Identification and validated quantification of drugs of abuse, medicaments and their metabolites in blood and hair using liquid chromatographic – tandem mass spectrometric techniques in forensic toxicology and therapeutic drug monitoring

Dissertation
zur Erlangung des Grades
des Doktors der Naturwissenschaften
der Naturwissenschaftlich-Technischen Fakultät III -
Chemie, Pharmazie, Bio- und Werkstoffwissenschaften
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von

Kristina Yasmin Rust

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Herrn Chemielaborant

Dietmar “Karl” Bregel

Choose to be me,

to be free,

to be my way...

Samu Haber

TABLE OF CONTENT

1 GENERAL PART

1.1 ANALYTICAL CHALLENGES IN FORENSIC TOXICOLOGY

1.1.1 Introduction

A forensic toxicologist has to deal with different questions every day. Post-mortem cases – was there a poisoning? Driving under the influence of drugs (DUID) cases – which drug was involved? Driving ability assessments – is the suspect abstinent? Drug facilitated crimes – was there an influence by the time of the offense? Seizures of drugs – what kind of drugs are involved? Are there (toxic) cutting substances?
Finding every important detail, however small it might be, is a great challenge in forensic toxicology. This might be a small amount of a toxic compound or a new substance. Sometimes there are only traces, but following these traces might lead to the answer of the case. Being up to date is important, because constantly new drugs are conquering the market [1,2]. These new drugs have to be covered, making the development of new analytical methods an indispensable task in forensic toxicology.
These analytical methods can also be used in clinical toxicology and therapeutic drug monitoring (TDM). Therefore, cooperation of experts in those fields might be valuable for both sides.

1.1.2 Drug classes

In the presented work, drugs of abuse with a special focus on new psychoactive substances as well as medicaments such as benzodiazepines including the so-called “z-drugs” and phosphodiesterase type 5 enzyme inhibitors such as the “Viagra” ingredient sildenafil have been chosen as showcase analytes. The methods that have been developed for their determination were used to answer actual forensic problems.

1.1.2.1 Drugs of abuse

Drugs of abuse represent an important class in forensic toxicology as their possession, their consumption and dealing are strictly forbidden. For roadside testing

in driving under the influence of drugs cases, oral fluid and urine roadside immunoassay tests are widely used. Positive tests must be confirmed in blood by a second independent method (most often mass spectrometric methods after chromatographic separation). Cannabinoids, opioids, cocaine, amphetamines and designer drugs such as methylenedioxymethylamphetamine (MDMA) are the main categories. Their determination is included in the daily routine in a forensic laboratory. There are immunoassays (IA) for screening and gas chromatography (GC) or liquid chromatography (LC) mass spectrometry (MS) methods for quantitative analysis [3,4,5,6]. Past consumption can be determined by hair analysis, which can also prove abstinence in driving ability assessments.

The class of drugs of abuse is growing constantly. There are different groups of new psychoactive substances that are coming up to be used in clubs or at music events. They are sold via internet and disguised as ‘bath salts’, ‘research chemical powders’ or ‘plant food’. One group is formed for example of the piperazines, such as benzylpiperazine (BZP), trifluoromethylphenylpiperazine (TFMPP) and meta-chlorophenylpiperazine (mCPP). Another group includes cathinone and the other beta-keto-amphetamines such as methylone, butylone, ethylone, methcathinone and mephedrone (4-MMC) (for the structures of the typical piperazine BZP and 4-MMC, as representative of the cathinone derivatives, see Figure 1). The pyrrolidinophenone methylenedioxypyrovalerone (MDPV) is also a derivative of cathinone. Information about the pharmacology and toxicological effects are rare; some studies, mainly case reports, have been published recently [7,8,9,10,11,12,13,14]. Additionally, little is known about the prevalence of these psychoactive substances. One reason is that they do not interact with the common immunoassays, consequently these screening tests are blind for these substances. Besides, the portfolios of the analyzing laboratories often do not contain new psychoactive substances. This results in a high number of unreported cases.

Another problem with these new psychoactive substances is that they are often not listed in the narcotic act. “Nulla poena sine lege” – if there is no law, one can’t be punished. Therefore, it is important to keep an eye on the drug market and identify new psychoactive substances in order to be able to schedule them under controlled substances act. Consequently, development of new analytical methods for their determination is one of the pressing tasks of a forensic toxicologist.

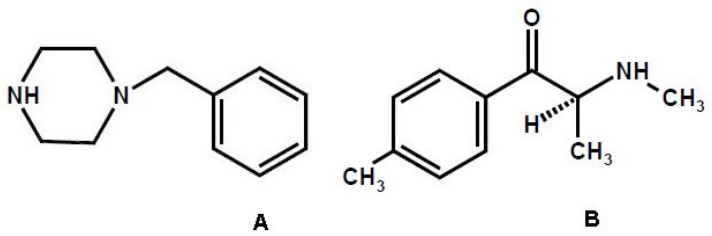

Figure 1: Structures of the piperazine BZP (A) and cathinone derivative 4-MMC (B).

1.1.2.2 Medicaments

Medicaments are a big heterogenic group and some have a high influence, e.g. on driving ability, either alone or in combination with alcohol. These are for example sedatives, hypnotics, neuroleptics or antidepressants. Substances acting on the benzodiazepine receptor system are often prescribed for therapy of anxiety and sleeping disorders or epilepsy and there is a high potential for abuse. The so-called 'z-drugs', namely zolpidem, zopiclone and zaleplon, have no structural relation to the benzodiazepines (examples given in Figure 2), but they are agonists of the benzodiazepine-1 (omega-1) receptor. The sedating effects of both groups, benzodiazepine and z-drugs as well as their side effects, such as lack of concentration, can reduce the ability to drive a car [15,16,17]. Unfortunately, they also found their way into the category of rape drugs and are connected to drug facilitated crimes [18]. Acute influence of drugs and medicaments can be determined in blood and in urine; past consumption can be shown using hair analysis. Therapeutic drug monitoring in blood(plasma) is widely used to check the compliance of a patient and to adjust dosage.

A group of medicaments of both clinical and forensic interest are inhibitors of the human cGMP-specific phosphodiesterase type 5 enzyme (PDE 5), such as sildenafil (Viagra®, Revatio®), vardenafil (Levitra®) and tadalafil (Cialis®, Adcirca®) (see Figure 3). As treatment for erectile dysfunction [19], there might be a connection to 'mors in actu' cases (death during sexual intercourse). Being a so-called life-style medicament, the risk of fake products on the black market is high. Sildenafil and tadalafil are also licensed for treatment of pulmonary arterial hypertension [20], because PDE 5 is also present in lung blood vessels and therefore its inhibition leads to selective pulmonary vasodilatation.

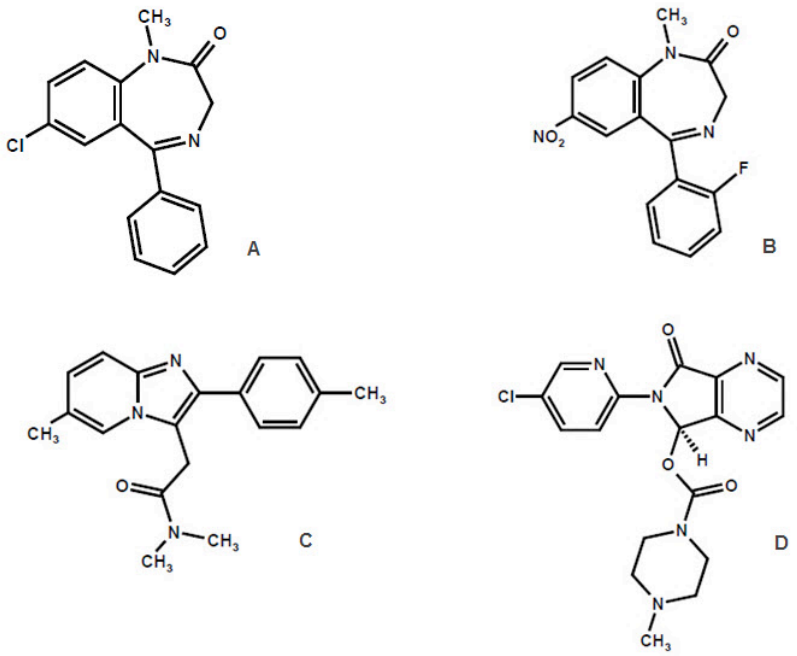

Figure 2: Structures of the benzodiazepines diazepam (A) and flunitrazepam (B) and of the z-drugs zolpidem (C) and zopiclone (D)

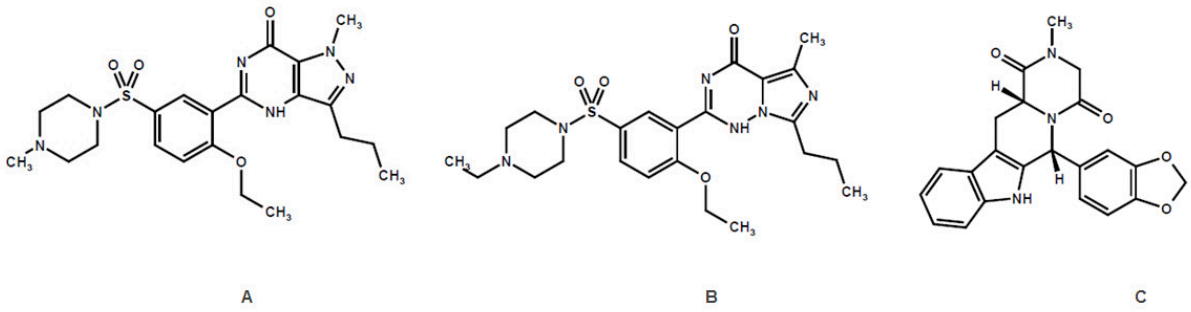

Figure 3: Structures of the PDE5 inhibitors sildenafil (A), vardenafil (B) and tadalafil (C)

1.1.3 Biological matrices

In the presented work, blood as the most common biological matrix in forensic toxicology as well as hair as the most common alternative matrix have been used to answer specific forensic questions. Some peculiarities of the different biological matrices in forensic toxicology are discussed in this chapter.

In forensic toxicology determination of xenobiotics in different biological matrices is necessary. The most common are blood (plasma, serum), urine and nowadays also

alternative matrices such as hair and oral fluid. Additionally, gastric content and tissue samples (muscle, liver, kidney, brain) have to be worked up, especially in post mortem cases when blood or urine are no longer available. Sometimes even perinatal matrices, such as meconium, have to be used to assess drug intake during pregnancy.

The best correlation between the drug concentration and the resulting pharmacologic effects is given in **blood** (plasma, serum), which is therefore useful for interpretation e.g. for assessment of impairment [5]. Drawbacks are often the limited volumes of blood for analysis and that the collection of blood has to be done by a professional.

Urine has a great advantage for that it can be obtained non-invasively and high volumes are often available [5]. Concentrations are higher in urine because of physiological concentration in the kidneys. Unfortunately, not in all cases parent compounds can be found in urine, because they have been metabolized. In order to analyze the metabolites of a specific compound, the metabolic pathways must be either known or established to assure which structure is of interest. Additionally, metabolites are partly excreted in conjugated form. These conjugates must either be cleaved or included into the analytical procedure [21].

In **hair** drugs are incorporated and stored for a long time [22]. Hair is like a recorder, it plots the consumption behavior depending on hair length. Therefore, retrospective investigation over months to years is possible. Assessment of acute impairment is however not possible. A great advantage compared to blood and urine is that hair can be stored at ambient temperature, so no space in freezing compartments is needed avoiding freeze-thaw stability problems. If no head hair is available, the use of body hair, e.g. chest hair, is also possible. A weakness of hair as analytical matrix is the risk of external contamination with drug powder or smoke. To differentiate between actual ingestion of a substance and external contamination, analysis of metabolites might be necessary. The Society of Hair Testing (SoHT) gives further recommendations [23].

Oral fluid can be obtained non-invasively like urine, but much more easily and less intimate. These advantages make oral fluid a valuable matrix for roadside drug testing. Oral fluid testing is also used in controlled trials in other fields, e.g. smoking cessation [24].

1.1.4 Analytical toxicology

The presented work focuses on the use of modern liquid chromatography tandem mass spectrometry (LC-MS/MS) technology for answering forensic questions. Besides the use of modern instruments, sample preparation, identification criteria and validation must be considered.

1.1.4.1 Sample preparation

Before these different matrices can undergo a toxicological analysis, a good sample preparation is necessary. The most common ones are liquid-liquid extraction (LLE), solid-phase extraction (SPE) and protein precipitation. The aim of a sample preparation is a high concentration of analytes of interest and a loss of everything that might interfere [25]. This is of special importance when using LC-MS/MS because this technique is prone to matrix effects especially when applying electrospray ionization (ESI) [26]. Matrix effects can cause both higher signals of the analyte (ion enhancement) and lower signals (ion suppression). Therefore a good sample clean-up is mandatory.

For hair samples, the sample preparation is more complicated. First, decontamination is necessary to get rid of cosmetic treatments and external contamination. Second, the incorporated substances must be extracted. This can be done in different ways, e.g. extraction with methanol, aqueous acids or buffer solutions; digestions with enzymes or sodium hydroxide [22]. After the extraction or digestion, a LLE or SPE is recommended.

Another important point in sample preparations is the use of an internal standard. For LC-MS/MS procedures the use of stable-isotope-labeled (SIL) internal standards, e.g. deuterium labeled compounds, is recommended because they can compensate for analytical problems (e.g. matrix effects, recovery) [27].

1.1.4.2 Analytical techniques

Analytical procedures in forensic toxicology comprise identification steps followed by (validated) quantification. At last, interpretation of the results by the forensic toxicologist is needed and an expert report is given. Routinely, samples are prescreened using immunoassays [6]. These results must be confirmed by another method. In the last decades, GC-MS was the gold standard for that purpose [28]. GC-

MS shows good sensitivity and gives spectral information for identifying analytes [28]. Many GC-MS methods for the determination of drugs of abuse in blood have been published [6].

In recent years, starting in the 1990s, the LC-MS(/MS) technique is establishing itself in the field of analytical toxicology [29]. Especially for analytes which do not perform good with GC-MS, such as non-volatile, hydrophilic and thermo labile analytes [5]. Peters gives an overview over the recent developments and published methods using LC-MS(/MS) [5]. When using a tandem mass spectrometer quantification is done best employing the multi reaction monitoring (MRM) mode. The first quadrupole monitors the mass of a given compound and the third quadrupole scans for fragment ions that had been selected in previous experiments. It provides a high selectivity and sensitivity, because only the analytes of interest are scanned. The use of internal standards is recommended as given above (see 1.1.4.1).

Nowadays, new analytical methods should be fully validated, at least if the analytes are to be determined on a regular basis. Full validation for a single analyte which might appear once a year or even less is not necessary, but its detection is indispensable for an unknown screening in clinical and forensic toxicology. An overview over the recent published approaches for toxicological drug screening using LC-MS is given by Maurer [30].

A Q-Trap-LC-MS/MS is usually not suitable for a full-scan screening, but it is suitable for a multi target screening (MTS). With an MTS one can combine the MRM mode with a spectral method such as enhanced product ion scan (EPI). Using information dependent acquisition (IDA) with a survey scan in the MRM mode, the sample is analyzed for a given number of analytes. If an analyte is tested positive, the signal triggers the second experiment, which delivers an EPI spectrum of the corresponding analyte. Mueller et al. developed that kind of method for 301 drugs [31] and Dresen et al. improved the MTS in order to detect and identify 700 drugs simultaneously using scheduled MRM (sMRM) transitions [32]. The unambiguous identification, e.g. with EPI, is especially important in cases with substances, that have similar transitions in the MRM mode. Allen showed that O-desmethylvenlafaxine, the metabolite of the antidepressant venlafaxine, can cause false positive results for tramadol due to partly identical transitions [33].

1.1.4.2.1 Identification criteria

In clinical and forensic toxicology widely accepted criteria for mass spectrometry-based compound identification are still under discussion [5,34,35]. For the mass spectrometric identification of drug residues in foodstuffs, guidelines can be found in Commission Decision of 12 August 2002 of the European Communities [36]. This document describes criteria for mass spectrometric detection. When using for example selected ion monitoring (SIM) or MRM, a system of identification points (IP) is applied. A minimum of 3 IP are requested. One can achieve that with working with 3 SIM, which give 1 IP each, or with 2 MRM, which accounts for 1.5 IP each. For an unambiguous identification the combination of MRM and EPI is a good approach [37] that has also been followed in the presented work.

1.1.4.2.2 Method validation

A forensic toxicologist must be able to rely on his analytical results for evaluation and interpretation. Therefore it is essential that his analytical procedures provide him with correct results. Method validation can guarantee that. The German Society of Toxicology and Forensic Chemistry (GTFCh) developed guidelines for method validation in forensic toxicology [38]. Detailed descriptions of validation experiments especially for LC-MS have also been published [39]. The first part of a validation includes experiments concerning selectivity, matrix effects, extraction recovery, process efficiency, processed sample stability and linearity. The second part consists of experiments to evaluate the accuracy and precision and freeze and thaw stability. Peters stated that dealing with matrix effects is one of the key issues in LC-MS/MS [5]. Matuszewski et al. described an experimental approach in which matrix effects; recovery and process efficiency can be determined simultaneously [40]. This approach has also been used in the presented work.

1.2 AIMS AND SCOPES

Forensic toxicological analyses have different challenges, e.g. uncommon analytes, different matrices and new releases on the illicit drug market. LC-MS/MS analysis is a useful tool to tackle these problems [5,28]. For quantitative purposes a determination in the MRM mode is appropriate and an unambiguous identification can be achieved through EPI spectra.

Therefore, the aims of the presented studies were:

- Development and validation of a quantitative method for the determination of phosphodiesterase-5 inhibitors and two of their metabolites in blood plasma with an application to therapeutic drug monitoring (TDM) and forensic cases

- Development and validation of a quantitative method for the determination of benzodiazepines and z-drugs in hair by using the MRM-EPI mode and application to driving ability assessments

- Extension of the classical drug screen by developing a qualitative screening method for new psychoactive substances in hair and application to retrospective studies for assessment of prevalence

2 PUBLICATIONS OF THE RESULTS

The results of the studies were published in the following papers:

2.1 Detection and Validated Quantification of the Phosphodiesterase Type 5 inhibitors Sildenafil, Vardenafil, Tadalafil and two of their Metabolites in Human Blood Plasma by LC-MS/MS – Application to Forensic and Therapeutic Monitoring Cases [41] (DOI: 10.1097/FTD.0b013e31827318b8)

2.2 Detection and Validated Quantification of 21 Benzodiazepines and 3 "Z-drugs" in Human Hair by LC-MS/MS [42]

(DOI:10.1016/J.FORSCIINT.2011.07.052)

2.3 Prevalence of New Psychoactive Substances: a Retrospective Study in Hair [43] (DOI:10.1002/dta.1338)

3 CONCLUSIONS

A fully validated method for the determination of three phosphodiesterase-5-inhibitors and two of their metabolites in blood plasma has been developed and has also been applied to forensic cases and in therapeutic drug monitoring. Patients with pulmonary arterial hypertension and sildenafil treatment were monitored. Not only the concentration of sildenafil (Revatio®) could be used for therapeutic drug monitoring; the concentration of norsildenafil, the main metabolite, was monitored too.

For the determination of 21 benzodiazepines and 3 z-drugs in hair a method has been developed that was also fully validated according to international guidelines. After a two-step extraction procedure, an unambiguous identification and quantification was possible using the MRM-IDA-EPI mode. This method has been successfully applied to driving ability assessments and is now in routine use in the forensic hair laboratory and has been used in hundreds of cases.

The drug market is changing constantly and therefore it is important to be up to date. The extension of the classic drug screen was used for a retrospective study, which was based on reanalyzing hair samples of 2009 and 2010 from driving ability assessment cases for new psychoactive substances. This retrospective study proved that these new drugs are actually in use and that their prevalence is high. In 37% of the cases such drugs were found. At least the most common ones should be included in screening procedures in clinical and forensic toxicology. Thus, LC-MS/MS has proven to be a useful tool for tackling particular problems in forensic toxicology.

4 SUMMARY

The presented studies are dealing with analytical challenges in forensic toxicology and how the LC-MS/MS technology can be a useful tool for solving those analytical problems. Two quantitative methods have been developed and validated. First, a method for the determination of PDE5-inhibitors in blood plasma has been set up that could be used for forensic cases and in therapeutic drug monitoring for patients suffering from pulmonary arterial hypertension and sildenafil treatment. Second, a method was developed for quantification of 21 benzodiazepines and three z-drugs in hair and has been applied in hundreds of driving ability cases. For both methods the MRM-mode was used; in the second method the information dependent acquisition (IDA) was integrated using an EPI scan for an unambiguous identification. To deal with a changing drug market, an extension of the classic drug screen was developed and a retrospective study in hair of 2009's and 2010's driving ability cases could reveal the prevalence of new psychoactive substances and gave useful hints which drugs should be integrated in daily routine procedures.

In the presented work, LC-MS/MS has successfully been used for tackling particular problems in forensic toxicology. Validated quantification of different classes of drugs in blood as well as in hair and even screening for new psychoactive substances in hair were possible employing this versatile technique emphasizing the role of LC-MS in forensic toxicology.

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6 ABBREVIATIONS

4-MMC	Mephedrone (4-Methylmethcathione)
BZP	Benzylpiperazine
cGMP	Cyclic guanosine monophosphate
DUID	Driving under the influence of drugs
EPI	Enhanced product ion (scan)
ESI	Electrospray ionization
EtG	Ethyl glucuronide
GC	Gas chromatography
GTFCh	Gesellschaft für Toxikologie und Forensische Chemie
IA	Immunoassay
IDA	Information dependent acquisition
IP	Identification point
LC	Liquid chromatography
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LLE	Liquid liquid extraction
mCPP	meta-Chlorophenylpiperazine
MDMA	Methylenedioxymethylamphetamine
MDPV	Methylenedioxypyrovalerone
MRM	Multi reaction monitoring
MS	Mass spectrometry
MTS	Multi target screening
PDE5	Phosphodiesterase type 5
SIL	Stable isotope labeled
SIM	Selected ion monitoring
sMRM	Scheduled multi reaction monitoring
SoHT	Society of Hair Testing
SPE	Solid phase extraction
TDM	Therapeutic drug monitoring
TFMPP	Trifluoromethylphenylpiperazine
z-drugs	zolpidem, zopiclone, zaleplon

7 ZUSAMMENFASSUNG

Im Rahmen dieser Dissertation wurde anhand von Studien gezeigt, dass die LC-MS(/MS) Technologie ein nützliches Werkzeug für die Bearbeitung der analytischen Herausforderungen in der forensischen Toxikologie ist. Zuerst wurde eine Methode zur Bestimmung von PDE5-Hemmern im Blutplasma aufgesetzt, die in forensischen Fällen und im Therapeutischen Drug Monitoring Anwendung fand. Als zweites konnte eine Methode zur Bestimmung von 21 Benzodiazepinen und den Z-Hypnotika in Haaren entwickelt werden, die in Fahreignungsbegutachtungs-Fällen eingesetzt wurde. Für beide Methoden wurde der MRM-Modus benutzt; die zweite Methode wurde durch die „Information Dependent Acquisition“ und den EPI-Scan für eine gesicherte Identifizierung erweitert. Das klassische Drogenscreening wurde um neue psychoaktive Substanzen erweitert. Eine retrospektive Studie in Haaren von Fahreignungs-Fällen aus 2009 und 2010, konnte die hohe Prävalenz dieser Substanzen aufzeigen und Hinweise geben, welche Substanzen in die Routine zu integrieren sind.

In der vorliegenden Arbeit wurde die LC-MS/MS Technik erfolgreich eingesetzt, um einige der speziellen Fragestellungen der forensischen Toxikologie zu beantworten. Mit dieser Technik konnten sowohl validierte quantitative Bestimmungen verschiedener Analyten in Blut und Haaren als auch eine gerichtete Suchanalyse auf neue psychoaktive Substanzen in Haaren verwirklicht werden, was die wichtige Rolle dieser vielseitigen Technik für die forensische Toxikologie unterstreicht.