula" together with a library search, both using the HighResNPS subset PCDL. The "Find by formula" search lead to positive findings that were based on MS1 spectral information. The criterion was a mass error less than ±5 ppm and a score above 80 where the scoring was taking the mass match, isotope spacing and isotope abundance into account. In the case when MS/MS spectra were acquired for the precursor ion of a detected compound, these MS/MS spectra were compared with those in the PCDL. The comparison was done both by reverse search (the peaks in the PCDL are compared with the MS/MS spectra) and by forward search (the peaks in the MS/MS spectra are compared with the PCDL). The threshold library match was set to 1 (of max. 100) for both forward and reverse score. As the maximum number of fragment ions per library spectrum was three, the lowest resulting reverse score of a match was 33.

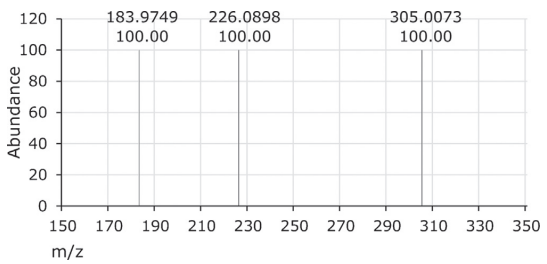

Fig. 1. Library spectrum of flubromazepam.

A filter in the software was applied in order to distinguish compounds with MS/MS spectra (category 1) and without MS/MS spectra (category 2). Category 1 compounds found by the algorithm "Find by Formula" could be evaluated further by comparing the acquired MS/MS with the library spectrum. If there was no agreement based on the MS/MS comparison the compound was considered a false positive. If there was a match, a visual evaluation comparing the acquired spectrum with the library spectrum was undertaken to rule out false positive matches due to fragments of low abundance e.g. from contaminants. The LOIs estimated for the compounds selected in the validation applies for category 1 compounds.

For category 2 compounds, no MS/MS data had been acquired and fragment confirmation could not be done. Thus, only the MS signal could be used to evaluate the quality of the findings. Without the MS/MS spectra identification parameter the number of potential positives would have been large and included noisy signals and bad peak shapes. A peak area threshold of 5×10^4 was applied to limit the number of findings to investigate. Consequently, higher detection limits were expected for these compounds compared to category 1 compounds. In order not to miss any important findings, a mass accuracy limit of ±10 ppm and mass match score above 80 was first applied (criterion a). This was tested with 42 random data files and gave 74 findings. After investigating the results and filtering out findings due to interferences and background signal, only compounds with mass accuracy better than ±5 ppm and mass match score above 95 were left. These two thresholds were consequently used as criterion b. Finally, a third factor was added to criterion b, an RT restriction of 1.5 min, as the compounds in the groups under investigation are highly likely to elute after this time period (criterion c). The number of findings in the 42 random data files as a function of criterion a, b or c are illustrated in Fig. 2. Criterion c (mass accuracy better than ±5 ppm, mass match score higher than 95 and RT 1.5 min. or more) was applied for all category 2 compounds. A compound appearing in several data files in the same batch was considered an isomer originating from the chemicals used or as endogenous molecules with equal theoretical masses. The risk of accepting false positives is higher for category 2 than for category 1 findings, especially if thresholds and limits are set too wide.

Any new finding was further evaluated by comparing acquired MS/MS spectra with other sources (e.g. mzCloud[1]) or alternatively by analysing a reference standard, if available at the laboratory. Due to variations in the RTs over the time period the samples were

[1] https://www.mzcloud.org.

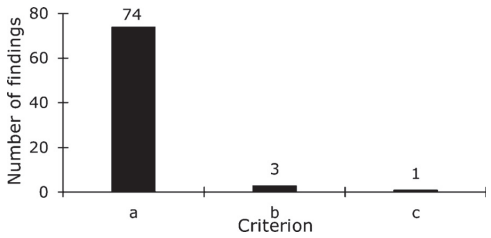

Fig. 2. Number of category 2 findings in 42 random data files as a function of criterion a (peak area threshold of 5×10^4, mass accuracy limit of ±10 ppm and mass match score above 80), criterion b (mass accuracy limit reduced to ±5 ppm and mass match score above 95) or criterion c (mass accuracy better than ±5 ppm, mass match score higher than 95 and RT 1.5 min. or more).

originally analyzed, RT deviations up to 0.5 min. were tolerated when comparing these samples to reference standards. If a consistency in fragments or RTs was observed, the finding was reported to a person with access to the original case report. If a presumably novel moiety was identified and a reference standard was available, this standard was analysed and RTs and MS/MS spectra were compared.

3. Results and discussion

3.1. Validation of original analytical method

3.1.1. Instrument sensitivity and limit of identification

The instrument response and RT variation over time was expressed by plotting the peak area and RT of the internal reference standards extracted from one calibrator from each analytical run (Fig. S1 in supplementary material). Morphine-d3 showed an RT difference (maximum – minimum) of 0.28 min. and a mean peak area of 2.7×10^5 (standard deviation (SD) 1.3×10^5). Codeine-d3 showed an RT difference of 0.35 min. and a mean peak area of 4.4×10^5 (SD 1.6×10^5). Benzoylecgonine-d3 showed an RT difference of 0.32 min. and a mean peak area of 7.8×10^5 (SD 3.9×10^5). Finally, griseofulvin showed a RT difference of 0.44 min. and a mean peak area of 2.8×10^5 (SD 1.4×10^5). The peak areas of internal reference standards in the data files are not only reflecting the variation in instrument response but also variation in extraction efficiency and matrix effects over time. This gives a more relevant expression compared to a direct injection of a neat performance test sample.

LOIs were estimated for a representative group of synthetic cannabinoids, synthetic opioids and designer benzodiazepines (Table 2). LOIs are unknown for new compounds but the experiment indicated that synthetic cannabinoids could be detected if present above approximately 10–20 ng/mL, synthetic opioids above 1 ng/mL and designer benzodiazepines above 10 ng/mL. Electrospray ionization is best suited for analysis of compounds with medium-to-high polarity but is not optimal for all compounds [28]. The LOIs in Table 2 are only estimates of the instrument sensitivity through the acquisition period. As seen by the results from the internal reference standards, the peak areas varied during the period due to e.g. instrument condition and periodic maintenance. How this in turn affected the LOIs is difficult to determine, as the value is not only a result of signal intensity, but also the automatic selection of precursor ions based on the DDA settings. If the compound still is among the precursors selected for fragmentation it will probably be identified. Given the peak area threshold applied to detect category 2 substances, a higher concentration must be present in order to detect them as compared to category 1 substances. A review of the data files from the LOI experiments shows that a peak area of 5×10^4 generally corresponds to two- or threefold the concentration of the LOI of category 1 substances (see Table S2 in supplementary material). These data also indicate that mass match score of 95 is achieved for most compounds when a peak area around 5×10^4 is measured.

Table 2
Retention time (RT), limit of identification (LOI), recovery (RE) and matrix effect (ME) for a selection of compounds in the three groups of new psychoactive substances included in the present study.

Substance	RT [min]	LOI [ng/mL]	RE [%]	ME [%]
Synthetic cannabinoids				
MDMB-CHMICA	14.0	10	68	97
AB-CHMINACA	11.9	20	91	107
BB-22	14.3	10	57	86
JWH-018	14.4	2	51	85
PB-22	13.8	10	68	89
THJ-018	14.8	10	32	69
Synthetic opioids				
Fentanyl	7.1	1	87	132
Remifentanil	5.4	1	94	123
Cyclopropylfentanyl	7.5	1	82	128
Para-fluorofentanyl	7.2	0.5	88	124
Furanylfentanyl	7.3	0.5	100	124
Acetylfentanyl	6.0	1	100	127
Designer benzodiazepines				
Deschloroetizolam	8.8	2	107	119
Diclazepam	10.7	5	87	110
Etizolam	9.3	2	110	121
Flubromazepam	9.1	10	110	72
Flubromazolam	8.5	5	113	121
Pyrazolam	6.4	10	114	122
Meclonazepam	9.3	10	110	105

3.1.2. Recovery and matrix effects

Major differences were observed in the estimated RE (%) of the synthetic cannabinoids, with values ranging from 32% (THJ-018) to 91% (AB-CHMINACA) (Table 2). The remaining compounds had REs above 82%. All compounds showed an ME between 69% and 127% demonstrating that both ion-suppression and ion-enhancement occur. ME values with relatively little deviation from 100% for the studied compounds indicate that severe ion suppression is unlikely for other compounds in these groups.

3.2. Retrospective data file analysis

A total number of 1314 data files (242, 252, 273, 242 and 305, respectively, from the years 2014 to 2018) were processed with the new PCDL. The retrospective analysis revealed six new findings of category 1 in addition to two compounds (fluorofentanyl and cyclopropylfentanyl) that had been reported when the data files were processed with the original method, but only after seized material had become available (Tables 3 and 4). In addition there were 35 possible findings of category 2 (Table 5) not reported when the data files were processed with the original method.

3.2.1. Category 1 findings

Flubromazepam was detected in two data files from 2015 and 2016 respectively. There was a mass match score in both data files higher than 95, a mass accuracy better than 3.46 ppm and an RT deviation of less than 0.07 min. The mass match can be visualized by the resemblance of the spectrum of flubromazepam and the theoretical pattern indicated by the boxes in Fig. 3. The three diagnostic fragments in the library spectrum were also found in the MS/MS data acquired from the precursor in the two data files (see Fig. 4A). An additional comparison of the MS/MS spectra from the data file and the analysis of a reference standard showed good

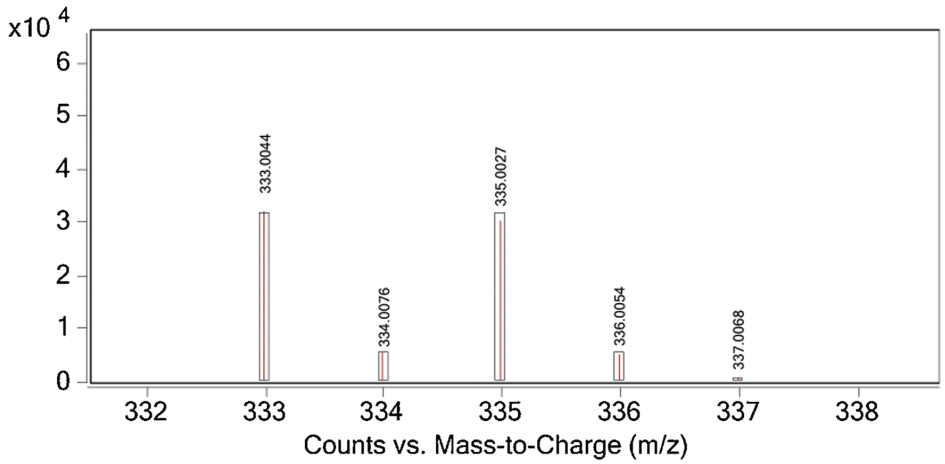

Fig. 3. MS1-spectrum of flubromazepam extracted from a data file (red lines) with theoretical isotopic pattern illustrated by the black boxes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

agreement also for additional fragment masses (see Fig. 4B). Flubromazepam was first described in 1962 and is a highly potent and incompletely evaluated benzodiazepine structurally related to phenazepam [29,30]. Flubromazepam started to emerge in online shops in Europe in 2012. In Norway it was detected in seized material by the Norwegian National Criminal Investigation (KRIPOS) for the first time in 2013.

Phenibut was detected in a data file from 2015 and showed a mass match score higher than 85, a mass accuracy of −1.63 ppm and an RT deviation of 0.12 min. compared to a reference standard analysed in 2018. Evaluation of the RT over time showed that a deviation up to 0.5 min. could be expected due to change of analytical column lot and tubing. Phenibut is a neuropsychotropic drug with possible cognition enhancing effects that was discovered and introduced into clinical practice in the 1960s Soviet Union [31]. The drug is widely used in Russia and is claimed to have various clinical effects, e.g. to relieve tension and anxiety and to improve sleep. Phenibut can cause dependency. It is not scheduled or classified as a medicinal drug in Norway and is not for legal sale. Private import is prohibited by law. KRIPOS did not detect phenibut in any cases before 2019. Our laboratory reported detection of phenibut in seized material and biological samples for the first time in 2016, and it has since then been part of the routine analytical repertoire at our laboratory.

Fluorofentanyl was detected in one data file from 2016 with a mass match score higher than 97, a mass accuracy of −0.21 ppm and good agreement in the diagnostic ions. Analysis of reference material showed an RT deviation of less than 0.05 min. Moreover, a compound with molecular formula $C_{23}H_{28}N_2O$ was detected in a data file from 2018 with mass match score higher than 96 and mass accuracy of 2.87 ppm. The diagnostic fragments of *m/z* 105.0699 and 188.1434 showed that the compound most probably was a fentanyl analogue and the software suggested either cyclopropylfentanyl, methacrylfentanyl or crotonylfentanyl. These three compounds share the same formula and diagnostic fragments. Consequently, they are not possible to distinguish from each other based on category 1 criteria only, but analysis of reference material showed good RT agreement (deviation 0.01 min.) with cyclopropylfentanyl. In fact, fluorofentanyl and cyclopropylfentanyl had already been confirmed by targeted analysis of the data files based upon information from analysis of seizures from the scene requested by the police [32,33]. However, as these compounds would not have been detected originally if we had not known which substances to suspect, they are included in the present material.

Identification of flubromazepam, phenibut, fluorofentanyl and cyclopropylfentanyl (of category 1) was based on the mass accuracy of the monoisotopic MS signal, presence of diagnostic fragment ions and, finally, RT agreement. Fulfilment of these criteria gave the highest level of confidence that can be achieved in a retrospective review when re-analysis of the actual specimen is not possible. Detection and confirmation of compounds with HR-MS can be divided in different levels of confidence based on information available from the data acquisition, as suggested by

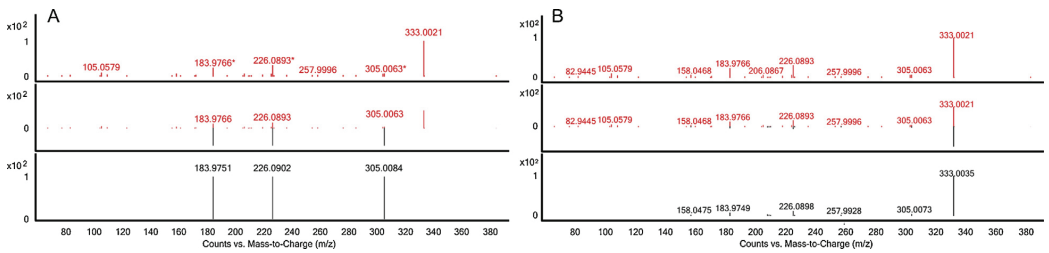

Fig. 4. (A) Acquired MS/MS-spectrum of flubromazepam with diagnostic fragments marked with asterisk (at the top), library spectrum from PCDL (at the bottom) and a comparison (in the middle). (B) Acquired MS/MS-spectrum (at the top), full MS/MS-spectrum from a flubromazepam reference standard (at the bottom) and a comparison (in the middle).

Table 3
New compounds found after applying category 1 criteria, including identification data and case information.

Compound (year)	Molecular formula	Retention time sample/reference standard (Δ min)	Mass match score	Diagnostic fragment	Mass (calculated)	Mass accuracy [ppm]	First reported in Norway	Case information
Flubromazepam (2015)	$C_{15}H_{10}BrFN_2O$	9.08/9.15 (−0.07)	95.55		314.0049	3.46	2013[a]	Male, approx. 30 yrs. old. History of drug abuse, found dead after drug use.
				$C_{14}H_{11}FN_2$	226.0901	−3.29		Ethanol, amphetamine, metamphetamine, methylenedioxymetamphetamine, metylenedioxyamphetamine, diazepam, desmetyldiazepam, 7-aminoclonazepam, alprazolam, pregabalin, mephedrone, buprenorphine, norbuprenorphine and gamma-hydroxybutarate found in blood.
				C_7H_7BrN	183.9756	5.25		
				$C_{14}H_{11}N_2FBr$	305.0084	−7.10		
Phenibut[c] (2015)	$C_{10}H_{13}NO_2$	1.53/1.65 (−0.12)	85.36		180.1019	−1.63	2016[b]	Same subject as above.
				C_9H_9	117.0699	1.81		
				$C_{10}H_9O$	145.0648	−16.67		
Flubromazepam (2016)	$C_{15}H_{10}BrFN_2O$	9.21/9.15 (0.06)	97.45		333.0033	0.24	2013[a]	Female, approx. 50 yrs. old. History of drug abuse, found dead at home.
				$C_{14}H_{11}FN_2$	226.0901	3.48		Ethanol, paracetamol, gabapentin, pregabalin, tramadol, O-desmethyltramadol, amitriptyline, nortriptyline, sertraline and chlorprothixene found in blood.
				C_7H_7BrN	183.9756	0.95		
				$C_{14}H_{11}N_2FBr$	305.0084	16.4		
Fluorofentanyl[d] (2016)	$C_{22}H_{27}FN_2O$	7.18/7.17 (0.01)	97.73		355.2180	−0.21	2016[b]	Male, approx. 20 yrs. old. Found dead at home with drug paraphernalia.
				$C_{13}H_{18}N$	188.1434	−5.42		7-aminoclonazepam, diazepam, desmethyldiazepam, alprazolam, tetrahydrocannabinol and gamma-hydroxybutyrate found in blood.
				C_8H_9	105.0699	−3.56		
				$C_{14}H_{17}FNO$	234.1289	−9.92		
Cyclopropylfentanyl[d] (2018)	$C_{23}H_{28}N_2O$	7.46/7.47 (−0.01)	96.53		349.2274	2.87	2017[a]	Male, approx. 30 yrs. old. Found dead at home with pills on site.
				$C_{13}H_{18}N$	188.1434	−2.52		Morphine, morphine-3-glucuronide, morphine-6-glucuronide, buprenorphine, norbuprenorphine, pregabalin, amphetamine, methylenedioxymetamphetamine, metyhlenedioxyamphetamine, benzoylecgonine, 7-aminoclonazepam and tetrahydrocannabinol found in blood.
				C_8H_9	105.0699	1.05		
				$C_{15}H_{18}NO$	228.1383	−4.46		

[a] Detected in seized material by the Norwegian National Criminal Investigation.
[b] Detected in seized material by our department.
[c] 4-amino-3-phenylbutyric acid.
[d] Reported originally but included here to illustrate method suitability.

Schymanski et al. [34]. In that approach, level 5 through level 1 requires increasing information from the MS signal to diagnostic fragments and RTs [34]. Findings of category 1 in our retrospective method can be compared to a situation close to level 1. Level 1 requires confirmation with a reference standard, which was the case with our new findings, but as long as the sample and standard are not analysed simultaneously, a definite confirmation is not achieved.

In a retrospective approach co-identification of metabolites can further strengthen the confidence of a finding. Searches for the major metabolites of the detected compounds were done in the relevant data files. Metabolites from published in vivo and in vitro studies were selected [29,35–37]. Neither of the metabolites of fluorofentanyl were detected in the data file containing this compound. In the data file containing cyclopropylfentanyl the *N*-dealkylated metabolite and two hydroxylated metabolites were detected. The metabolites of flubromazepam found in literature to be the most abundant (hydroxylated flubromazepam and debrominated flubromazepam) were not detected in any of the two positive samples. The metabolism of phenibut has to our knowledge not been studied, and no putative target metabolites have been described in the literature.

Three other positive category 1 findings could be refuted after further investigation (Table 4). For methoxyacetylfentanyl the RT deviation compared with the reference standard was significant, indicating that the compound rather was an isomer of methoxyacetylfentanyl with similar fragmentation patterns. There were no other described fentanyl analogues with identical molecular formula. The presence of fragments of *m/z* 105.0699 and 188.1434 was however a strong indicator that the compound consisted of the piperidine and phenyl moiety characteristic to fentanyl itself as well as many fentanyl analogues. Metabolites of fentanyl hydroxylated at the alkyl or phenetyl moeity have the same monoisotopic mass as methoxyacetylfentanyl and the diagnostic fragments 105.0699 and 188.1434 will be the same (Fig. 5).

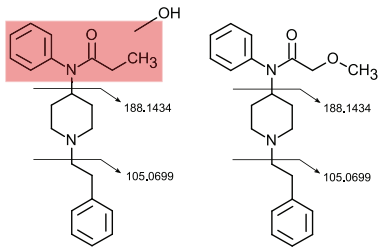

Fig. 5. Fragmentation of hydroxyfentanyl (left) and methoxyacetylfentanyl (right). The superimposed area indicates position of hydroxyl-group.

Table 4
New compounds found after applying category 1 criteria, but refuted as "false positive" findings.

Compound (year)	Molecular formula	Retention time (RT) sample/reference standard (Δ min)	Mass match score	Diagnostic fragment	Mass (calculated)	Mass accuracy [ppm]	Comment
Methoxyacetylfentanyl (2016)	$C_{22}H_{28}N_2O_2$	5.13–5.75 (−0.62)	91.36		353.2224	0.20	RT not in agreement with reference standard. Monoisotopic mass and diagnostic fragments suggest fentanyl hydroxylated at the alkyl or phenetyl moiety
				$C_{13}H_{18}N$	188.1434	−3.8	
				C_8H_9	105.0699	−17.23	
				$C_9H_{12}N$	134.0964	Not found	
JWH-167 (2014)	$C_{21}H_{23}NO$	11.82–13.86 (−2.06)	95.32		306.1852	0.90	RT not in agreement with reference standard
				$C_{14}H_{16}NO$	214.1226	7.94	
				C_7H_7	91.0542	−9.63	
				$C_{13}H_{18}N$[a]	188.1434	−11.05	
Tilidine (2015)	$C_{17}H_{23}NO_2$	9.24–5.56 (3.28)	90.87		274.1802	1.65	RT not in agreement with reference standard
				$C_{15}H_{17}O$	229.1223	Not found	
				$C_{12}H_{11}$	155.0855	−5.88	
				C_7H_7	91.0542	45.1	

[a] Diagnostic fragment from mzCloud.

Fentanyl was reported in the original analysis of the sample, which explains the presence of a metabolite. Thus, it could be concluded that the finding was caused by fentanyl intake.

Category 1 findings of JWH-167 and tilidine were detected in one data file each, from 2014 and 2015, respectively. The fragments in the MS/MS spectra were in relatively good agreement with the diagnostic fragments from the library spectrum, and in addition the m/zCloud database was consulted and showed agreement with one additional fragment. Reference standards were acquired to compare RTs and significant RT differences clearly showed that neither JWH-167 nor tilidine were present. These examples of false positive results illustrate the importance of having access to the reference substance in order to check RT conformity.

3.2.2. Category 2 findings

A total of 35 possible category 2 findings was the result when applying criterion c (better than 5 ppm mass accuracy, mass match score higher than 95 and RT 1.5 min. or later). The initial findings are presented in Table 5. A further evaluation of RT, signal-to-noise ratio and chromatographic peak shape for every finding was done. The metabolite AB-FUBINACA M3 (#13−15), carfentanil (#17 and 18), tilidine (#35) and three of four findings of phenibut (#31, 32 and 34) could be disproved due to large RT deviations from reference standards. Based on the RTs of other synthetic cannabinoids analysed with the same chromatographic conditions (see Table 2) findings of synthetic cannabinoids with RTs less than 5 min. were regarded as highly unlikely and removed from the list. This was the case for 5-fluoro-PY-PINACA (#3 and 4), 5-fluoro-3,5-AB-PFUPPYCA (#5), AB-BICA (#9 and 10) and MA-CHMINACA (#20). 5-fluoro-AB-PINACA N-(4-hydroxypentyl) (#2), a metabolite and presumably more polar compound than its parent substance, is likely to have a shorter RT. Still, it will probably not elute as early as 3.6 min. A similar limit of 4 min. was applied on the synthetic opioids which lead to the rejection of 3-fluoro methoxyacetyl fentanyl (or ocfentanyl) (#1) and two findings of N-methyl norcarfentanil (#25 and 26). The signal-to-noise ratio was 3 or less for AB-CHMINACA 3-carboxylindazol (#11 and 12), N-methyl norcarfentanil (#27) and PB-22 3-carboxyindole (#30). The initial finding determined as benzyl carfentanil (#16) was disproved due to poor peak shape. A category 2 compound found in one or more data files, and also found with the same RT in other data files having MS/MS spectra acquired but no library match, was likewise rejected. One such example was ohmefentanyl, which was found in two data files with RTs of 7.8 min. The ion could also be found in other data files with the same RT but with acquired MS/MS spectra not in agreement with the PCDL. This strongly indicated that the two findings of ohmefentanyl (#28 and 29) were false positives. The same was the case with presumable findings of AB-FUBINACA (#6–8), JWH-200 analog 1 (or A-796260) (#19) and methoxyacetylfentanyl (#21–24).

Thus, after reviewing the 35 suggested category 2 findings, only one finding of phenibut (#33) remained. As no MS/MS spectra were available for library comparison, this finding could, however, not be confirmed with the same degree of confidence as those of category 1.

3.3. Strengths and weaknesses

The PCDL constructed in this study is based on data acquired on instruments from different manufacturers and based on different principles. A previous study has shown that libraries constructed from data acquired on either Orbitrap or QTOF can be used interchangeably by both instruments providing that suitable collision energies are applied [38,39]. An essential feature of the PCDL is the mass accuracy of the diagnostic fragments. In HighResNPS the masses of the fragments are added by either typing the formula, selecting the correct formula from a drop-down list of common fragments or typing the theoretical mass of the acquired fragment. This ensures that mass errors from the acquisition are not transferred to the database. A second important setting is the choice of collision energy applied when acquiring the diagnostic fragments that are added to the database. The collision energy applied can either be discrete (e.g. 10, 20 and 40 eV) or ramped, providing a combined result. Information on the choice of strategy used in the individual entry was not present in the database. In the Auto MS/MS method used in this study, the collision energy was a voltage correlated to the mass of the precursor. Potentially this can result in differences in relative abundance when comparing a library spectrum and an acquired MS/MS spectrum. However, the settings in the retrospective reprocessing algorithm ensure a hit even if only one of the diagnostic fragment ions could be found in the acquired spectrum.

The risk of false negative samples will always be present when searching for compounds that have not been subject to specific evaluation of LOI, which is the case for the majority of the compounds in the PCDL. In addition, the instrument response has been shown to fluctuate to some extent during the period of data acquisition. Due to the relatively high LOIs and low recoveries

Table 5
The 35 suggested findings after applying category 2 criteria, with retention time (RT) and an evaluation of whether the identification was correct or not based on the RT and the signal-to-noise ratio (S/N).

Suggested finding #	Compound	Year	RT [min]	Correct identification?
1	3-fluoro-methoxyacetyl fentanyl (or ocfentanyl)	2015	3.77	No, fentanyl analogue with RT under 4 min is not likely
2	5-fluoro-AB-PINACA N-(4-hydroxypentyl)	2018	3.59	No, synthetic cannabinoid with RT under 5 min is not likely
3	5-fluoro-PY-PINACA	2016	2.58	No, synthetic cannabinoid with RT under 5 min is not likely
4	5-fluoro-PY-PINACA	2018	2.63	No, synthetic cannabinoid with RT under 5 min is not likely
5	5-fluoro-3,5-AB-PFUPPYCA	2014	3.52	No, synthetic cannabinoid with RT under 5 min is not likely
6	AB-FUBINACA	2016	10.30	No, reference standard showed RT of 10.3 min. A large number of additional data files contain the same ion with same RT but with fragment ions not in agreement with library spectra
7	AB-FUBINACA	2016	10.31	No, reference standard showed RT of 10.3 min. A large number of additional data files contain the same ion with same RT but with fragment ions not in agreement with library spectra
8	AB-FUBINACA	2017	10.44	No, reference standard showed RT of 10.3 min. A large number of additional data files contain the same ion with same RT but with fragment ions not in agreement with library spectra
9	AB-BICA	2014	3.13	No, synthetic cannabinoid with RT under 5 min is not likely
10	AB-BICA	2017	3.79	No, synthetic cannabinoid with RT under 5 min is not likely
11	AB-CHMINACA 3-carboxylindazol	2014	4.12	No, chromatogram shows S/N < 3
12	AB-CHMINACA 3-carboxylindazol	2014	3.92	No, chromatogram shows S/N < 3
13	AB-FUBINACA M3	2014	4.89	No, reference standard showed RT of 11.0 min
14	AB-FUBINACA M3	2015	4.77	No, reference standard showed RT of 11.0 min
15	AB-FUBINACA M3	2017	3.54	No, reference standard showed RT of 11.0 min
16	Benzyl carfentanil	2015	10.91	No, poor chromatography
17	Carfentanil	2015	11.87	No, reference standard showed RT of 7.7 min
18	Carfentanil	2016	11.77	No, reference standard showed RT of 7.7 min
19	JWH-200 analog 1 (or A-796260)	2017	6.15	No, other data files contain the same ion with same RT but with fragment ions not in agreement with library
20	MA-CHMINACA	2014	2.86	No, synthetic cannabinoid with RT under 5 min is not likely
21	Methoxyacetylfentanyl	2014	6.34	No, reference standard showed RT of 5.7 min. Other data files contain the same ion with same RT but with fragment ions not in agreement with library spectra
22	Methoxyacetylfentanyl	2018	6.59	No, reference standard showed RT of 5.7 min. Other data files contain the same ion with same RT but with fragment ions not in agreement with library spectra
23	Methoxyacetylfentanyl	2018	6.58	No, reference standard showed RT of 5.7 min. Other data files contain the same ion with same RT but with fragment ions not in agreement with library spectra
24	Methoxyacetylfentanyl	2018	6.59	No, reference standard showed RT of 5.7 min. Other data files contain the same ion with same RT but with fragment ions not in agreement with library spectra
25	*N*-Methyl norcarfentanil	2014	2.90	No, fentanyl analogue with RT under 4 min is not likely
26	*N*-Methyl norcarfentanil	2016	2.72	No, fentanyl analogue with RT under 4 min is not likely
27	*N*-Methyl norcarfentanil	2018	6.20	No, chromatogram shows S/N < 3
28	Ohmefentanyl	2018	7.84	No, other data files contain the same ion with same RT but with fragment ions not in agreement with library spectra
29	Ohmefentanyl	2018	7.85	No, other data files contain the same ion with same RT but with fragment ions not in agreement with library spectra
30	PB-22 3-carboxyindole	2016	6.61	No, chromatogram shows S/N ~ 3.6
31	Phenibut	2014	6.39	No, reference standard showed RT of 1.65 min
32	Phenibut	2014	2.94	No, reference standard showed RT of 1.65 min
33	Phenibut	2016	1.58	Yes, probably since reference standard showed RT of 1.65 min
34	Phenibut	2016	4.11	No, reference standard showed RT of 1.65 min
35	Tilidine	2018	4.34	No, reference standard showed RT of 1.65 min

among the synthetic cannabinoids in the validation, the risk of false negatives appears to be more likely in this group. It should also be emphasised that the two large NPS groups cathinones and phenetylamines were left out of this study in order to limit the extent of investigated compounds.

Applying the method on our data files has shown that identification of ions that were not selected for fragmentation (category 2) clearly requires a manual re-evaluation. The list of category 2 findings was significantly longer than category 1 findings, but still 35 potential positives out of 1314 data files is a manageably low number. The peak area threshold of 5×10^4 was important to keep the number of potential category 2 findings low, but will at the same time result in higher detection limits for these compounds. All except one of the potential category 2 findings could be disproved after a careful evaluation of the RTs and signal-to-noise ratios in the chromatogram. The need for a manual evaluation of category 2 findings is a limitation of the DDA approach. If DIA had been used there would have been few presumable findings where the MS1 signal was detected but no fragment ions were available. DIA, on the other hand, is limited by co-eluting compounds being fragmented at the same time resulting in complicated high energy spectra. The pattern can be even more complex by co-eluting compounds sharing the same fragments. DDA generates MS/MS spectra from a known precursor which minimizes the risk of "contaminating" fragments from co-eluting compounds. On the other hand, there is a limit to the number of co-eluting precursors which can be isolated and fragmented. The many category 2 findings also show the importance of having the fragmentation information in order to do an efficient retrospective analysis. Re-analysis of case samples was not possible in this study due to ethical restrictions. Consequently, the presumable category 2 finding of phenibut could not be confirmed. In real forensic case work the sample could have been re-analysed with a targeted MS/MS method where the precursor ion of phenibut is prioritized for fragmentation experiments. If a match with a library spectrum was achieved the finding would have been of category 1.

4. Conclusion

Data files from UHPLC-QTOF-MS analysis of 1314 forensic post mortem samples from the period 2014 to 2018 were retrospectively re-evaluated. The re-evaluation was performed using a PCDL with compounds within the groups of synthetic cannabinoids, synthetic opioids and designer benzodiazepines. In total, five new substances were identified with the highest degree of confidence possible with a retrospective approach. The identification relied on available MS/MS spectra from the acquisition and matching with the diagnostic fragment ions in the library spectrum. In addition, RT agreement with a reference substance was decisive in order to filter out false positives. The number of new findings were lower than expected and mainly originated from the first half of the time period investigated, indicating that our laboratory has been able to keep the analytical library fairly up to date.

It is important to emphasise that new and highly potent drugs like fluorofentanyl and cyclopropylfentanyl can escape attention if not specifically searched for. Detection in biological samples is in many cases dependent on information about the likely drug candidates - either from indirect sources such as labelling on seized drug packages, which may be imprecise, or preferably from direct analysis of the ingested substance. In Norway, biological samples are analysed by toxicology laboratories, whereas impounded drugs are submitted for analysis at a central police laboratory. There are no organizational connections or traditions for exchange of information between these two types of institutions. If it had not been for the availability of seizures in the two cases involving fluorofentanyl and cyclopropylfentanyl these would not have been detected with our original screening method.

The presented method proved to be a relatively easy and convenient approach to search for new compounds retrospectively. The use is not limited to retrospective analysis and can easily be applied as a supplement to the standard screening method with little extra effort, especially when the routine screening workflow gives a negative result but the circumstances suggest a more thorough investigation. The PCDL can be updated at regular time intervals or when important compounds are added to HighResNPS.com.

Note

After the completion of this study a Public Compound Database and Library version of the complete highresnps database has been made available for download from the website highresnps.com.

CRediT authorship contribution statement

Per Ole M. Gundersen: Conceptualization, Methodology, Formal analysis, Investigation, Writing - original draft, Writing - review & editing. **Sebastian Broecker:** Software, Methodology, Writing - review & editing. **Lars Slørdal:** Resources, Data curation, Writing - review & editing. **Olav Spigset:** Conceptualization, Supervision, Resources, Writing - review & editing, Project administration. **Martin Josefsson:** Conceptualization, Supervision, Methodology, Writing - review & editing.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at https://doi.org/10.1016/j.forsciint.2020.110274.

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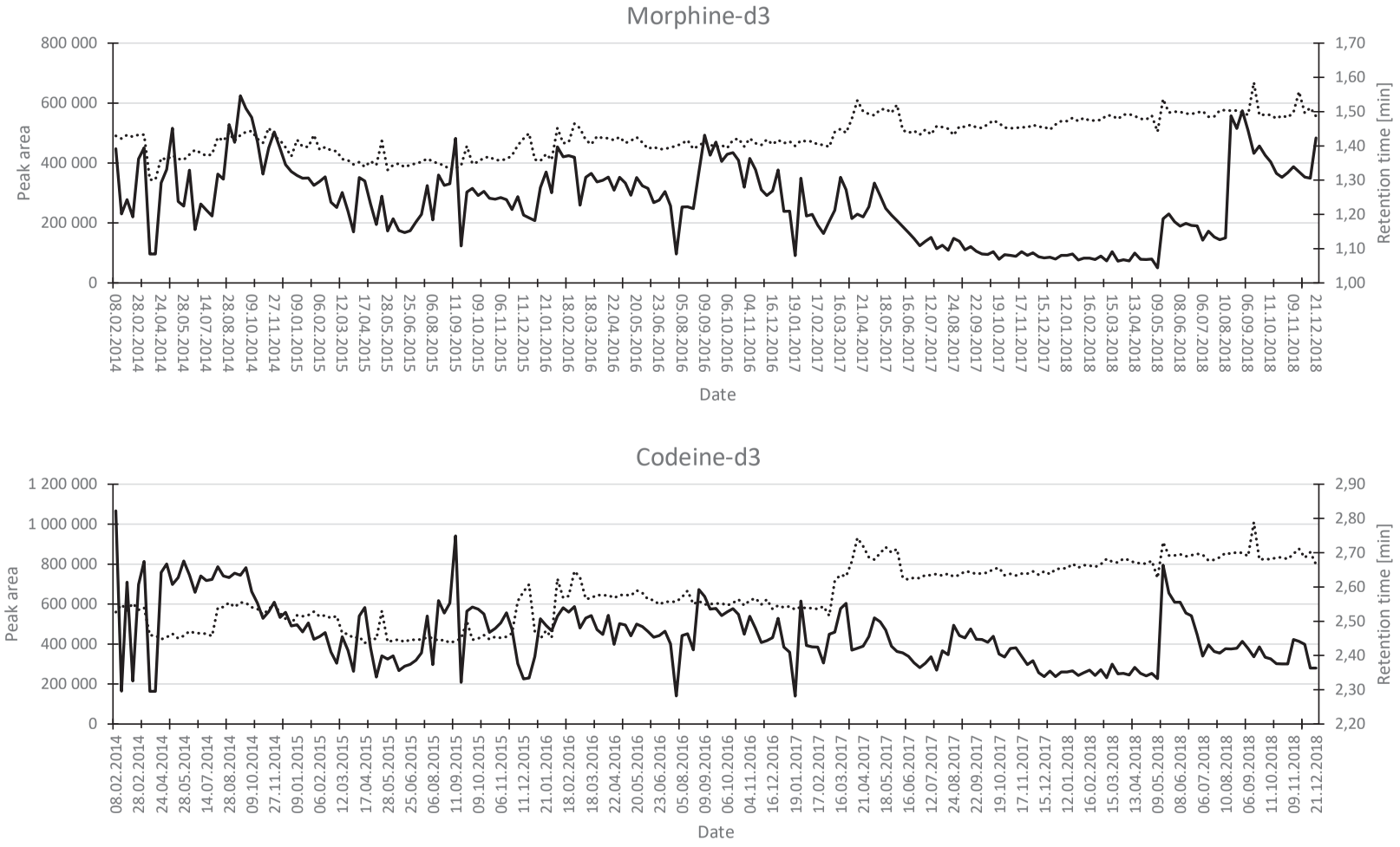
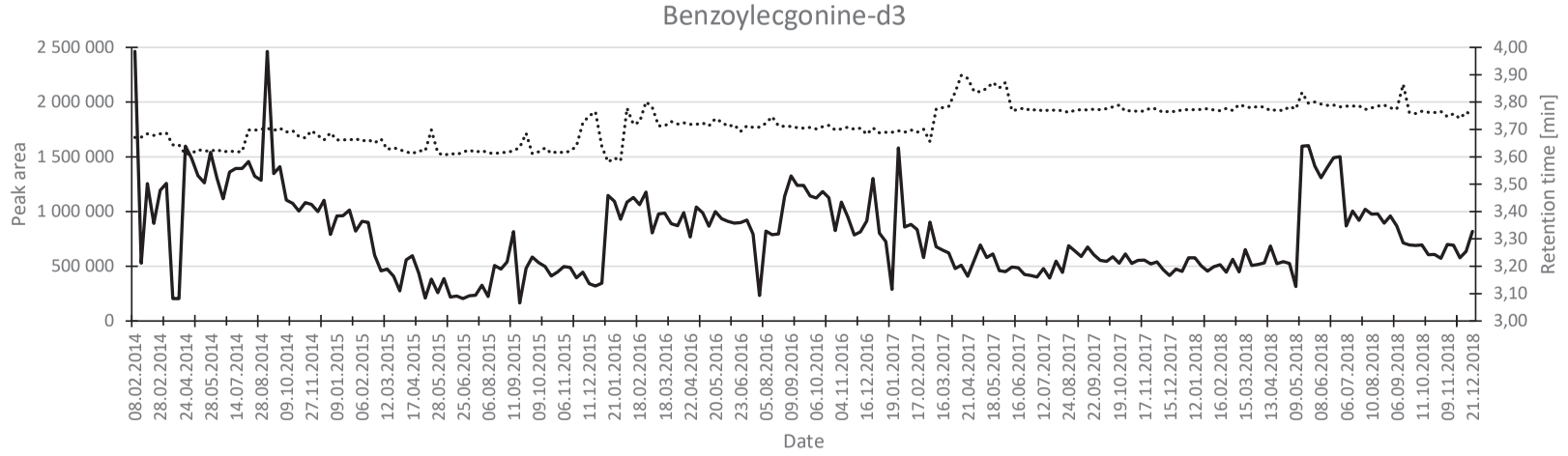
Supplementary material – Paper III

Table S1 is available through online version at https://doi.org/10.1016/j.forsciint.2020.110274

Figure S1: Peak area (solid line) and retention time (dotted line) of the internal reference standards morphine-d3, codeine-d3, benzoylecognine-d3 and griseofulvin in one calibrator from all analytical runs from 2014 to 2018




Benzoylecgonine-d3
Peak area
2 500 000
2 000 000
1 500 000
1 000 000
500 000
0
Retention time [min]
4,00
3,90
3,80
3,70
3,60
3,50
3,40
3,30
3,20
3,10
3,00
08.02.2014
28.02.2014
24.04.2014
28.05.2014
14.07.2014
28.08.2014
09.10.2014
27.11.2014
09.01.2015
06.02.2015
12.03.2015
17.04.2015
28.05.2015
25.06.2015
06.08.2015
11.09.2015
09.10.2015
06.11.2015
11.12.2015
21.01.2016
18.02.2016
18.03.2016
22.04.2016
20.05.2016
23.06.2016
05.08.2016
09.09.2016
06.10.2016
04.11.2016
16.12.2016
19.01.2017
17.02.2017
16.03.2017
21.04.2017
18.05.2017
16.06.2017
12.07.2017
24.08.2017
22.09.2017
19.10.2017
17.11.2017
15.12.2017
12.01.2018
16.02.2018
15.03.2018
13.04.2018
09.05.2018
08.06.2018
06.07.2018
10.08.2018
06.09.2018
11.10.2018
09.11.2018
21.12.2018
Date

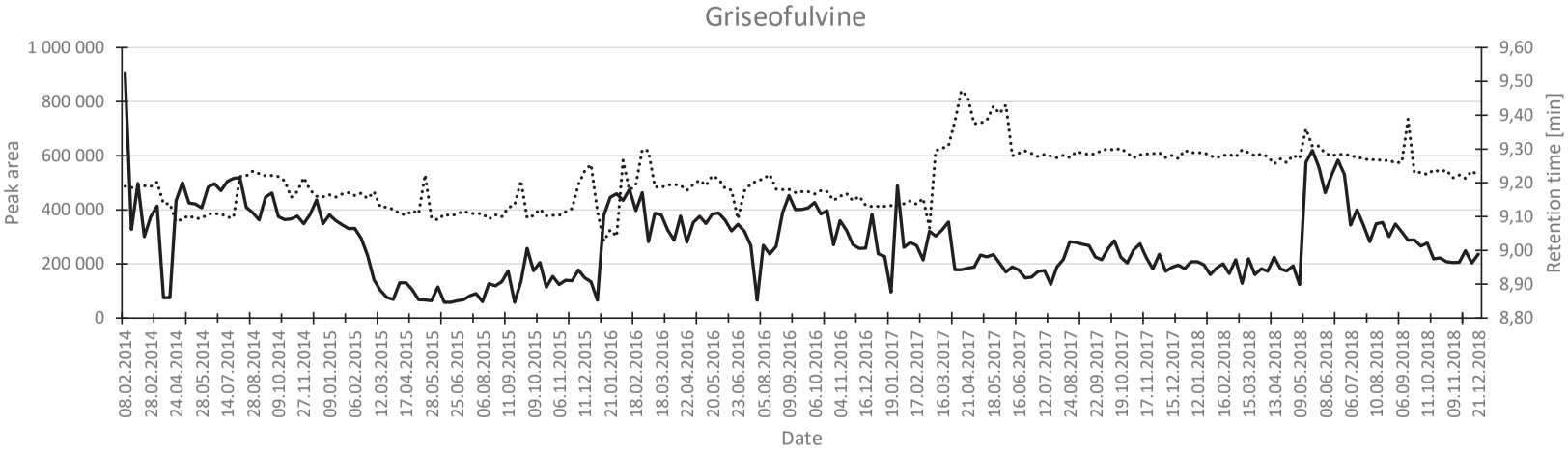

Griseofulvine
Peak area
1 000 000
800 000
600 000
400 000
200 000
0
Retention time [min]
9,60
9,50
9,40
9,30
9,20
9,10
9,00
8,90
8,80
08.02.2014
28.02.2014
24.04.2014
28.05.2014
14.07.2014
28.08.2014
09.10.2014
27.11.2014
09.01.2015
06.02.2015
12.03.2015
17.04.2015
28.05.2015
25.06.2015
06.08.2015
11.09.2015
09.10.2015
06.11.2015
11.12.2015
21.01.2016
18.02.2016
18.03.2016
22.04.2016
20.05.2016
23.06.2016
05.08.2016
09.09.2016
06.10.2016
04.11.2016
16.12.2016
19.01.2017
17.02.2017
16.03.2017
21.04.2017
18.05.2017
16.06.2017
12.07.2017
24.08.2017
22.09.2017
19.10.2017
17.11.2017
15.12.2017
12.01.2018
16.02.2018
15.03.2018
13.04.2018
09.05.2018
08.06.2018
06.07.2018
10.08.2018
06.09.2018
11.10.2018
09.11.2018
21.12.2018
Date

Table S2: The approximate peak area and mass accuracy score achieved at the concentrations corresponding to limit of identification (LOI) and 2x LOI for the compounds included in the validation.

Compound	**Conc [ng/ml]**	**Peak area (approx.)**	**Mass accuracy score**
Acetylfentanyl	1 (LOI)	35000	97
	2	85000	98
Furanylfentanyl	0.5 (LOI)	15000	90
	1	30000	96
Parafluorofentanyl	0.5 (LOI)	15000	97
	1	32000	98
Cyclopropylfentanyl	1 (LOI)	30000	84
	2	85000	97
Remifentanil	1 (LOI)	20000	96
	2	50000	93
Fentanyl	1 (LOI)	35000	81
	2	100000	95
Deschloroetizolam	2 (LOI)	20000	92
	5	40000	97
Diclazepam	5 (LOI)	20000	92
	10	45000	98
Etizolam	2 (LOI)	19000	94
	5	38000	97
Flubromazepam	10 (LOI)	30000	92
	20	60000	97
Flubromazolam	5 (LOI)	20000	89
	10	45000	96
Pyrazolam	10 (LOI)	30000	95
	20	-[1]	-[1]
Meclonazepam	10 (LOI)	16000	92
	20	-[1]	-[1]
MDMB-CHMICA	10 (LOI)	15000	96
	20	35000	99
AB-CHMINACA	20 (LOI)	50000	82
	-[2]	-[1]	-[1]
BB-22	10 (LOI)	50000	97
	20	80000	99
JWH-018	2 (LOI)	20000	93
	5	30000	96
PB-22	10 (LOI)	35000	93
	20	100000	99
THJ-018	10 (LOI)	25000	98
	20	80000	99

[1]Information missing

[2]Concentrations higher than 20 ng/ml were not tested