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BIOANALYTICAL STUDIES OF DESIGNER BENZODIAZEPINES

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Bioanalytical studies of designer benzodiazepines

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ABSTRACT

The fast appearance of benzodiazepine analogues, referred to as new psychoactive substance (NPS) or designer benzodiazepines, requires the continuous update of detection methods in order to keep up with the latest drugs on the recreational drug market. Moreover, as usually only limited information on toxicity and excretion patterns of these new drugs exists, this needs to be evaluated to report on adverse effects and to determine suitable targets for drug testing.

Urine drug testing usually involves screening using immunoassay followed by confirmation of positive screening results using mass spectrometric (MS) methods. We studied the detectability of designer benzodiazepines in urine using commercial immunoassays and demonstrated that most designer benzodiazepines can be detected by immunoassay. It is thus important to update confirmation methods to include designer benzodiazepines. We also developed a liquid chromatographic–tandem MS (LC–MS/MS) confirmation method for designer benzodiazepines in urine using direct dilution of samples and hydrolysis of conjugates. Subsequently, a further improved screening and confirmation method using LC–high-resolution MS (LC–HRMS(/MS)) was developed. HRMS screening is performed in full scan and is a generic method that can easily include new analytes. LC–HRMS/MS confirmation only requires re-injection of the sample.

Many samples from drug dependent patients with a positive immunoassay screening result for benzodiazepines but not containing prescription medicines detected a designer benzodiazepine instead. Comparable results were obtained for acute intoxication cases from emergency wards in the STRIDA project. In total, 28 designer benzodiazepines were covered by the analytical method and 17 of these were detected in the samples. Classification of a designer benzodiazepine as a narcotic substance generally meant that it was removed from the NPS market and replaced with another novel benzodiazepine. It was further demonstrated that intoxications by designer benzodiazepines might cause central nervous system depression.

Studies on metabolic patterns of five designer benzodiazepines using urine samples from confirmed intoxication cases identified suitable analytical targets for urine drug testing, instead of or together with the parent compounds and both with and without hydrolysis of conjugated forms.

In summary, the results demonstrated frequent use of designer benzodiazepines in Sweden and in cases of acute intoxication that they might cause serious adverse effects. This underlines the importance of including designer benzodiazepines and/or metabolites thereof in drug testing. Screening for designer benzodiazepines can be performed by immunoassay or LC–HRMS, and confirmation methods can make use of direct dilution of urine samples followed by hydrolysis and direct injection into LC–MS or LC–HRMS systems.

LIST OF SCIENTIFIC PAPERS

I. **Pettersson Bergstrand M**, Helander A, Hansson T, Beck O. Detectability of designer benzodiazepines in CEDIA, EMIT II Plus, HEIA, and KIMS II immunochemical screening assays. Drug Test Anal. 2017;9:640-645

II. **Pettersson Bergstrand M**, Helander A, Beck O. Development and application of a multi-component LC-MS/MS method for determination of designer benzodiazepines in urine. J Chromatogr B Analyt Technol Biomed Life Sci. 2016;1035:104-10.

III. **Pettersson Bergstrand M**, Beck O, Helander A. Urine analysis of 28 designer benzodiazepines by liquid chromatography–high-resolution mass spectrometry. *In manuscript*

IV. Meyer MR, **Bergstrand MP**, Helander A, Beck O. Identification of main human urinary metabolites of the designer nitrobenzodiazepines clonazolam, meclonazepam, and nifoxipam by nano-liquid chromatography-high-resolution mass spectrometry for drug testing purposes. Anal Bioanal Chem. 2016;408:3571-91

V. **Pettersson Bergstrand M,** Meyer MR, Beck O, Helander A. Human urinary metabolic patterns of the designer benzodiazepines flubromazolam and pyrazolam studied by liquid chromatography-high resolution mass spectrometry. Drug Test Anal. 2018;10:496-506

VI. Bäckberg M, **Pettersson Bergstrand M**, Beck O, Helander A. Clinical characteristics of new designer benzodiazepines – intoxication cases from the Swedish STRIDA project. *In manuscript*

CONTENTS

LIST OF ABBREVIATIONS

CEDIA	Cloned enzyme donor immunoassay
EMIT	Enzyme multiplied immunoassay technique
ESI	Electrospray ionization
HRMS	High resolution mass spectrometry
HEIA	Homogenous enzyme immunoassay
KIMS	Kinetic interaction of microparticle in solution
LC	Liquid chromatography
MS	Mass spectrometry
NPS	New psychoactive substances
NSI	Nano-electrospray ionization
UHPLC	Ultra high performance liquid chromatography
UHPLC-MS/MS	Ultra high performance liquid chromatography tandem mass spectrometry

1 BACKGROUND

In 2015, around 29.5 million people world-wide were estimated to suffer from illicit drug use that required treatment [1]. Such abuse has a large impact on society and the individual, with e.g. associated health problems and complications at the workplace [2]. Treatments for substance abuse problems can be both psychosocial and pharmacological and drug testing can be used as a valuable tool for detection and monitoring treatment progress.

1.1 DRUG TESTING

Drug testing can be performed using different matrices, e.g. blood, breath, hair, oral fluid and urine. Urine has become widely used for drug testing purposes and testing is first performed using screening (commonly by immunochemical methods) followed by confirmation with a more selective method (e.g. mass spectrometry). Drug testing is usually focused on traditional drugs of abuse but as of a few years back additional testing has become increasingly important due to the introduction of a large amount of new substances of abuse into the recreational drug market [3].

1.2 THE NEW PSYCHOACTIVE SUBSTANCE (NPS) PHENOMENOM

Violation of the law by distributing, manufacturing or possessing a narcotic substance can result in long prison sentences. To circumvent the risk of penalty, substances with structures similar to drugs classified as narcotic substances (e.g. amphetamine, benzodiazepines, cocaine, heroin) have been synthetized [4]. Due to this modification they usually have similar effects, but can be sold legally in many countries [4]. The European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) refer to these analogs as new psychoactive substances (NPS) with the definition that such substances include “a new narcotic or psychotropic drug, in pure form or in preparation, that is not controlled by the United Nations drug conventions, but which may pose a public health threat comparable to that posed by substances listed in these conventions” [5].

In order to monitor the appearance of such structural variants, EMCDDA started to register these substances in the European Union Early Warning System. This system has registered >600 substances and >70% of them were added to the list during the past 5 years, with benzodiazepines and opioids increasing more and more (Figure 1) [3]. Around 20 benzodiazepines were monitored by the EMCDDA in 2016, with 6 new reported in 2016 alone (Figure 1) [3]. The large rise in NPS has become a challenge for drug testing laboratories since detection methods have to keep up with the latest drugs on the market. To confirm intake of a new drug, comparison with a reference material of this drug is usually needed. Due to the fast turn-over of these new drugs, such material is rarely present until just before the drug is replaced by another analogue of the classical drug. Furthermore, detection of NPS in biological samples is also complicated by the fact that little is usually known about their excretion patterns in humans [6].

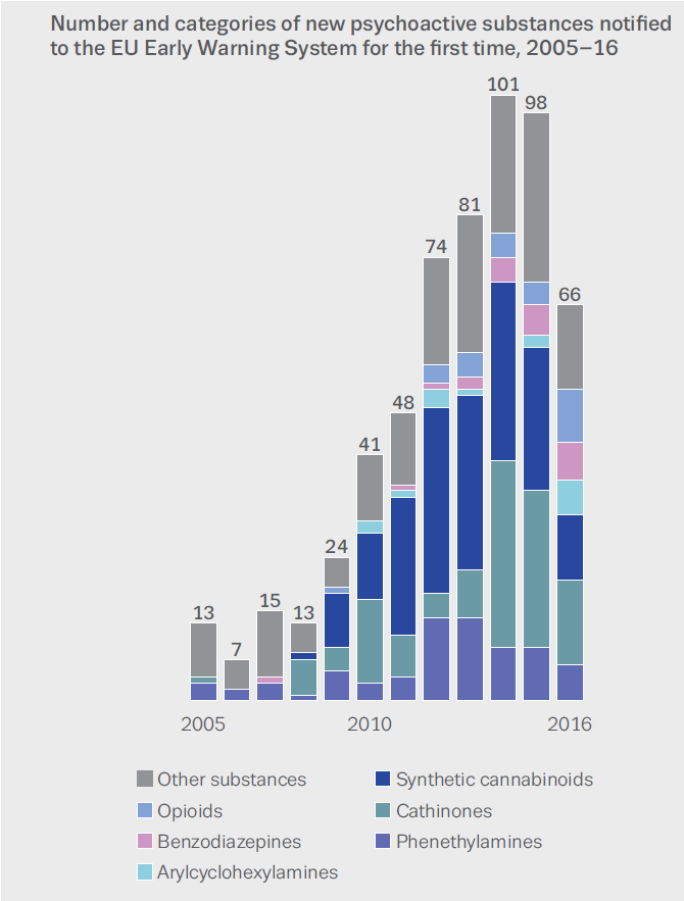

Figure 1: Number of new psychoactive substances reported to the EU Early Warning system from 2005 to 2016. The number of newly reported benzodiazepines (purple) have increased steadily since 2011, with six new registered in 2016 (re-print from [3] with permission).

Another issue with NPS is that as most of them have not been tested on humans before their appearance onto the recreational drug market nothing is known about their adverse effects [4]. Furthermore, NPS are commonly synthetized in clandestine laboratories with poor quality controls where contaminants and differences in doses may be found in the "products", thus increasing the risk for adverse effects [4]. A substance where several severe intoxications have been noted is 3,4-methylenedioxypyrovalerone (MDPV) [7], which was banned by the European Union (EU) in 2014 and as a result is illegal to manufacture or market within the EU [8]. Other bans have been implemented in an attempt to reduce the number of NPS appearing on the recreational drug market [8]. These bans consist of countries amending their laws on controlled substances to include either a generic control (control of a substance cluster), analogue control (control of all substances with "chemical similarities" to listed substances) and individual listing (only control of listed substances) [8]. Currently, Sweden has the individual listing system and several substances included in this list are benzodiazepines (referred to as either designer or NPS benzodiazepines) [9].

1.3 BENZODIAZEPINES AND DESIGNER BENZODIAZEPINES

Benzodiazepines are drugs used to treat anxiety, insomnia and epilepsy [10]. Prescription frauds of these drugs are common in Europe [11] and a number of studies have shown that these drugs are commonly abused [12]. Furthermore, benzodiazepines are in many cases abused simultaneously with other substances, e.g. opioids to increase the effects of the opioids [12].

The designer/NPS benzodiazepines are structural variants of prescription benzodiazepines and throughout this thesis all benzodiazepines currently not used as pharmaceuticals in Sweden are referred to as "designer benzodiazepines". We have chosen this definition since Sweden has added several benzodiazepines to their narcotics list in the past few years and also since phenazepam was recently added as a substance in the Convention on Psychotropic Substances of 1971 [13], thus complicating the designation.

1.4 METABOLISM

The disposition of drugs within the body can be divided into four phases: absorption from site of administration, distribution within the body, metabolism and excretion/elimination [10]. Metabolism is the step in which the drug is modified to become more easily eliminated [10]. Due to this, urine usually only contains small amounts of unchanged drug. Consequently, bioanalytical methods for measurements in urine require measurement of metabolites and/or very low detection limits of the parent compound.

Metabolism of benzodiazepines generally consist of two steps; oxidation and glucuronidation [14]. Additionally, other important steps include N-demethylation and for benzodiazepines containing a NO_2 group, as in the case of flunitrazepam, nitro reduction [14, 15] followed by acetylation [15]. These previously mentioned metabolic steps have also been detected for designer benzodiazepines (**Paper IV, V**) [16-27].

Demethylation, oxidation and reduction steps are referred to as phase I (or functionalization) reactions since a new functional group is added to the drug [28]. It is apparent from the benzodiazepines reported in a review on their metabolism [14] that the cytochrome P450 (CYP) enzymes are important metabolizing enzymes for the phase I reactions of benzodiazepines. The highest amount of CYP enzymes can be found in the liver and due to the large amount of different isoenzymes, a classification system depending on "family", "subfamily" and individual gene has been applied [29]. This system is used to describe the isoenzyme responsible for a certain metabolic reaction and some that are important in human drug metabolism include CYP2C19, CYP2D6 and CYP3A4 [29].

Acetylation, glucuronidation and sulfation are instead referred to as phase II (or conjugation) reactions [28]. These reactions are performed by acetyltransferases, UDP-glucuronyltransferases (UGTs) and sulfotransferases (SULTs), respectively [28].

Inter-individual differences can be found in the metabolism of drugs and these differences can depend on genetics (polymorphism) or environmental factors (inhibition or reduction of

the enzyme responsible for the metabolic reaction due to the presence of another compound) [10]. Thus, inter-individual differences in metabolic patterns needs to be taken into consideration when developing bioanalytical methods for detection of NPS to select the optimal bioanalytical targets.

1.5 METHODS FOR ANALYZING DRUGS OF ABUSE

Immunochemical assays have the advantage of being performed on high capacity automated analyzers that can include a large panel of tests. Disadvantages include poor specificity and target of substance classes rather than a specific compound for many assays, thus making it difficult to determine the drug ingested [30]. Due to this, as previously mentioned, drug testing is a two-step process usually involving screening with an immunochemical assay followed by confirmation with a more specific methodology e.g. mass spectrometry (MS) [31]. However, due to the high number of emerging NPS, high resolution mass spectrometry (HRMS) methods are preferred for screening since it is faster to include new substances with this technique compared to commercial immunoassays [32].

For detection of benzodiazepines in biological samples, the use of liquid chromatography-mass spectrometry (LC–MS) has increased [33]. Although chromatographic separation methods (LC more than gas chromatography (GC)) are most common, other techniques (e.g. capillary electrophoresis, capillary electrochromatography) have also been applied [33]. Many of these separation techniques have also been utilized in recently published articles for detection of designer benzodiazepines in biological samples [19, 22, 24-26, 34-42].

1.5.1 Immunochemical assays

As previously mentioned, immunochemical assays are usually used for drug screening. In **Paper I** we investigated the detectability of designer benzodiazepines using commercially available immunoassays. The techniques used are described below.

1.5.1.1 Cloned Enzyme Donor Immunoassay (CEDIA), Enzyme Multiplied Immunoassay Technique (EMIT) and Homogenous Enzyme Immunoassay (HEIA)

In EMIT [30] and HEIA [43], if a drug is present in the patient sample, both the drug present in the sample and the enzyme-labeled drug can bind to an antibody (Figure 2) [30, 43]. The enzyme-labeled drug not bound to the antibody can then react with the substrate and produce a product with an absorbance at a certain wavelength, resulting in an increased absorbance [30]. On the other hand, if the drug is not present in the investigated patient sample, the enzyme-labeled drug binds to the antibody, resulting in an inability of the enzyme to cleave the substrate (Figure 2) [30, 43]. Consequently, a lower absorbance is observed.

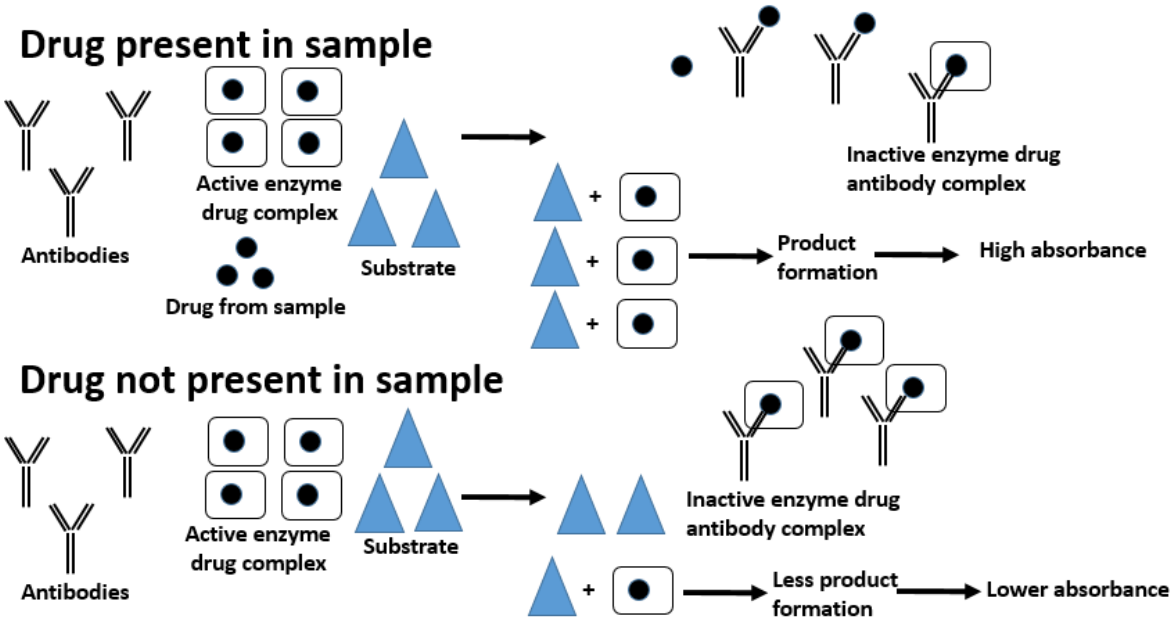

Figure 2: Principle of the homogenous enzyme immunoassay (HEIA) and enzyme multiplied immunoassay technique (EMIT).

The CEDIA technique uses the same principle as EMIT and HEIA, but with two inactive enzyme fragments (a drug labeled enzyme donor, ED and an enzyme acceptor, EA) [30]. When ED binds to EA an active enzyme is formed. The active enzyme can cleave a substrate, in turn forming a product that absorbs light. As a result, if drug is present in the patient sample an increase in absorbance will be observed, otherwise a lower response will be detected [30].

1.5.1.2 Kinetic Interaction of Microparticle in Solution (KIMS)

The principle of KIMS is that if the drug is not present in a patient sample when measured using KIMS, an antibody will bind to a drug labeled microparticle, resulting in aggregate formation and an increase in absorbance [30]. However, if the drug is present in the patient sample, both the drug present in the sample and the drug labeled microparticle can bind to the antibody. Thus, the drug labeled microparticles that are not bound to the antibody cannot form aggregates and there will be a decrease in absorbance [30].

1.5.2 Sample preparation and separation techniques

Before instrumental analysis a sample must be prepared with a suitable procedure. The sample preparation step must either separate components in a sample because of a more unselective detection (e.g. UV) or transfer the compound of interest from the biological matrix to a suitable extract. A common sample preparation procedure is liquid-liquid extraction, which uses a mixture of two different solvents to separate the compound of interest from matrix components [44]. Another extraction technique is solid phase extraction (SPE). In SPE the biological fluid is added to a cartridge containing a solid phase that adsorbs

components in the sample. This is followed by the addition of a liquid to wash out interferences in the sample and finally an additional liquid is added to elute the compounds of interest. These two extraction techniques have the advantage of achieving a sample clean up and also enables enrichment of the analytes to increase sensitivity. A faster alternative is to add e.g. an organic solvent or an acid to the biological samples to precipitate proteins present (e.g. plasma proteins), then centrifuge the samples and inject the supernatant into the separation system [44].

For urine samples, an alternative to extraction, that has been possible due to the combination of ultra high performance liquid chromatography (UHPLC) columns with a high separation efficiency and highly selective detection techniques, is to simply dilute the sample and inject it into the UHPLC–MS/MS system. A recent review demonstrated the application of dilution of samples combined with LC–MS in drug testing [45]. Authors pointed out the possibility of using this combination to develop fast, simple methods including substances with a larger variety in polarity as compared to GC-MS [45].

The chromatographic separation of components present in a sample is achieved by differences in distribution between a mobile phase and a stationary phase [44]. When GC is applied, the liquid sample is vaporized in the injector and then transported into the separating column using a carrier gas (mobile phase). Derivatizations are often performed to increase the thermostability and volatility of analytes but it should be noted that in many cases, the optimization and utilization of these derivatization procedures can be time consuming [44].

In LC, the mobile phase is a liquid phase. The most common LC separation mode is the "reversed phase", in which the stationary phase is non-polar (e.g. octadecylsilane/C18) and the mobile phase is polar [44]. The polar mobile phases generally include a buffer and an organic solvent that is miscible with water (e.g. acetonitrile, methanol). The separation is performed using either a constant mixture of the components in the mobile phase (isocratic elution) or a mixture that is changed during the chromatographic run (gradient elution). The latter is usually used in multi-methods that include analytes with a large variety in polarity in order to optimize the analysis time [44]. An advantage of using the reversed phase mode is that polar liquids (e.g. diluted urine) can be directly injected into the LC system.

A major progress in LC analysis was the introduction of UHPLC. In UHPLC it is possible to perform the separations with stationary phases of smaller particle sizes (that gives a higher separation efficiency) due to instrumentation that allows for a higher back-pressure [46]. An additional LC technique that required amendment of instrumentation is nano-LC [47]. Nano-LC includes columns with a more narrow inner diameter, thus enhancing the sensitivity of the separations due to less bandbroadening [48].

1.5.3 Mass spectrometry (MS)

For LC–MS analysis, the investigated compounds are present in the liquid phase when exiting the LC column, and the analytes need to be converted into the gas phase before entering the high vacuum inside the MS [49]. This conversion is performed in the interface

between the mass spectrometer and the LC. In the MS the ions are separated according to mass/charge (*m/z)* ratios and their intensities are recorded using a detector.

A common interface used in LC–MS analysis is electrospray ionization (ESI). ESI is an ionization process where a liquid is sprayed from a capillary tube (where charges are picked up from an added potential over the capillary) following the addition of a heated gas resulting in evaporation of the spray into smaller droplets [49]. These droplets are then additionally broken down by evaporation which causes an even smaller droplet size (Figure 3). This process continues and finally, desorption of charged molecules occurs (Figure 3) [49]. Furthermore, when no sample clean-up is performed (as in the direct dilution approach), the amount of substances competing for charges increase resulting in a lower ionization efficiency for some compounds [45]. This difference in ionization efficiency is known as matrix effect and needs to be carefully validated.

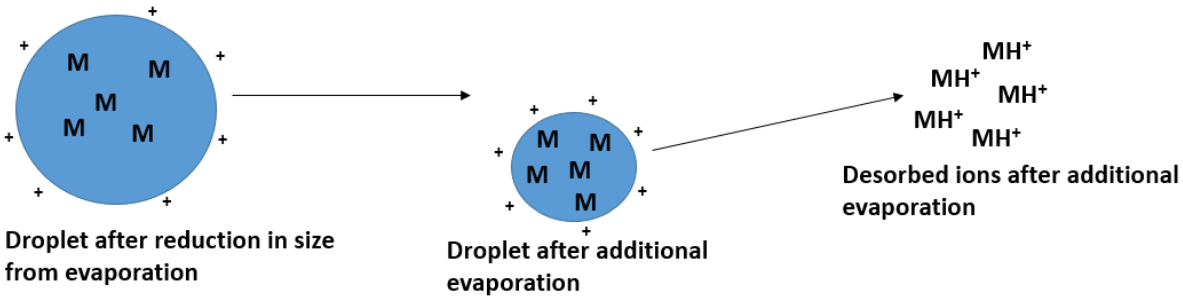

Figure 3: Evaporation of droplets to desorbed ions in electrospray ionization (all ions are drawn as MH^+ ions for simplicity, however other adducts e.g. Na^+ are also possible).

One way of increasing the selectivity and sensitivity in MS methods is to use triple quadrupole instruments in tandem mass spectrometry (MS/MS) mode. The triple quadrupole mass spectrometer is a low unit resolution mass spectrometer consisting of three quadrupole analyzers (Q1–Q3) connected together in a sequence (Figure 4) [49].

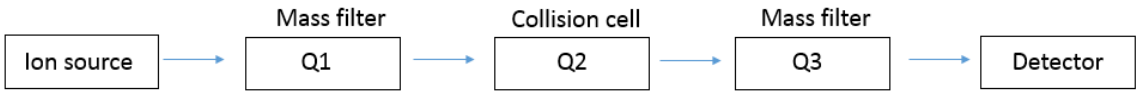

Figure 4: Schematics of a triple quadrupole mass spectrometer showing the travel directions of the ions.

Each quadrupole consists of four rods to which potentials are applied. In MS/MS mode, ions generated from the ion-source travel to the first quadrupole where, due the generated electric field in the quadrupole, only ions of a selected *m/z* ratio will pass through to the second quadrupole [49]. This selection is the result of ions with a selected *m/z* ratio being drawn

towards the charged rods without touching the rods since the potential of the rods are interchanging. The second quadrupole contains an inert collision gas that can collide with the ions supplied from the selection in the first quadrupole, which then allows for fragmentation of the ions. These fragmented ions are thereafter introduced into the third quadrupole where a selection according to *m/z* is performed in the same manner as in the first quadrupole [49]. As a result selective and sensitive methods can be developed since both the selection of ions with a certain *m/z* in Q1 and Q3 and the collision energy in Q2 can be optimized for a specific compound.

A way to further increase the selectivity of MS methods is to use high resolution mass spectrometry (HRMS). The high resolving power of HRMS instruments allow determination of single elemental compositions, thus making them superior to triple quadrupole instruments in identification of unknown substances. A high resolution mass analyzer that was first described in 2000 but is now considered a "mainstream" mass spectrometer, is the orbitrap mass spectrometer [50]. These instruments consists of a C-trap that injects the ions into the orbitrap analyzer (Figure 5). Once the ions are injected into the orbitrap mass analyzer, they will start to oscillate around the central electrode due to a strong electrical field inside the orbitrap (Figure 5) [50]. The resulting current is then measured and transformed to frequencies and intensities of each *m/z* using Fourier transformation, resulting in the mass spectrum (Figure 5) [49]. The orbitrap has the advantage of yielding almost no background noise from other components present in the sample while on the other hand background noise can be found in TOF analyzers (another type of HRMS instrument), thus affecting the detection limits [51].

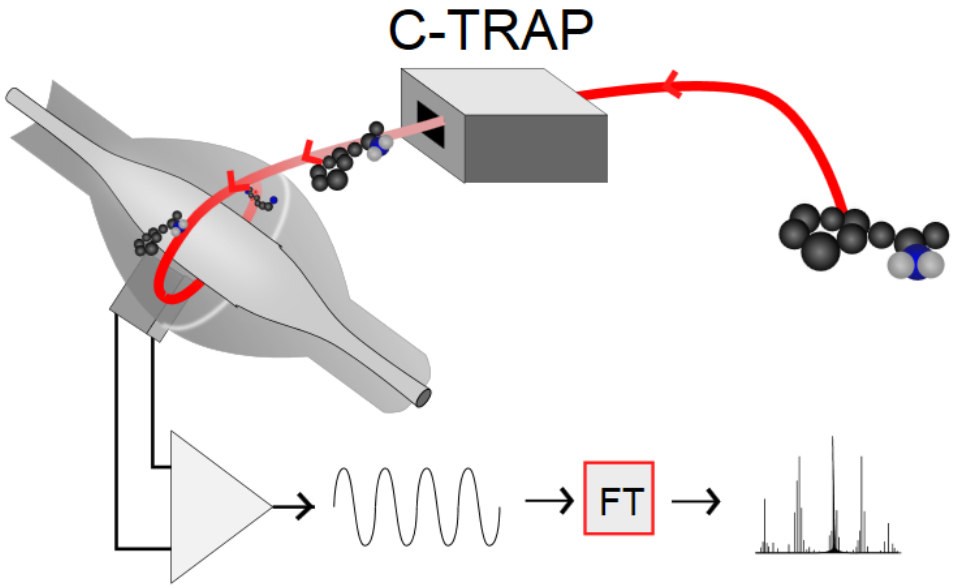

Figure 5: Schematics showing the transfer of ions from the C-trap to the orbitrap mass analyzer where ions oscillate around the central electrode resulting in a current that is transformed into the mass spectrum using Fourier transformation.

1.6 METHODS FOR ASSESSING METABOLIC PATTERNS OF NPS

Urine analysis of NPS is complicated since when NPS first appear on the drug market little or no knowledge regarding their excretion patterns in humans are known [6]. Consequently, it is essential to perform such studies in order to evaluate the metabolites present and to compare their presence in the sample with parent drug to determine analytical targets suitable for drug testing. An important factor in assessing the metabolic patterns of NPS is the limitations in performing controlled human studies due to ethical problems, and as a result several in vitro studies, animal studies, self-ingestion experiments and studies using samples from confirmed intoxication cases have been applied to circumvent this issue [6]. Many of these techniques have been utilized to determine the metabolic patterns of designer benzodiazepines (**Paper IV, V**) [16-27, 52].

1.6.1 In vitro studies

In vitro studies have been performed using both subcellular fractions and intact hepatocytes [29]. Subcellular fractions include e.g. human liver microsomes (HLM) and human liver cytosols (HLC). HLM are prepared by breaking up eukaryotic cells and isolating certain enzymes by centrifugation, with the result of HLM present as sediments and HLC as supernatant. HLM include enzymes located at the endoplasmic reticulum such as CYPs and UGTs, while HLC instead includes soluble enzymes such as SULTs and catechol-*O*-methyltransferase [29].

Hepatocytes have the advantage of containing enzymes responsible for both phase I and II metabolism and co-substrates needed for metabolic reactions [29]. However, commercially available cryopreserved hepatocytes are more expensive and entail more complex incubation media compared to the subcellular fractions mentioned above [29]. Furthermore, enzyme activities can vary remarkably from lot to lot.

1.6.2 In vivo studies

In vivo studies to determine the excretion pattern of NPS in urine have included investigations of samples obtained from drug users, animal- and self-experiments [6]. These techniques have the advantage of representing living organisms as well as enabling collection of the biological matrix of interest [6].

Animal experiments can be designed to mimic typical human doses [6]. On the other hand, as the excretion pattern may differ between humans and other animals, high dose investigations might be necessary. The higher dose would then increase the possibility to detect major human metabolites that are only present in low amounts in the animal when scaling to human doses is performed [6].

In some countries, self-experiments are possible without ethical approval [16, 17, 19, 26, 52]. Here, the authors ingest the drug and then provide the biological samples for metabolism investigations. However, for the designer benzodiazepines, samples from such controlled studies have only been collected from one individual in each study [16, 17, 19, 26, 52], thereby potentially missing inter-individual differences in metabolic patterns.

Several NPS metabolism studies have been performed using samples obtained from drug users [6]. In these cases, urine metabolites can be corroborated by the presence of parent compounds in the urine (**Paper IV, V**) or by paired blood samples from the same patient. However, it is important to keep in mind the possibility of co-ingestions of structurally related compounds by the patient, making in vitro studies or animal studies good complements [6]. On the other hand, if samples are present from several patients, inter-individual difference are more likely to be detected and thus the certainty for the suggested analytical targets increase.

2 AIMS

The aims of this doctoral project were to:

a) Investigate the detectability of designer benzodiazepines in urine using different bioanalytical techniques (immunochemistry and mass spectrometry)
b) Investigate the occurrence of designer benzodiazepines in Sweden
c) Determine the clinical characteristics of designer benzodiazepines from analytically confirmed intoxication cases

The specific aims were:

Paper I

- To determine the detectability of designer benzodiazepines using commerically available immunoassays by determining cross-reactivities of parent compounds in spiked urine samples and by analyzing urine samples from intoxication cases

Paper II

- To develop and validate an LC-MS/MS method for detection of designer benzodiazepines in urine
- To apply this method for analysis of patient urine samples to investigate occurrence of designer benzodiazepines in Sweden

Paper III

- To develop and validate an LC-HRMS method for detection of designer benzodiazepines in urine
- To apply this method for analysis of patient urine samples to confirm method suitability

Paper IV

- To determine the human metabolic patterns of clonazolam, meclonazepam and nifoxipam and suggest metabolites suitable as targets for drug testing purposes

Paper V

- To determine the human metabolic patterns of flubromazolam and pyrazolam and suggest metabolites suitable as targets for drug testing purposes

Paper VI

- Study the occurrence of designer benzodiazepines in Sweden
- Investigate the clinical features of designer benzodiazepines
- Investigate the pattern of abuse among designer benzodiazepine users (in relation to multi-intoxications and time of national legislation)

3 MATERIALS AND METHODS

3.1 STUDY SAMPLES

The urine samples used for all studies were either de-identified aliquots collected from the routine drug testing service at the Department of Clinical Pharmacology, Karolinska University Laboratory originating from drug dependence clinics (ethical permit no. 00-230) or from the STRIDA project (ethical permit no. 2013/116-31/2). STRIDA (*S*amverkansprojekt avseende *T*oxicitetsutredning och *R*iskbedömning av *I*nternet*D*roger baserat på kliniska *A*nalyser) is a project that monitors the occurrence and health hazards of NPS in Sweden [53] with samples originating from patients presenting in emergency units all over Sweden.

3.2 SAMPLE PREPARATION

Urine samples were analyzed either directly (in all immunochemical assays) or after dilution (LC-MS analysis). The dilution applied for LC-MS analysis was performed either with (**Paper I-III, V-VI**) or without hydrolysis (**Paper IV, V**) of the samples. For hydrolysis, 170 µL internal standard (IS) in 10 mmol/L ammonium acetate and 30 µL β-glucuronidase was added to 50 µL urine. When no hydrolysis was performed, urine samples were diluted 1:5 with IS in 0.1% formic acid in Milli-Q water.

3.3 INSTRUMENTATION

The CEDIA, EMIT II Plus and HEIA immunochemical assays investigated were performed on an Olympus AU680 instrument (Beckman Coulter, Brea, CA, USA) and the KIMS II assay on a Cobas 6000 (c501) instrument (Roche Diagnostics, Mannheim, Germany).

In **Paper I and II** an ACQUITY UPLC system with a Xevo TQ tandem mass spectrometer (Waters, Milford, MA, USA) operating in positive electrospray mode was used. Additionally, in **Paper I and III-VI**, an UHPLC-HRMS system (Dionex Ultimate UHPLC System coupled to a Q-Exactive system equipped with a heated electrospray ionization (HESI)-II source (Thermo Scientific, Waltham, MA, USA)) was utilized. Apart from the UHPLC-HRMS system mentioned above, **Paper IV** also used an EASY-nLC system coupled to a Q-Exactive equipped with an EASY-Spray source (Thermo Scientific).

3.4 VALIDATION

The method developed in **Paper II** was validated with the EMA guideline [54] using the following parameters: accuracy and precision, carry-over, dilution integrity, linearity, limit of detection (LOD), lower limit of quantification (LLOQ), matrix effects, selectivity and stability. These parameters (apart from dilution integrity) were also validated in **Paper III** but using an in-house guideline instead. The validation performed in **Paper IV** included investigation of intra accuracy and precision, LLOQ, LOD, matrix effect and selectivity and was less extensive compared to **Paper II-III** as the focus of **Paper IV** was the metabolism studies.

4 RESULTS AND DISCUSSION

The analytical work used four immunochemical assays (CEDIA, EMIT II Plus, HEIA and KIMS II), a triple quadrupole mass spectrometry method and several orbitrap mass spectrometry methods in order to gain knowledge on the detectability, occurrence and metabolic patterns of designer benzodiazepines.

4.1 DETECTION OF DESIGNER BENZODIAZEPINES USING IMMUNOCHEMISTRY

The study in **Paper I** was performed to evaluate a number of commercial immunochemical assays for detection of designer benzodiazepines in urine.

Paper I concluded that, using a 200 ng/mL cut-off, all compounds gave a response above the cut-off in the KIMS II assay when concentrations were ≤500 ng/mL, while for the CEDIA assay this was the case for all investigated compounds (Figure 6) except flutazolam. Whereas when using the EMIT II Plus and HEIA assays only 10 of the 13 investigated designer benzodiazepines had a response above the cut-off at the highest concentration investigated (1000 ng/mL).

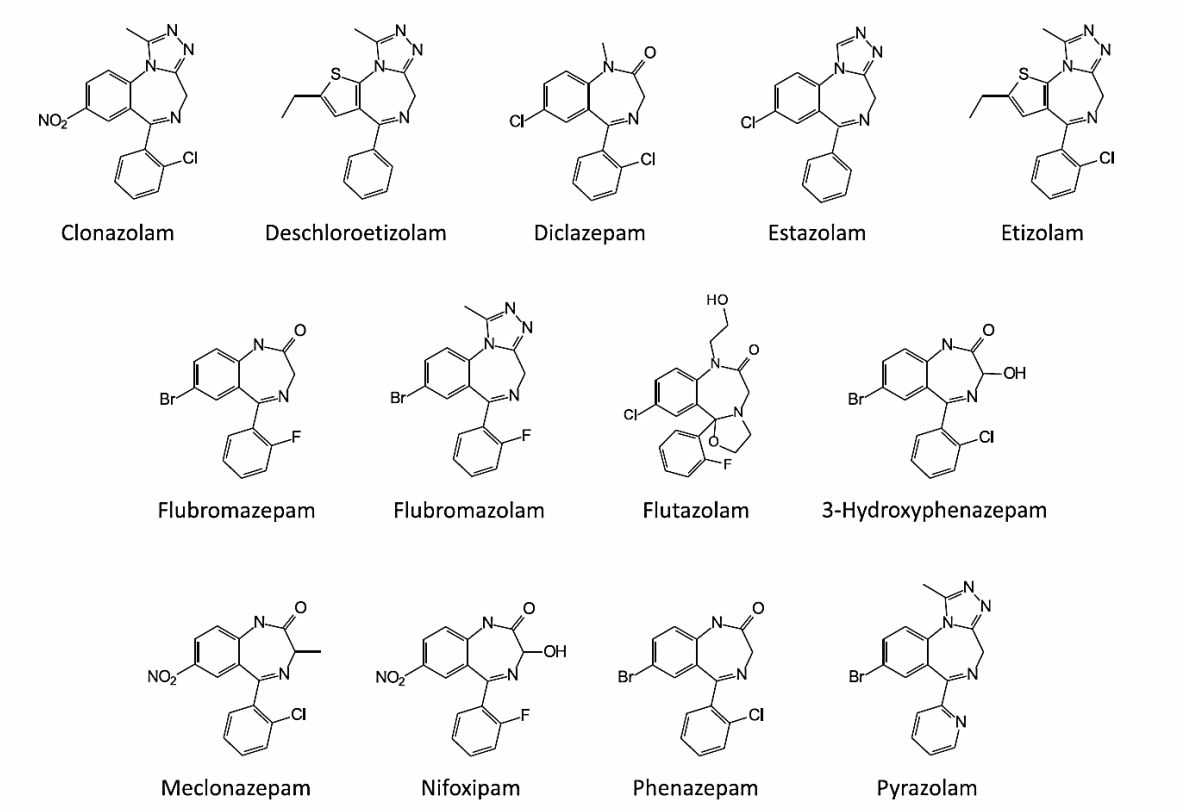

Figure 6: Chemical structure of the 13 benzodiazepines investigated in Paper I (re-printed from Paper I with permission).

In agreement with our study, Kyle et al [55] detected a mean cross-reactivity of 142% for phenazepam using the SIMENS EMIT II Plus assay (as compared to our 133%). They also performed studies of phenazepam using other immunoassays, showing cross-reactivities >80% in all but the enzyme linked immunosorbent assay (ELISA) [55]. Additionally, ELISA detection of phenazepam and other designer benzodiazepines (diclazepam, etizolam, flubromazepam and pyrazolam) has been studied in blood [56]. These authors concluded that the ELISA Immunalysis assay is sensitive enough to detect etizolam and pyrazolam in postmortem cases and that the benzodiazepines tested had a good cross-reactivity [56].

When urine samples confirmed to contain pyrazolam were investigated using immunochemistry in **Paper I**, the concentration determinations from the LC-MS/MS analysis of the samples suggested presence of metabolites (due to a higher assay response than expected from the cross-reactivity experiments) although at that time none had been detected [52]. Consequently, we later performed a study to investigate the metabolic pattern of pyrazolam in human urine samples (**Paper V**). The results from this study (**Paper V**) confirmed presence of pyrazolam metabolites.

None of the samples confirmed to contain only meclonazepam were detected in the HEIA assay, most likely due to the low parent concentrations found **(Paper I**) and since this assay has a low cross-reactivity of 7-aminometabolites [57] and **Paper IV** noted 7-amino-meclonazepam as one of the major metabolites. Additionally, only one third of the urine samples confirmed to contain etizolam were detected using the HEIA assay, suggesting that not only the parent compound, but also the metabolites have a low cross-reactivity using this assay.

Studies on the metabolic patterns of flubromazepam [16] and nifoxipam (**Paper IV**) indicated that the main metabolites found in urine are glucuronides. This is most likely the reason for the low reactivity of patient samples containing only one of these compounds in the EMIT II assay, since this assay generally has a low cross-reactivity of glucuronides and does not contain a reagent that can hydrolyze conjugates [58]. This is in agreement with the high detectability rate of patient samples with a confirmed intake of designer benzodiazepines in the immunoassays containing hydrolysis of the samples (CEDIA and KIMS II immunoassays).

The good detectability of designer benzodiazepines in the CEDIA assay (**Paper I)** is somewhat different from the results obtained in self-ingestion experiments by the group of Auwärter [16, 17, 19, 52]. They could detect intake from self-ingestions of diclazepam, flubromazepam, flubromazolam and pyrazolam in urine samples, albeit only in one of the samples collected from ingestion of flubromazepam [16, 17, 19, 52]. Their calculated cross-reactivity of diclazepam and flubromazepam was 136% and 79% in urine [16, 17], in comparison to our results in **Paper I** of 141% and 164%. One possible explanation for the differences obtained for flubromazepam might be variances between different lots, which has been noted as one of the drawbacks of immunoassay [30]. In serum samples obtained from the self-ingestions, only flubromazepam was detected [16, 17, 19, 52]. A low detectability

was also noted using the Fluorescence Polarization Immunoassay (FPIA) [16, 17, 52]. The reason for this could be the low doses ingested in the self-administration studies (only one tablet or capsule, apart from the case of pyrazolam where two tablets were ingested) [16, 17, 19, 52].

The results from **Paper I**, in addition to previous studies [55, 56] showed that most designer benzodiazepines can be detected using commercially available immunoassays. Consequently, it is important to update confirmation methods to cover also these compounds, otherwise there is a risk of reporting "false" negative results.

4.2 DETECTION OF DESIGNER BENZODIAZEPINES USING LIQUID CHROMATOGRAPHY MASS SPECTROMETRY

To ensure a high quality in drug testing, efficient and sensitive methods are needed. Thus, we chose to use LC coupled to mass spectrometry when developing our detection methods for designer benzodiazepines (**Paper II and III**).

In Paper II, our aim was to develop an LC-MS/MS method for the detection of designer benzodiazepines in urine and also to apply this method to urine samples sent to the laboratory for drug testing to obtain an indication of their occurrence in Sweden.

An important step in assuring high quality methods for drug testing is validation of the method. In **Paper II** we applied the EMA guideline on bioanalytical method validation [54]. As this guideline focus on quantitation rather than qualification, additions were needed in order to enhance identification certainty. Therefore, an extra selected reaction monitoring transition (qualifier ion) per analyte investigated and a ratio between the ion transitions used for quantification and qualification was added [59]. Furthermore, to comply with the EMA guideline **Paper II** included rather extensive precision and accuracy experiments even though urine concentration determinations is less important since concentrations are dependent on many factors (e.g. urine pH and hydration status of the body) [60]. Due to these differences, drug testing in urine is by tradition only reported as either positive or negative (i.e. above or below a certain cut-off value). Recommendations of such cut-off values exists for some benzodiazepines [61], however these might not be suitable for designer benzodiazepines. Thus, by using a more thorough validation we would enhance the certainties of our concentration determinations and the results from our patient sample analysis could be used to set up cut-off values for drug testing of designer benzodiazepines.

The method developed in **Paper II** included dilution of urine and enzymatic hydrolysis (10 min), centrifugation (5 min) and injection directly into the LC-MS/MS system. As the total run time of the method was 4 min, the developed method is a fast way to analyze for designer benzodiazepine intoxication of the 11 benzodiazepines included in the method. A drawback of the method was that the CV (%) of the IS normalized matrix factor was not within the accepted values for clonazolam, flutazolam and nifoxipam.

When 390 patient urine samples that were screened positive with CEDIA and confirmed not to contain any prescription benzodiazepines were analyzed with the developed method, 40% were detected to contain at least one designer benzodiazepine. All designer benzodiazepines included in the method except diclazepam, flutazolam and phenazepam were detected. Phenazepam was, however, detected in samples from the STRIDA project. Additionally, amendment of urine drug testing cut-off values was demonstrated for several designer benzodiazepines (e.g. clonazolam, flubromazepam and meclonazepam) as many of these were found in concentrations lower than the cut-off value for benzodiazepines at our laboratory (50 ng/mL) (**Paper II**, Table 1).

Table 1: Number of confirmed cases and concentration ranges found in the patient samples from Paper II (re-printed with permission from Paper II)

Substance	Concentration range (ng/mL)	Number of samples (N)
Pyrazolam	32-920	9
Flubromazepam	2.7-30	14
Meclonazepam	1.6-190	45
Etizolam	5.8-270	11
Nifoxipam	10-2800	4
Deschloroetizolam	130	1
Clonazolam	7.3-23	8
Phenazepam	26-70	3*
Flubromazolam	5.4-1500	96

** These samples were obtained from the STRIDA project and are therefore not included in the 40% reported as positive designer benzodiazepine intoxications*

In conclusion, the method developed in **Paper II** was efficient and was able to detect intake of designer benzodiazepines in 40% of urine samples screening positive with CEDIA that were confirmed not to contain a prescription benzodiazepine. The high number of detected cases indicate a rather common use of designer benzodiazepines in Sweden, and the importance of including designer benzodiazepines in confirmation methods for drug testing.

The aim of Paper **III** was to develop a sensitive and more flexible method for determination of designer benzodiazepines in urine that enables fast inclusion of new designer benzodiazepines appearing on the recreational drug market. This method was applied on urine samples from drug dependence clinics and acute intoxication cases suspected to involve NPS benzodiazepines (STRIDA samples).

To increase the identification certainty, both a screening method (LC–HRMS) and a confirmation method (LC–HRMS/MS) was developed. The screening method will enable fast inclusion of new analytes and retrospective investigation of already analyzed samples and will most likely result in less false positive screening result as compared to immunochemical assays, as has been noted in a previous study using drugs from different substance classes [62]. Also, this method is generic and could be applied to analysis of other drug classes as well. The confirmation method (LC–HRMS/MS) was developed to increase the certainty of the identification and is easily implemented since it only requires re-injection of samples with a positive result in the LC–HRMS screening method. Furthermore, since the sample preparation for the LC–HRMS method only included dilution of urine followed by hydrolysis (20 min) and the total run time was 5 min, screening of the urine samples will be fast.

In **Paper III**, we performed a method validation using in-house guidelines instead of the EMA guideline. This was decided since three samples per QC level was deemed enough, however the between-run variability was determined over a longer time period as compared to **Paper II** to better reflect routine analysis work. The stability studies of **Paper III** was more extensive than in **Paper II**, as results from **Paper II** indicated a difference in analyte stability between different urines. The extensive stability experiments noted that nifoxipam was the most unstable analyte and using the obtained stability data, it is not recommended to store patient urine samples longer than one day at room temperature or one week at 4 °C. Stability experiments were not performed at -20 °C since extensive studies for nifoxipam had already been done at that temperature (**Paper II)** and our results from **Paper III** suggested that nifoxipam is the most unstable of the 28 analytes investigated. Carry-over was investigated for all analytes from injection of blank urine after injection of a standard containing 1000 ng/mL. The peak area in these blank urines were <20% of the LLOQ peak area for all anlytes in both the screening and the confirmation method, except for pyrazolam and tetrazepam. The blank urine peak areas of pyrazolam were always lower than the LLOQ peak area, while for tetrazepam a response similar to the peak area at the LLOQ level was detected. Due to this, it is recommended to re-analyze positive samples of tetrazepam if they have a concentration around LLOQ and were analyzed directly after a sample with a concentration around 1000 ng/mL. Again, the precision and accuracy experiments were rather extensive in **Paper III** as a result of our wish to use concentrations found in patient samples to determine suitable cut-off values for designer benzodiazepines in urine drug testing.

When the developed screening and confirmation methods from Paper **III** was applied to analysis of patient samples from drug dependence clinics and from acute intoxication cases presenting in emergency wards 16 of the 28 benzodiazepines were detected. These included clobazam, clonazolam, deschloroetizolam, diclazepam, estazolam, etizolam, flubromazepam, flubromazolam, flunitrazolam, 3-hydroxyflubromazepam, 3-hydroxyphenazepam, ketazolam, meclonazepam, metizolam, nifoxipam, and pyrazolam. Also among these two sample sets a

rather high number (~30%) of cases were detected, again noting the importance of including designer benzodiazepines in drug testing analysis.

*Table 2: Concentration ranges found in **Paper III** in urine samples from drug dependence clinics (DDC) and from intoxication cases in emergency wards (STRIDA samples).*

Substance	Concentration found in DDC samples (ng/mL)	Number of DDC samples detected (N)	Concentration found in STRIDA samples (ng/mL)	Number of STRIDA samples detected (N)
Clobazam	86	1	-	-
Clonazolam	-	-	10-132	9
Deschloroetizolam	-	-	5	1
Diclazepam	-	-	8	1
Estazolam	30	1	8	1
Etizolam	-	-	40-1000	5
Flubromazepam	5	1	5-89	11
Flubromazolam	15-61	6	5-1081	53
Flunitrazolam	5-121	24	-	-
3-Hydroxy-flubromazepam	191-410	2	89-11580	10
3-Hydroxy-phenazepam	84-16549	5	258-12089	5
Ketazolam	-	-	-	1
Meclonazepam	-	-	5-126	10
Metizolam	5-14	3	8	1
Nifoxipam	149	1	973-3635	2
Pyrazolam	-	-	6-1995	11

The results in **Paper III** confirmed results from **Paper II** showing that the 50 ng/mL cut-off value is too high for several of the designer benzodiazepines (e.g. clonazolam, flubromazepam, flunitrazolam, meclonazepam and metizolam, Table 2). On the other hand, for 3-hydroxyflubromazepam (a metabolite of flubromazepam [16]), the 50 ng/mL cut-off seems sufficient enough (Table 2), and thus our obtained LLOQ of 50 ng/mL is acceptable. An example of a patient sample from ingestion of flubromazepam is presented in Figure 7.

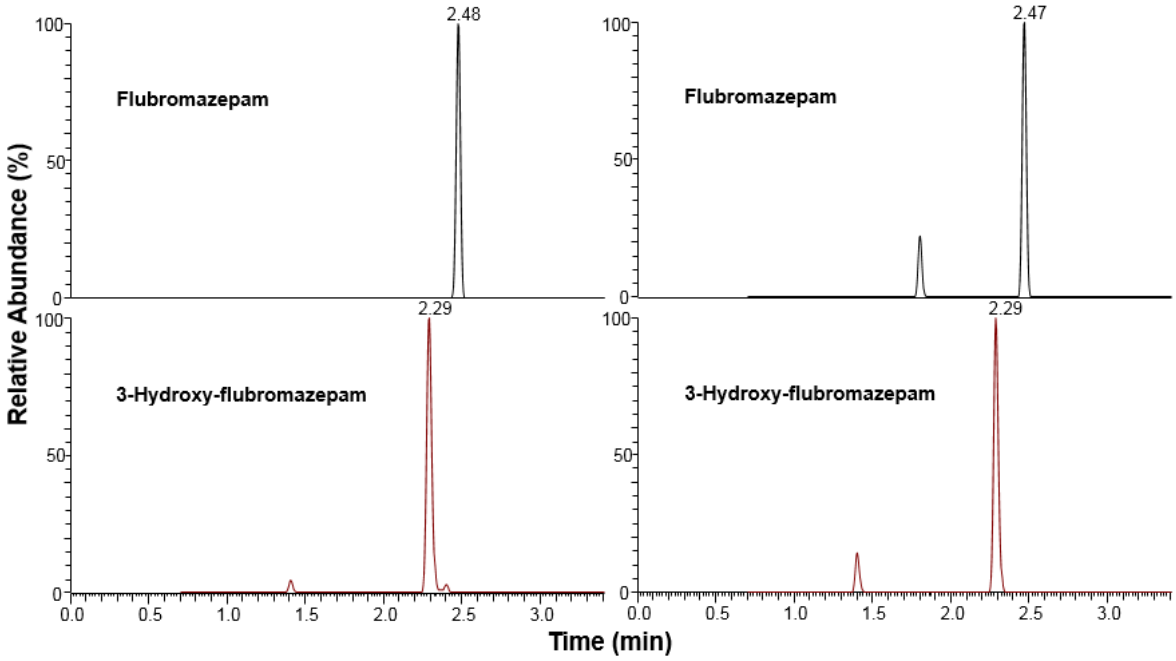

*Figure 7: Chromatograms from the screening method in **Paper III** showing a patient sample containing 7.4 ng/mL flubromazepam and 260 ng/mL 3-hydroxyflubromazepam (left) and a standard spiked at 5 ng/mL flubromazepam and 50 ng/mL 3-hydroxyflubromazepam (LLOQ levels of the analytes, right).*

In comparison to the method developed in **Paper III**, the method developed in **Paper II** and a GC-MS method developed by Meng et al for detection of four designer benzodiazepines in urine [37] had similar lower limit of quantification values (LLOQ). The GC–MS method was optimized for extraction of the four designer benzodiazepines included in the study, resulting in a faster extraction time than the hydrolysis times of our direct dilution methods [37]. However, a disadvantage of their method is the larger volume of urine (1mL) used in the sample preparation [37], as this would not be optimal in routine analysis work. Also, due to the extensive differences in polarity between different benzodiazepines [60] analysis of benzodiazepines with a lower lipophilicity might be difficult using their GC–MS method. Thus, our screening method developed in **Paper III** could be used instead since it allows fast inclusion of new analytes to the already existing method containing 28 benzodiazepines (Figure 8). Furthermore, the combined screening and confirmation method developed in

Paper III has the advantage of fast screening of an unlimited number of analytes for which a confirmation can be performed directly after the screening result on the same instrument by only re-injecting the sample.

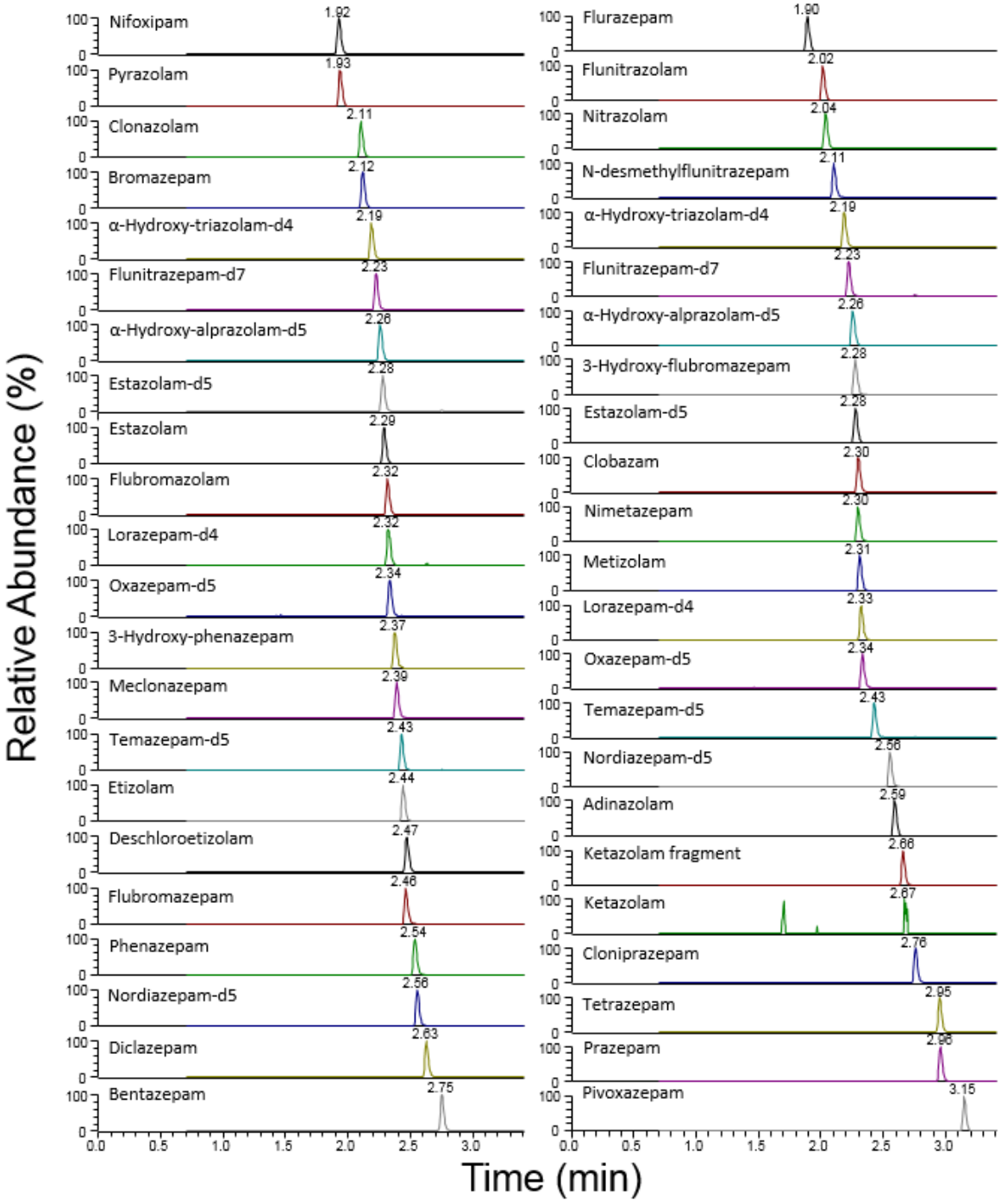

*Figure 8: Chromatograms showing the retention time (RT) of all analytes included in **Paper III** in a screening method run of the highest standard (1,000 ng/mL). Ketazolam is here shown as both M+H$^+$ ion (RT 2.67) and its in-source fragment at m/z 285.0789 (RT 2.66).*

Another study investigating the detection of designer benzodiazepines in blood also used HRMS as a mean for fast inclusion of new analytes to the detection method [34]. This study included both a targeted and an untargeted approach based on another HRMS technique coupled to a quadrupole [34]. One advantage of this method is that it uses an HRMS mode that allows obtaining MS/MS spectra without previously determining the parent ions used for recording the spectra [34]. However, since spectra are obtained simultaneously from several incoming ions, sensitivities of such methods are usually lower than for the ones where a parent ion is predetermined. Thus, for detection of compounds where a low amount is expected in the sample (as is the case of several designer benzodiazepines, **Paper II and III**), an approach with a predetermined parent ion would be preferable.

In summary, the LC–HRMS(/MS) method developed in **Paper III** offered sensitive detection of a large number of benzodiazepines. It can be used for fast screening and confirmation of benzodiazepine intake by simply preparing the sample once and it enables easy inclusion of new analytes. When applied to urine samples from drug dependence clinics and acute drug intoxication cases, it revealed a high prevalence (~30%) of designer benzodiazepine use, thus underlining the importance of including these benzodiazepines in urine drug testing. This prevalence number was similar to our previous study. When patient sample results from **Paper II and Paper III** were combined, ~38% of patient samples from drug dependence clinics that were positive in the CEDIA assay and confirmed not to contain prescription benzodiazepines instead contained designer benzodiazepines.

4.3 METABOLIC PATTERNS OF DESIGNER BENZODIAZEPINES

Both low resolution MS [16-21, 52] and HRMS (**Paper IV, V**) [16, 18-20, 22-27, 52] have been used to determine the metabolic pattern of designer benzodiazepines, and as mentioned in 1.5.3, the advantage of using HRMS is the ability to determine elemental compositions of unknown substances, resulting in more reliable interpretations in metabolite structure elucidations. Thus, both metabolic studies included in this thesis (**Paper IV, V**) were performed using HRMS.

The aim of **Paper IV** was to determine the metabolic patterns of clonazolam, meclonazepam and nifoxipam in humans by analyzing analytically confirmed intoxication cases. Furthermore, these results were used to determine suitable targets for urine drug testing.

Most recent studies of designer benzodiazepine metabolism have entailed analysis of the samples using LC-MS with electrospray ionization [16-27, 52]. As mentioned in section 1.5.3, there is a risk of large matrix effects that then would lead to a misinterpretation of the relative amount of metabolites present in the sample due to differences in ionization efficiency. In order to minimize this effect, we instead performed the analyses in **Paper IV** using using a low flow variant, nano-electrospray ionization (NSI). One reason to use NSI was the concept that the chromatogram peak areas correspond better with concentration values as compared to ESI [63, 64]. It has been suggested that this would be due to the smaller initial droplet sizes obtained in NSI compared to ESI, which would lead to lower

amount of break downs necessary for ionization. This would then result in smaller matrix effects due to reduction of the amount species present for ionization and thus provide peak areas that better correspond to actual concentration [64]. Another advantage of the instrumental step up used in **Paper IV** is the use of nano-LC coupled to NSI that, in theory, would result in improved separation efficiency and increased sensitivity compared to conventional UHPLC because of the lower inner diameters used in nano-LC (1.5.2).

Our studies in **Paper IV** using NSI underlined the need to involve metabolites of clonazolam, meclonazepam and nifoxipam to allow reliable drug testing of these compounds since the abundance of the parent compound was low compared with the acetamino and amino metabolites (Figure 9). Based on the relative abundance between the metabolites in several analytically confirmed intoxication cases (Figure 9), 7-aminoclonazolam, 7-acetamino-meclonazepam and nifoxipam glucuronide seems to be the most abundant metabolites.

Apart from human in vivo studies in our **Paper IV** and a later study [22], metabolism studies of meclonazepam have included in vitro studies in HLM, HLM combined with HLC, s9 and hepatocytes [20-23] and in vivo studies using mouse urine [22]. Results using only HLM detected a hydroxy metabolite [20] and reduction of the nitro group [20-22]. When incubations of HLM were combined with HLC, an acetamino metabolite, in addition to the previously mentioned metabolites, was detected [23]. This was also the case when s9 fractions were used [23].

Using hepatocytes, Vikingsson et al [22] confirmed the metabolites (amino and acetamino) we detected in **Paper IV**, using urine samples from analytically confirmed ingestions of meclonazepam. Furthermore, their study detected 13 candidate metabolites of meclonazepam using urine from paired blood samples with confirmed meclonazepam intake. However, mass spectra could only be obtained for the amino and acetamino metabolites. As a result, the certainties in chemical structure of the rest of the metabolites are lower. Furthermore, apart from the amino and acetamino metabolites only one additional metabolite (monohydroxy acetaminometabolite) was detected in mouse urine [22]. This imply that the main targets in urine testing should be the amino and acetamino metabolites, which confirm our previously reported suggestions from **Paper IV**. It has been suggested [22] that the reason the acetamino metabolite was not detected when incubations were performed only with HLM were that it is likely that this metabolite is formed by the cytosolic enzyme NAT2, as in the case of clonazepam.

The other study using human urine samples [22] had higher area ratios for the amino metabolite in three of their four investigated urine cases, while in our previous study with NSI (**Paper IV**) the area was higher for the acetamino metabolite in four of seven cases (Figure 9). Authors of the other meclonazepam study suggested that their detected differences in ratio between the amino and acetamino metabolites could be due to polymorphism of NAT2 [22]. However, as the matrix effects cannot be evaluated due to lack of reference material, the matrix effects could also be the reason for the differences. Furthermore, as the acetamino metabolite is a conjugated metabolite of the aminometabolite, variance in intensity

might also derive from differences in time between ingestion and sampling. On the other hand, as the patient samples collected from the STRIDA project are collected from patients presenting in emergency wards a sampling close to ingestion can be assumed. The results from the relative distribution of the parent compound and the metabolites in the STRIDA samples (Figure 9, individual no 3–5) suggest that the conjugation of the aminometabolite to the acetaminometabolite is rather fast.

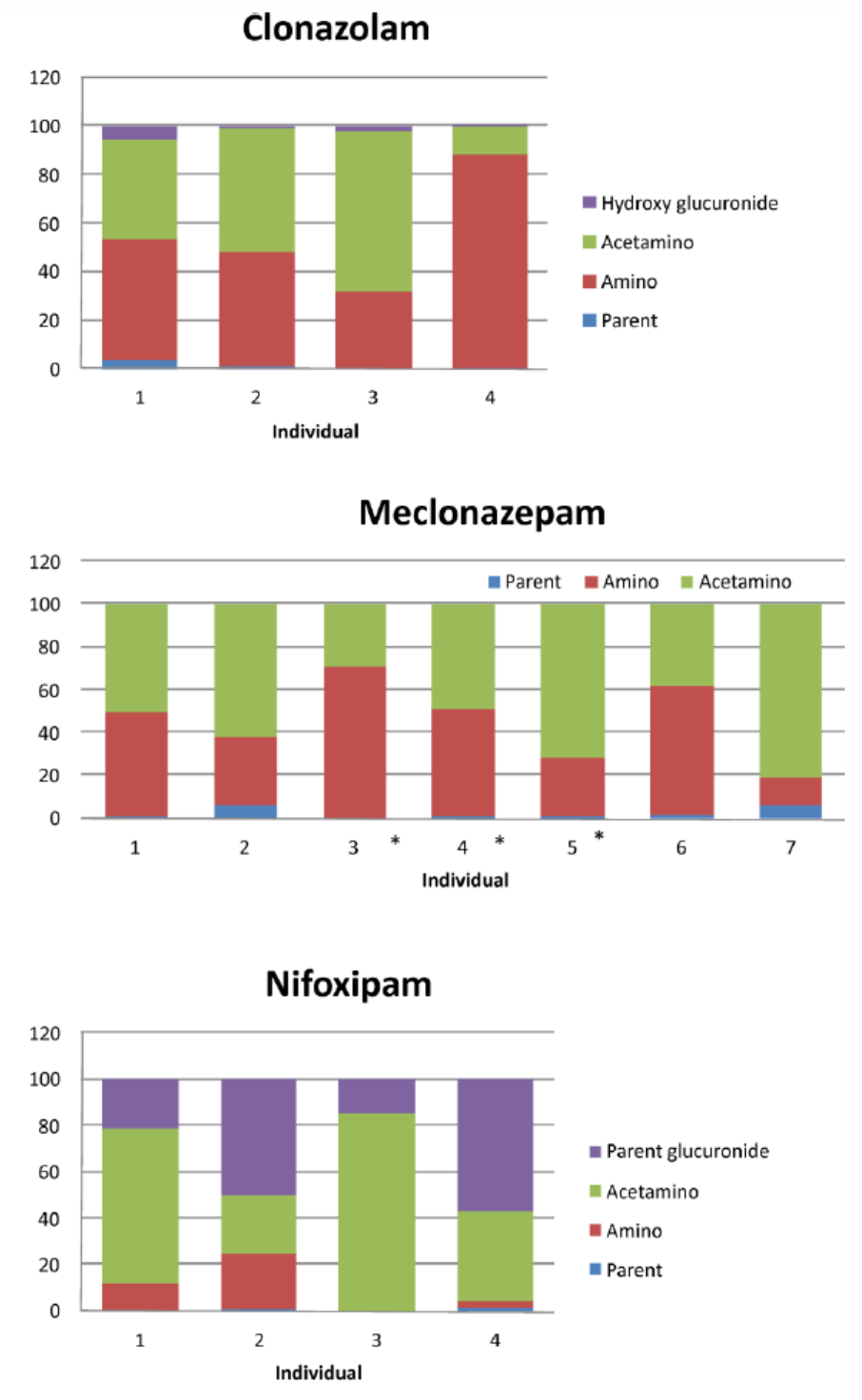

Figure 9: Relative areas (%) of clonazolam, meclonazepam, nifoxipam and metabolites compared to the total area of all analytes in the samples. STRIDA samples are marked with an asterix (re-printed from Paper IV with permission).

To conclude, as the metabolites of meclonazepam had a higher peak area compared to the parent compound in two separate investigations, **Paper IV** and [22], these metabolites can therefore be considered suitable targets for urine drug testing. The most abundant analytes in our NSI studies using cases from several analytically conformed intoxication cases were 7-aminoclonazolam, 7-acetaminomeclonazepam and nifoxipam glucuronide. Thus, urine drug testing should focus on these three metabolites rather than, or in addition to, the parent compound. On the other hand, as nifoxipam is a metabolite of flunitrazepam [65] caution must be taken when interpreting such results even though both nifoxipam and its metabolites are present in the sample.

The aim of **Paper V** was to further investigate the metabolic pattern of flubromazolam and pyrazolam and recommend suitable targets for urine drug testing by analyzing several urine samples from analytically confirmed intoxication cases.

A method validation was performed in **Paper V** as we wanted to compare the concentrations of parent compound in our study samples using a sample preparation with and without hydrolysis. Since the metabolic pattern studies were the focus of **Paper V**, the validation was not as extensive as in **Paper II and III**. Matrix effects were ≤16% at the two investigated concentration levels for both sample preparation methods applied in **Paper V**, suggesting only minor interferences in ionization of the parent compounds from other components present in the sample.

Extensive metabolic investigations have been performed for flubromazolam (**Paper V**) [19-21, 24, 25]. The HLM studies of flubromazolam detected mono hydroxy metabolites [20, 21, 25], di-hydroxy metabolites [20, 21, 25], hydroxy glucuronides [25] and a parent glucuronide [25]. However, one of the in vitro studies could not detect any metabolites in HLM and only small signals of two hydroxy metabolites in hepatocytes [24]. The same study could on the other hand detect several metabolites (hydroxy metabolites, di-hydroxy metabolites, hydroxy glucuronides, di-hydroxy glucuronides and parent glucuronides) in vivo in urine samples from mice [24]. Furthermore, several studies have been performed using urine samples from analytically confirmed intoxication cases (**Paper V**) [19, 24, 25]. Only one study could detect a low signal of a di-hydroxy metabolite in these cases [24], noting the importance of confirming results from HLM studies using human urine samples. In the results published from human urine samples, two hydroxy metabolites, at least one hydroxy glucuronide and at least one parent glucuronide have been detected (Figure 10, **Paper V**) [19, 24, 25].

Hydrolysis of the urines samples was recommended due to the high abundance of glucuronides (Figure 10, **Paper V**) [19, 24]. The advantage of hydrolyzing urine samples during investigations of flubromazolam intake was confirmed in **Paper V**, where it was shown that the concentrations of flubromazolam increased as much as 2–19 fold when hydrolysis was applied. If hydrolysis is performed, a hydroxy metabolite together with the parent compound should be used as targets in urine drug testing. If no hydrolysis is applied, the best targets for drug testing are parent compound together with a parent glucuronide and/or a hydroxy glucuronide.

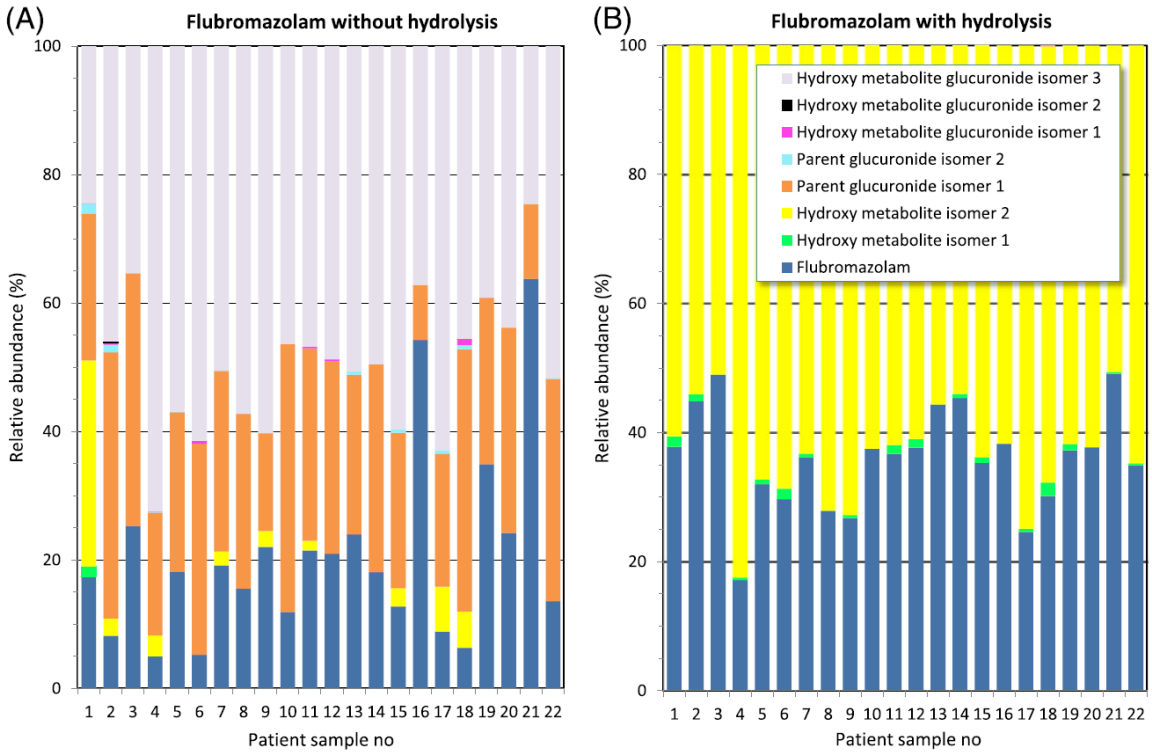

Figure 10: Relative areas (%) of flubromazolam and metabolites compared to the total area of all analytes in the samples without hydrolysis and with hydrolysis (re-printed from Paper V with permission).

The first metabolic study of pyrazolam did not detect any metabolites neither in HLM nor in samples from self-administration studies [52]. As mentioned previously, due to the results obtained in **Paper I** suggesting the presence of metabolites, we performed a confirmation study using urine samples from several analytically confirmed intoxication cases. Our results in **Paper V** contradicted these previous data as phase I hydroxy metabolites and glucuronide conjugates of both the parent compound and the phase I metabolites were found (Figure 11). Possible reasons for the differences include inter-individual excretion patterns and dose differences between the self-ingestion study and consumed amount by patients using the drug. Also, even though metabolites of pyrazolam were detected, their relative intensities were much lower in the patient samples as compared to the parent compound (both with and without hydrolysis). Although ESI was used as an ionization technique in this study and the estimated abundances of the metabolites might be highly affected by matrix effects, no significant increase in parent concentration was detected after hydrolysis was performed. For pyrazolam, if hydrolysis is not performed, either parent glucuronide and parent compound or only parent compound should be used as bioanalytical targets in urine drug testing. When hydrolysis is applied, the parent compound and a hydroxy metabolite are the best targets.

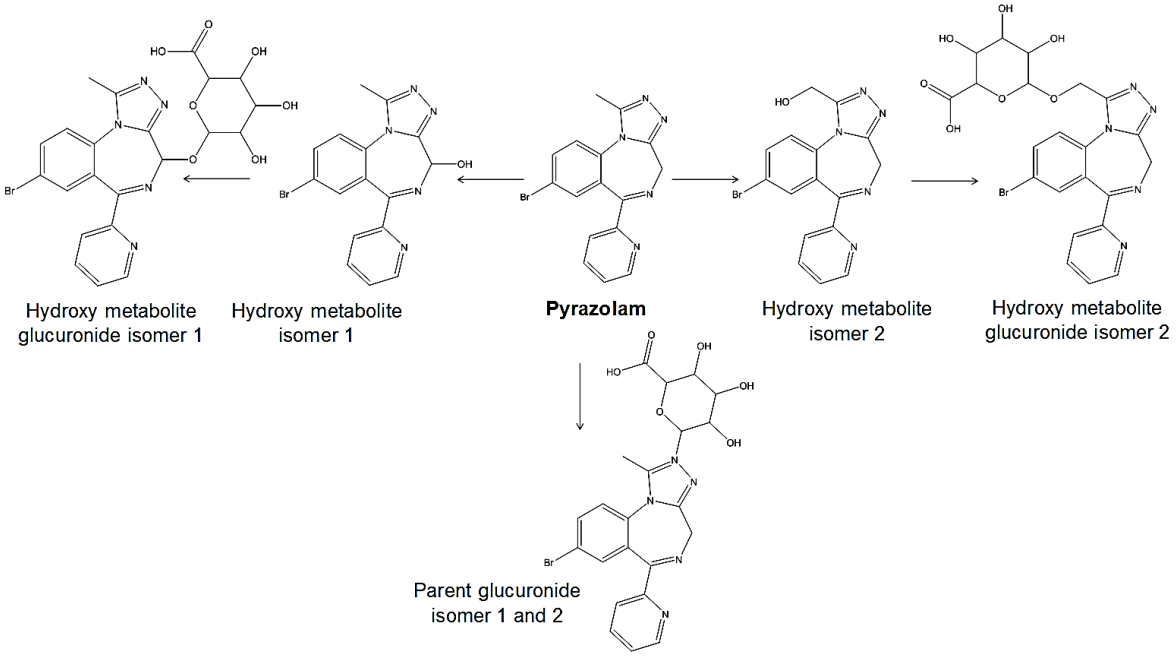

Figure 11: Proposed metabolic pathway of pyrazolam. Main targets in drug testing include parent compound and parent glucuronide (without hydrolysis) or parent compound and hydroxy metabolite isomer 2 (with hydrolysis).

In summary, **Paper V** noted that flubromazolam samples should be hydrolyzed before urine analysis in order to increase detection of both parent compound and phase I metabolites. Good targets for urine drug testing in such cases are parent compound and a hydroxy metabolite. For pyrazolam hydrolysis is not necessary due to the high parent concentration and a good additional target to increase the certainties of the identification is the parent glucuronide.

4.4 PREVALENCE OF DESIGNER BENZODIAZEPINES IN SWEDEN

The aim of **Paper VI** was to study the occurrence of designer benzodiazepines in Sweden, investigate patterns of abuse among designer benzodiazepine users (in relation to multi-intoxications and time of national legislation) and to investigate clinical features of designer benzodiazepines. This was performed by analyzing patient samples collected from intoxicated patients admitted to hospitals due to exposure of NPS or unknown drugs.

The study in **Paper VI** was made possible due to the highly selective methods developed in **Paper II and III**. Among the 1913 urine samples investigated in **Paper VI**, from patients presented to hospitals for emergency treatment from 2012 to 2016, 11% were confirmed to contain a designer benzodiazepine. The most common designer benzodiazepine detected was flubromazolam (detected in 42% of all designer benzodiazepine intoxications, Figure 12). In most cases, the designer benzodiazepines disappeared from the market after they became classified as narcotics in Sweden (Figure 12).

Furthermore, designer benzodiazepines were mostly ingested together with substances from other classes (stimulants, depressants and mixtures from several classes of drugs at 11, 29 and 50%, respectively). A large co-ingestion of designer benzodiazepines and prescription benzodiazepines was detected and as much as around one third of all investigated samples contained a prescription benzodiazepine. Unfortunately, as there was no standardization of written documentation or sampling time, it has not been possible to distinguish whether this derived from benzodiazepine treatment in critical care or misuse. Also, as some designer benzodiazepines are metabolites of prescription benzodiazepines or other designer benzodiazepines [66, 67] this complicates the bioanalytical investigations. One might also argue that since our detection method mostly target designer benzodiazepine parent compounds and not metabolites, many cases could probably go undetected due to the variation in sampling time. This could especially be the cases for diclazepam, clonazolam and meclonazepam intoxications, where low parent concentrations have been reported **(Paper II, III**) [17].

In agreement with our results from **Paper VI** (noting a large co-ingestion of designer benzodiazepines with prescription benzodiazepines), a study using driving under the influence of drugs (DUID) samples detected amphetamines and prescription benzodiazepines as the most common substances ingested with phenazepam [68]. The same study detected phenazepam in 3.5% of all positive DUID cases, however no cases were detected in workplace drug testing. Moreover, another larger study using samples from various criminal offenders (e.g. DUID cases) detected 0.3% designer benzodiazepines among the >22 000 cases investigated [35]. Again, this study demonstrated a large co-ingestion of prescription benzodiazepines (present in 66% of the designer benzodiazepine intoxications where other drugs were detected as well) and designer benzodiazepines [35]. A study with samples from nightclub patrons detected etizolam and phenazepam in oral fluid samples, however no data regarding co-ingestions was reported [36]. The cases found in these three studies (all performed in Nordic countries) demonstrated a much lower prevalence of designer benzodiazepines than our 11%. As our sample set is based on suspected intake of NPS presenting to hospitals, this might be the reason for the higher prevalence of designer benzodiazepines found in our study or it could also derive from a higher occurrence in Sweden in general as compared to other Nordic countries.

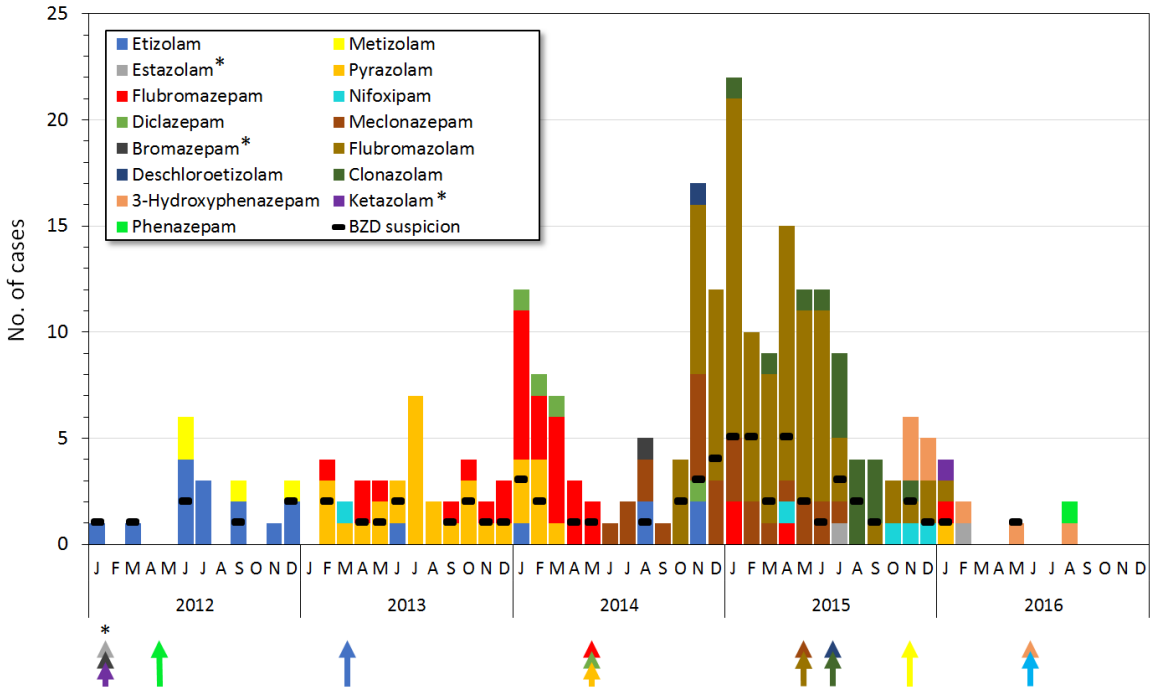

*Figure 12: Cases testing positive for designer benzodiazepines in the STRIDA project from 2012 to 2016. Black bars represent a suspicion or claimed exposure to any benzodiazepine (BZD), and arrows represent the time for classification (*substances banned before the start of the study).*

The main clinical sign detected in patients exposed only to designer benzodiazepines in our study was central nervous system (CNS) depression and the benzodiazepine antidote flumazenil was proven useful for severe intoxications. Furthermore, when analyzing the data from cases confirmed to only contain designer benzodiazepines, flubromazolam was indicated as especially hazardous since two of the three most severe ill patients had a confirmed flubromazolam intake. This agrees with a previously reported case study where a patient needed hospital treatment for several days [38].

In summary, designer benzodiazepines were detected in 11% of intoxication cases presenting in emergency wards in Sweden from 2012 to 2016 and most of these were multiple intoxications. Intoxications using designer benzodiazepines may result in CNS depression that can be treated using the benzodiazepine antagonist flumazenil. As most of the designer benzodiazepines investigated in **Paper VI** disappeared from the recreational drug market after being classified as narcotic substances, one might consider changing the classification system in Sweden from an individual listing system to one of the two other main systems (the generic or the analogue laws) applied for NPS [8].

5 CONCLUSIONS

Frequent use of designer or NPS benzodiazepines was demonstrated in Sweden, both from studies of urine samples from drug dependence units submitted for drug testing and samples from acute intoxication cases at hospitals. The following main conclusions can be drawn:

- Most designer benzodiazepines showed good cross-reactivity in commercial immunoassays targeting prescription benzodiazepines in urine samples.
- LC–MS methods for analysis of designer benzodiazepines in urine can be developed using a simple direct dilution approach followed by hydrolysis of conjugated forms prior to injection into the LC–MS system.
- In cases where laboratories have access to LC–HRMS, screening using diluted hydrolyzed urine samples injected directly into the LC–HRMS system combined with re-injection of positive cases in LC–HRMS/MS mode is an alternative to immunoassay screening followed by LC–MS confirmation.
- Metabolic patterns of the designer benzodiazepines clonazolam, flubromazolam, meclonazepam, nifoxipam and pyrazolam corresponded to those of prescription benzodiazepines, including e.g. hydroxylation, glucuronidation and, for compounds containing a nitro group, nitro reduction followed by acetylation.
- Parent compounds are generally suitable analytical targets in urine drug testing for designer benzodiazepines, except when containing a nitro group, where suitable targets instead are the amino and acetamino metabolites.
- Designer benzodiazepine use was indicated to be rather common in Sweden. About 38% of urine samples from drug dependence units showing positive immunoassay screening result but confirmed not to contain prescription benzodiazepines were demonstrated to instead contain designer benzodiazepines.
- Intoxication by designer benzodiazepines may result in central nervous system depression that can be treated using flumazenil.
- Designer benzodiazepines generally disappeared from the recreational drug market after being classified as narcotic substances but were replaced by novel variants.

Consequently, it is important to include new designer benzodiazepines in bioanalytical drug testing methods and to continuously update these detection methods.

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