Development of an Ultra High Performance Liquid Chromatographic-Tandem Mass Spectrometric Multi-Analyte Approach for Target Screening in Eight Different Matrices and Quantification in Three Different Matrices

Dissertation

zur Erlangung des Grades

des Doktors der Naturwissenschaften

der Naturwissenschaftlich-Technischen Fakultät III -

Chemie, Pharmazie, Bio- und Werkstoffwissenschaften

der Universität des Saarlandes

von

Deborah Montenarh

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Für Frau Elisabeth Rolles

“The only way to do great work is to love what you do. If you haven’t found it yet, keep looking. Don’t settle. As with all matters of the heart, you’ll know when you find it.“

Steve Jobs

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1 GENERAL PART

1.1 INTRODUCTION

In clinical and forensic toxicology, screening and quantification of different classes of drugs is a very important task to evaluate different topics e.g. chemical-induced diseases, acute intoxication or poisoning, toxic effects, driving under the influence of drugs, drug-induced impairment of legal responsibility, committing a crime under the influence of drugs, and also death caused by intoxication.[1-6] In these fields, the following drugs are usually involved: drugs of abuse such as amphetamines, designer drugs, opiates or cocaine, synthetic opioids, alkaloids such as atropine, and most recently, new synthetic drugs such as synthetic cannabinoids or phenethylamines, psychotropic drugs (e.g. antidepressants and neuroleptics), and benzodiazepines or Z-drugs.[7,8] In addition, several cases of sudden death associated with phosphodiesterase type 5 (PDE-5) inhibitors often in combination with heart diseases have been pulished.[9]

Opiates (e.g. morphine and derivatives) or synthetic opioids (e.g. tilidine) can cause sedative and analgesic effects but also some side effects such as sedation, respiratory depression, constipation, and a strong sense of euphoria.[10] The combination of these drugs with alcohol or benzodiazepines increases the rates of adverse events, intoxication, and death of patients.[11]

Over the last years cannabis users shifted to the consumption of herbal mixtures including synthetic cannabinoids as a more or less legal alternative to cannais.[12] Synthetic cannabinoids have the same, if not stronger, effects than cannabis.[12] The production of these herbal mixtures is mostly uncontrolled, thus, declaration of the ingredients is often insufficient, which can lead to problems. Consumers often do not know, which compounds have been added to the mixture.[13] Studies of synthetic cannabinoids over the last years show the importance of those substances in post-mortem forensic toxicology. Acute effects of synthetic cannabinoids as well as chronic exposure have to be considered.[13]

Phenethylamine containing two methoxy groups at positions 2 and 5 of the benzene ring and a substituted 4 position interact effectively with serotonin receptors, and act as agonists of the 5-HT2A receptor.[14] This group of substances was named 2C family y Alexander Shulgin.[14]

Benzodiazepines enhance the effect of the neurotransmitter gamma-aminobutyric acid (GAA), which may lead to sedative, hypnotic, anxiolytic, anticonvulsant, and muscle relaxing effects.[10] These effects may result in slow and uncertain reflexes and can therefore influence

driving aility.[15] In combination with alcohol and other CNS active substances, they can lead to severe poisonings or even death.[11,16] Three groups of benzodiazepines are known; short- (e.g. triazolam), intermediate- (e.g. flunitrazepam), or long-acting (e.g. nordazepam) enzodiazepines.[16] The so-called Z-drugs (e.g. zaleplon), also called non-benzodiazepines have a pharmacological activity similar to benzodiazepines. Z-drugs have demonstrated efficacy in treating sleep disorders as they have less influence on the sleep architecture but also known producing side effect such as pronounced amnesia and more rarely hallucinations, especially when used in large doses.[17]

Antidepressants are psychotropic drugs that were mainly used for treatment of depression, in obsessive-compulsive, panic, generalized anxiety disorder, chronic pain, lethargy, insomnia as well as in post-traumatic stress disorder.[18] In the group of antidepressants, several sub-groups are known such as tricyclic antidepressants (e.g. amitriptyline), tetracyclic antidepressants (e.g. mianserin), selective serotonin reuptake inhibitors (SSRIs, e.g. citalopram) or selective norepinephrine reuptake inhibitors (SNRIs, e.g. milnacipran), and monoamine oxidase A inhibitors (e.g. moclobemide).[19,20] In combination with substances like alcohol, benzodiazepines, neuroleptics, or opioids, the respiratory depressant effect can increase and cause acute poisonings.[10]

Neuroleptics, also named antipsychotics, are primarily used to treat psychosis such as hallucinations but also for the treatment of schizophrenia and the manic episodes of bipolar disorder.[18] Neuroleptics, especially the classic ones, can show severe side effects such as extrapyramidal symptoms and cardiovascular complications such as arrhythmia or ventricular fibrillation.[20,21] These side effects can result in sudden death, especially when overdosed. Therefore, neuroleptics play an important role in clinical and forensic toxicology.[22,23] Moreover, besides the central nervous system (CNS) effects, the vegetative side effects, such as accommodation disorder or hypotonic dysregulation can impair the driving ability. One can differentiate between first-generation neuroleptics such as droperidol and second-generation neuroleptics such as clozapine.[24] The latter produce less extrapyramidal disabilities such as parkinsonoid than first-generation.[24]

In clinical and forensic toxicology, the analytical strategy includes screening followed by quantification.[3,4] Screening for the above-mentioned drug classes is important and for some of them (e.g. benzodiazepines, antidepressants, and neuroleptics), quantification is also demanded to clarify different topics as already discussed above.[4] As the background of the individual cases as well as the profile of requirements may be very different, the toxicologist has to choose the best suitable biosample and method of analyses to clarify the questions.[4]

The best biosample to show the acute influence of a drug on the body is blood, which consists of cellular particles such as erythrocytes, leukocytes, thrombocytes.[26] Blood plasma includes clotting factors and results from centrifugation of blood after treatment with anticoagulants. Plasma without clotting factors is named “serum” and contains less fibrinogen but more phosphate and sodium ions.[26] However, depending on sampling, storage, and/or type of analysis, whole blood, plasma, or serum are used for analysis. For the interpretation of drug concentrations in blood comprehensive blood level lists can be helpful.[27,28] For abstinence programs in a rehabilitation center or for driving ability surveys, blood seems not to be the best suitable biosample because not the acute influence but the consumption behavior is of interest.[29,30] For this question, urine or hair samples are better biosamples because drugs can be detected even days or up to weeks/months after the intake.[29,30] Concentrations of analytes or their metabolites in urine can be higher than in blood, so that screening for them may give better results.[4] The collection of urine samples is accompanied by the invasion of the privacy as laboratory stuff has to attend the sampling site.[29] To circumvent this invasion of the privacy, hair samples can be collected.[29] It has been proposed that drugs are incorporated into hair by binding to intracellular components as melanins, lipids, and proteins, and therefore, provide a historical profile depending on the hair length.[31] As shown elsewhere, hair analysis can allow to distinguish between acute, chronic, and one-time substance use.[32,33] In post mortem cases, different biosamples are usually available.[34] To determine the link between compound concentrations and effect, whole blood is the main post mortem biosample, however, post mortem redistribution as well as instability of drugs have to be kept in mind for interpretation of post mortem analyte findings.[25,35] In post mortem cases where blood cannot be obtained, tissues such as liver or kidney are possible biosamples for analysis.

After collection of the best suitable biosample, it has to be analyzed by suitable approaches.[4,6] The first step is to find the right extraction procedure for each biosample.[4] For whole blood, plasma, serum, and post mortem blood, methods using liquid-liquid-extraction, solid-phase-extraction, dilution, and protein precipitation have been published.[34,36-46] Urine workup can be performed by enzymatic hydrolysis of the conjugates and liquid-liquid-extraction,[47] enzymatic hydrolysis and solid-phase extraction,[37] solid-phase extraction without cleavage of conjugates,[36] or dilution.[48] For hair samples, methods using methanolic extraction,[31] microwave-assisted hydrolysis and microwave extraction,[49] extraction with phosphate buffer,[50] extraction with acetonitrile[51] and solid-phase extraction[34,52] were published. For homogenized liver tissue, incubation with Subtilisin, followed by an extraction with butyl chloride was published.[53] Laloup *et al.* published one single extraction for three biosamples, blood, urine, and hair.[36] Maurer *et al*. published a work-up procedure for plasma, gastric content, and urine using diethyl ether-ethyl acetate (1:1).[54] Different extraction

procedures for every biosample and every drug group cost time and resources, therefore one extraction procedure for all above-mentioned drug classes and biosamples should be found to analyze each biosample in one analytical run. Different methods have been published for screening and quantification of different drug classes. Over the last years, more and more multi-analyte approaches were developed using different techniques, e.g. immunochemical analysis, gas chromatography-mass spectrometry (GC-MS), and/or liquid chromatography-mass spectrometry (LC-MS).[7,12,19,23,34,36,38-48,56-63]

The results of toxicological analyses and their interpretation can have serious legal consequences. Therefore, a screening or quantification method has to be validated and the quality of the method has to be controlled strictly and constantly by internal and external quality control. For screening and quantification methods, Peters *et al.*[64,65] described extensively, which parameters should be included in validation experiments for quantitative methods; selectivity, calibration model (linearity), recovery, matrix effects, stability, accuracy (bias), precision (repeatability, intermediate precision), the limit of detection (LOD) and lower limit of quantification (LLOQ). When using LC-MS(/MS), matrix effects, ion suppression and enhancement may cause problems and therefore has to be checked for.[38,66]

2 AIMS AND SCOPES

As mentioned above, a lot of procedures for screening and quantification of different drug classes using liquid chromatography-mass spectrometry (LC-MS/MS) or gas chromatography-mass spectrometry (GC-MS) in different biosamples have been published but none of them showed a screening method for over 100 drugs in eight different matrices, or validated quantification of various drugs in the three most commonly used biosamples, whole blood, plasma, and serum.

Therefore, the aims of the present dissertation are:

→ To develop a simple extraction and LC-MS/MS approach for screening and identification of over 100 analytes in eight different matrices

→ To develop and validate multi-analyte LC-MS/MS approaches for quantification of 33 antidepressants, 33 neuroleptics, and 19 benzodiazepines including Z-drugs in whole blood, plasma, and serum

3 PUBLICATION OF THE RESULTS

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

3.1 A SIMPLE EXTRACTION AND LC-MS/MS APPROACH FOR THE SCREENING AND IDENTIFICATION OF OVER 100 ANALYTES IN EIGHT DIFFERENT MATRICES. (DOI 10.1002/DTA.1657)[67]

3.2 Detection and quantification of benzodiazepines and Z-drugs in human whole blood, plasma, and serum samples as part of a comprehensive multi-analyte LC-MS/MS approach. (DOI 10.1007/s00216-013-7513-x)[68]

3.3 QUANTIFICATION OF 33 ANTIDEPRESSANTS BY LC-MS/MS-COMPARATIVE VALIDATION IN WHOLE BLOOD, PLASMA, AND SERUM. (DOI 10.1007/S00216-014-8019-X)[69]

3.4 Development and validation of a multi-analyte LC-MS/MS approach for quantification of neuroleptics in whole blood, plasma, and serum. (10.1002/dta.1923)[70]

4 DISCUSSION AND CONCLUSIONS

Development including full validation of the multi-analyte approaches has been published in four original papers presented in chapters 3.1-4 of this thesis.[68-70]

The multi-analyte multi-biosample approach (3.1) allowed for the first time screening and identification of 130 analytes from the most important drug classes in forensic toxicology in eight different biosamples. Only one single work-up approach, one column, one gradient, and one scheduled multi-reaction mode (MRM) method was used. The described extraction procedure was preferred over that described by Laloup *et al.,*[36] who already published one single extraction procedure for three biosamples, blood, urine, and hair, because all validated GC-MS procedures in the authors' laboratory, using also this extraction procedure, should be used in combination with the presented approach. For identification, three MRM transitions, the retention times as well as the enhanced product ion (EPI) spectra (if intensity > 5000 cps) were used. Some analytes needed three MRM transitions for the certain identification. For the application of the established plasma extraction procedure,[54] it had to be adjusted to pH 7.4 using Sörensen Buffer because of different pH values in the different biosamples such as whole blood, serum, and post mortem blood. As shown by the limit of detection (LOD) or recovery results, this extraction approach, which should also be used for liver tissue, gastric content, hair samples, and urine samples, was stretched to its limits especially for urine samples. The LODs in whole blood, plasma, serum, and post mortem blood should be lower than the lowest therapeutic concentration described in comprehensive blood level lists.[28,27] For 29 of 130 analytes, especially designer drugs, no therapeutic concentrations were found in those lists. For six out of the 101 remaining analytes, the LODs were in the range of the lowest therapeutic concentrations, for 16 analytes, in the therapeutic range, and for three analytes, in the range of the highest therapeutic concentrations.[27,28] In addition, the LODs for some analytes found in whole blood, plasma, and serum were not the same as in post-mortem blood and liver tissue. The LOD results were comparable to other multi-analyte methods for the respective biosample.[41,42,47-52,63] Recovery, matrix effect, and process efficiency tests were performed for 30 typical analytes covering each drug class and the whole retention window. Amitriptyline showed higher RE values in whole blood, post-mortem blood and liver tissue in comparison to the values found in plasma and serum. These results were reproducible but not explainable yet. A t-test was performed to compare the matrix effect (ME) values between plasma and the other mentioned biosamples. ME values for plasma vs. serum (2/30) and plasma vs. post-mortem blood (3/30) differed not significantly, but significant differences were found for plasma vs. whole blood (9/30), plasma vs. liver

tissue (11/30), plasma vs. hair (14/30), and plasma vs. urine samples (19/30). The significant differences for the ME values between the different biosamples resulted from the different endogenous components of the biosamples. Whole blood includes erythrocytes and leukocytes and coagulates within a few minutes into a half-solid mass named coagulum, which includes modified plasma, blood cells, and fibrin.[26] Serum which contains less fibrinogen than plasma but more phosphate and sodium ions is formed after centrifugation. If whole blood is treated with anticoagulants, plasma is formed after centrifugation.[26] Liver tissue includes hepatocytes, whole blood, fat tissue and bile.[71] Especially the presence of fat impeded the extraction of some analytes. Urine includes water, urea, electrolytes, creatinine and sometimes proteins.[71] Hair consists mostly of keratin, after the extraction procedure often fat arrears were detected, which could impede the extraction of some analytes.

As mentioned before, in clinical and forensic toxicology, the analytical strategy includes screening and identification followed by quantification.[4]
Therefore, for the three largest groups of drugs out of the previous described multi-analyte multi-biosample approach (benzodiazepines and Z-drugs (3.2), antidepressants (3.3), neuroleptics (3.4)) and for the most common biosamples in the author´s laboratory (whole blood, plasma, and serum), an LC-MS/MS approach for quantification was developed and fully validated. Special focus was placed on the matrix effect, recovery, and process efficiency and their statistical differences in-between the different biosamples. The validation consisted of selectivity, recovery, matrix effects, process efficiency with statistical data evaluation, cross talk, calibration model, accuracy, precision, processed sample stability, freeze/thaw stability, bench top stability, lower limit of quantification (LLOQ), limit of detection, and applicability in whole blood, plasma, and serum according to national guiudelines.[64,65] Furthermore, for the group of antidepressants, comparative validation between these three biosamples were done. For all analytes and biosamples a full six-point calibration was performed.

The LC-MS/MS method described in 3.2 allowed accurate and precise quantification of 16 benzodiazepines in whole blood, for 18 benzodiazepines in plasma, and for 17 benzodiazepines in serum. In 2015, Mata *et al.* also published an LC-MS/MS method for quantification of benzodiazepines in six different matrices using weak anion exchange tips with lower LOD results than presented in this dissertation, but no statistical data evaluation was performed to compare the RE, ME or PE values between the different biosamples.[44] In contrast, in our study, statistical data evaluation was performed to compare the RE values. Especially tetrazepam showed significant differences for RE values. The RE value in plasma samples was 235% compared to 78% (serum) and 85% (whole blood). The enhanced signal of tetrazepam in plasma samples compared to the signal in whole blood and serum samples

led to the conclusion that the tetrazepam concentration in a plasma sample could not be calculated by the serum or whole blood calibration curve. For other analytes of the benzodiazepine group, cross calibration seemed to be possible, e.g for bromazepam where no significant differences were found for the RE values. For benzodiazepines, no selectivity problems were observed, in contrast to the approach for antidepressants (3.3), where in the MRM transitions of amoxapine in plasma and serum samples an interfering signal caused by the internal standard was detected. As the interfering signal was only the signal of the qualifier, it was irrelevant for quantification.

Also for the group of antidepressants (3.3), a fully validated LC-MS/MS approach was developed for whole blood, plasma, and serum. In addition, accuracy and precision were determined for the first time for each biosample calculated over a calibration curve in the corresponding biosample as well as over a calibration curve made in another biosample. Cross-calibration for analytes in whole blood and serum calculated over a calibration curve made in plasma, for analytes in whole blood and plasma calculated over a calibration curve made in serum provided better results than analytes in plasma and serum calculated over a calibration curve made in whole blood. With the exception of three analytes in whole blood, two analytes in plasma, and five analytes in serum, all antidepressants fulfilled the validation criteria in the three biosamples. The assumption that calculation of benzodiazepines should only be done in the same biosample has been confirmed for antidepressants after cross-calibration experiments.

For 25 neuroleptics in whole blood, 19 in plasma, and 17 in serum (3.4), this LC-MS/MS method allowed accurate and precise quantification. In contrast to the approach for benzodiazepines and antidepressants, ME values for plasma vs. serum differed more significantly than between plasma vs. whole blood or serum vs. whole blood. A reason for that could not be found so far. No significant enhancement or suppression of co-eluting analytes from the same drug class was observed for the analyte pairs: aripiprazole/levomepromazine, benperidol/droperidol, buspirone/risperidone, chlorpromazine/perphenazine, chlorprothixene/zotepine, clozapine/ziprasidone, flupentixol/fluspirilene, pimozide/sertindole, and pipamperone/promethazine. Remane *et al.*[42] published significant enhancement or suppression for four analyte pairs using APCI (atmospheric pressure chemical ionization), so therefore separate spiking solutions for calibrators and QCs samples were prepared, which is time- and cost-intensive. In the presented method using ESI, one spiking solution for all analytes could be used. During three freeze/thaw cycles, all analytes were stable with exception of chlorprothixene, fluspirilene, and olanzapine. This is in accordance with already described. [23,42,45] As expected, in contrast to Kirchherr *et al*.,[19] who used a more sensitive apparatus, our LLOQs

are not as low as there published LLOQs. In summary, the three multi-analyte multi-biosample approaches (3.2-4) fulfilled the requirements for a validated assay for quantification of most of the analytes. Drug levels of authentic cases determined in an external laboratory with already published methods[41,42] could be confirmed with the new approaches and external quality control samples of proficiency tests were measured successfully. Thus, the method could be included into the forensic routine. However, the use of calibration curves, made with one type of biosample for quantification in other biosamples remains an unsolved problem. Regarding the fact that a lot of analytes with different chemical structures have been implemented in this multi-analyte multi-biosample approach, compromises concerning recovery, accuracy and precision and LOD had to be made in some cases.

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6 SUMMARY

In the present dissertation, the development of a multi-analyte multi-biosample LC-MS/MS approach with simple extraction is described for the screening and identification of over 100 analytes in eight different matrices. The analytes were extracted with one single liquid-liquid work-up approach from eight different matrices and separated with one column and one multi-reaction mode (MRM) method. In addition, three methods for quantification of 19 benzodiazepines including Z-drugs, 33 antidepressants, and 33 neuroleptics in three different matrices were developed, fully validated, and the results evaluated concerning cross calibration. The present studies showed that matrix effects and recovery results in different biosamples have an influence on the cross calibration between these biosamples. The applicability of the presented method was shown using authentic human samples and external quality control samples. Especially for the group of antidepressants, it was shown that cross-calibration between different biosamples could only be used for some analytes.

7 ZUSAMMENFASSUNG

Im Rahmen dieser Dissertation wurde eine LC-MS/MS Methoden für schnelles Screening von rund 100 Arzneistoffen und Drogen in acht verschiedenen Matrizes entwickelt. Für diese Vielzahl an Substanzen und Untersuchungsmaterialien wurden ein einziger Extraktionsansatz sowie eine analytische Säule und eine MRM-Methode zur Trennung verwendet. Zusätzlich wurden für die Quantifizierung von 19 Benzodiazepinen einschließlich sogenannter Z-Drugs, 33 Antidepressiva und 33 Neuroleptika in drei verschiedenen Blutpräparationen neue LC-MS/MS Methoden entwickelt, nationalen Richtlinien validiert und die Ergebnisse bezüglich Kreuz-Kalibration ausgewertet. Hierbei zeigte sich, dass Probleme mit Matrixeffekten und der Extraktionsausbeute bei einigen Substanzen in verschiedenen Untersuchungsmaterialien einen Einfluss auf die Kreuz-Kalibrierung zwischen diesen Untersuchungsmaterialien haben. Die Anwendbarkeit des vorgestellten Verfahrens wurde anhand authentischer Untersuchungsproben und externen Qualitätskontrollproben bestätigt. Vor allem für die Substanzklasse der Antidepressiva wurde gezeigt, dass die Kreuz-Kalibrierung zwischen verschiedenen biologischen Matrizes nur für ausgewählte Substanzen in Betracht gezogen werden kann.