Ultra High Performance Liquid Chromatographic-Tandem Mass Spectrometric Multi-Analyte Approach for Target Screening and Quantification in Human Blood Plasma: Development, Validation, and Application

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Die folgende Arbeit entstand unter der Anleitung von Herrn Professor Dr. Dr. h.c. Hans H. Maurer in der Abteilung Experimentelle und Klinische Toxikologie der Fachrichtung 2.4 Experimentelle und Klinische Pharmakologie und Toxikologie der Universität des Saarlandes in Homburg/Saar von Juni 2007 bis Februar 2011.

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Für alle,

die den Weg gemeinsam mit mir

gegangen sind!

Denn es ist nämlich weder die Erfahrung noch die Theorie allein geeignet, alles herauszufinden.

Galen

Table of Contents

1 GENERAL PART

1.1 INTRODUCTION

Intoxications and poisonings of drugs had occurred within living memory. Owing to the desire of detecting of and protecting from such poisons a natural scientific working field developed along side, analytical toxicology [1]. The major fields of analytical toxicology are screening, identification, confirmation, and quantification of foreign compounds such as drugs and/or poisons [2]. The gained analytical results are fundamental for the consecutive pharmacological and toxicological interpretation, needed for the advisory service in clinical toxicology. This helps to achieve an optimized drug treatment in such intoxications or poisonings cases.

Drug classes often reported to cause possible abuse, intoxication, and poisoning cases are psychotic drugs (antidepressants (AD) and neuroleptics (NL)), benzodiazepines (BZ) and Z-drugs, hypnotics, antiepileptics, analgesics, antihistamines, and cardiac drugs. ADs, NLs, and BZs are of special interest because of either their narrow therapeutic range or there high potential causing tolerance and dependence. Furthermore, therapeutic drug monitoring [3], compliance testing, and drug absence screening are also performed in clinical toxicology. Additionally, a possible Munchausen (by proxy) syndrome should be confirmed or denied.

To screen for different poisons and to evaluate the level of intoxication, body fluids of these intoxicated patients are often sent to a toxicology lab. Due to the strong relation between blood plasma concentrations and clinical symptoms, in most of the cases, blood is usually used for identification and quantification. As blood contains disturbing matrices, such as proteins and lipids, an appropriate sample preparation is needed prior to instrumental analysis [2, 4, 5]. Depending on the analytes to extract, solid-phase extraction [6-10], liquid-liquid extraction [6, 11-19] or online extraction [20] are normally used.

For screening, identification, and quantification, specific and sensitive analytical procedures covering many analytes of interest are needed, as the kind, number, and amount of poison(s) are not known in advance. Combining high separation power (gas chromatography) with high identification power (mass spectrometry) made such an intention possible and easier in handling [1, 21]. As a resultant of the new

developments in the liquid chromatography (LC)- mass spectrometry (MS) sector, many of so called multi-analyte screening procedures were published recently [2, 22-25]. The intention of such multi-analyte procedures is to monitor many analytes of interest with a single sample preparation and analyzing method. This makes it preferable, because they make the analytical process much simpler, faster, and more cost effective. Altogether, this is of importance because often only a limited amount of sample is available and the number of different analytes is unknown in the beginning of the analysis.

There are several strategies for selecting analytes to be included in particular multi-analyte procedures; according to chemical drug classes, in relation to pharmacological effects and/or the need for a quick and easy screening or quantification procedure. Several multi-analyte methods containing analytes from the same drug classes have been published for plasma and/or serum so far. For the detection of ADs, various GC-MS or LC-MS procedures have been described [8-10, 17, 18, 20, 26, 27]. Others have published methods for the detection of NLs [7, 19, 28], BZs [11-14, 14, 15, 29, 30], antiepileptics [31], oral antidiabetics [16], beta-blockers [32], and antihistamines [33]. Some working groups published multi-analyte methods arranged from compounds of different drug classes in relation to their pharmacological effects [13, 30, 34-36] and/or the need for a quick and easy screening or quantification procedure [6, 37, 38].

In clinical and forensic toxiclogy, there is also a strong need for precise and accurate quantification of these analytes. As the results of toxicological analysis can have serious clinical and/or legal consequences, the quality must be strictly controlled in method validation and quality control samples [2, 39-41]. Peters et al. [42, 43] 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) and the lower limit of quantification (LLOQ). Especially using LC-MS techniques, testing for matrix effects and other possible ion suppression or enhancement effects is necessary. The commonly used ionization (APCI and ESI) techniques in LC-MS are susceptible for such effects, which can cause serious problems during identification as well as quantification. Additional parameters which may be relevant include LOD, reproducibility, and ruggedness (robustness).

1.2 PHARMACOLOGY AND TOXICOLOGY

An overview of the pharmacology and toxicology of the most important drug classes are given in the following.

1.2.1 Antidepressants

ADs are widely used as a psychiatric medication for treatment of mood and anxiety disorders. As depression is associated with under representation and disbalances of the neurotransmitters serotonin, dopamine, and norepinephrine in the brain, all ADs are acting as enhancers of these neurotransmitters [44, 45]. These can be achieved by either inhibiting the re-uptake of the neurotransmitters or by inhibiting the degradation pathway/enzyme. According to their mode of action, ADs can be distinguished into: tri- and tetracyclic ADs, selective serotonin and/or norepinephrine re-uptake inhibitors and monoamine oxidase A inhibitors [46]. In Fig. 1, typical examples of the mentioned groups are shown, also representing the difference in chemical structures.

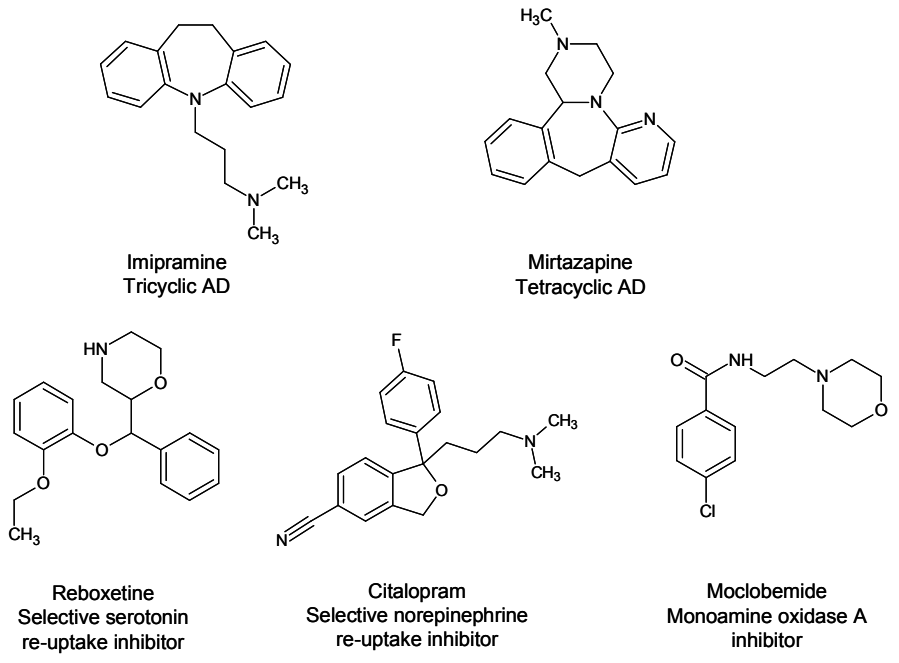


Fig. 1 Chemical structures of the different ADs

The tri- and tetracyclic ADs are not only acting as serotonin and norepinephrine re-uptake inhibitors, but also have effects on histamine H_1, muscarine, α-adrenaline and 5-HT_2 receptors. These can lead to unpleasant side-effects such as sedation, dry mouth, tachycardia, constipation, which often increases non-compliance in psychiatric patients. The newer generations of ADs are more specific reuptake inhibitors leading to more serotonin- and/or norepinephrine-related adverse effects such as extrapyramidal symptoms, sleeping disorders, tachycardia, and tremor. ADs alone, particularly the classic ones, and in combination with other drugs may cause severe poisoning after overdose.

1.2.2 Neuroleptics

Antipsychotic drugs also known as NLs are in use as a psychiatric medication to treat several mental disorders such as psychosis, schizophrenia, hallucinations, mania, sleeping disorders, and dementia of mood and anxiety disorders. Although the development of schizophrenia is not yet fully understood the neurochemical hypotheses of an overactivation of the dopaminergic system is well accepted. Nevertheless, the serotonergic system seems to be part of the pathogenesis of schizophrenia, as well. Therefore, NLs are all acting as dopamine (D) receptor antagonists, with prevalences to different subtypes, but also have effects on histamine H_1, muscarine, α-adrenaline, and 5-HT_2 receptors [44-46]. The classical NLs have prevalence on D_2-and D_1-receptors, causing extrapyramidal side-effects. They can also lead to side-effects such as sedation, hypotention, reflectoric tachycardia, dry mouth, obstipation, and accommodation caused by blocking the histamine H_1, muscarine and/or α-adrenaline receptors. The atypical neuroleptics act as D_2- or D_4- as well as 5-HT_2 receptor antagonists, causing no or slight extrapyramidal side-effects. The typical NLs are represented by phenothiazines, thioxanthenes, and butyrophenones. Atypical NLs, such as clozapine, olanzapine, zotepine, amisulpride, sulpiride, risperidone, ziprasidone, and aripiprazole cannot be sorted according to any specific structure [46]. Typical examples of both groups are depicted in Fig. 2.

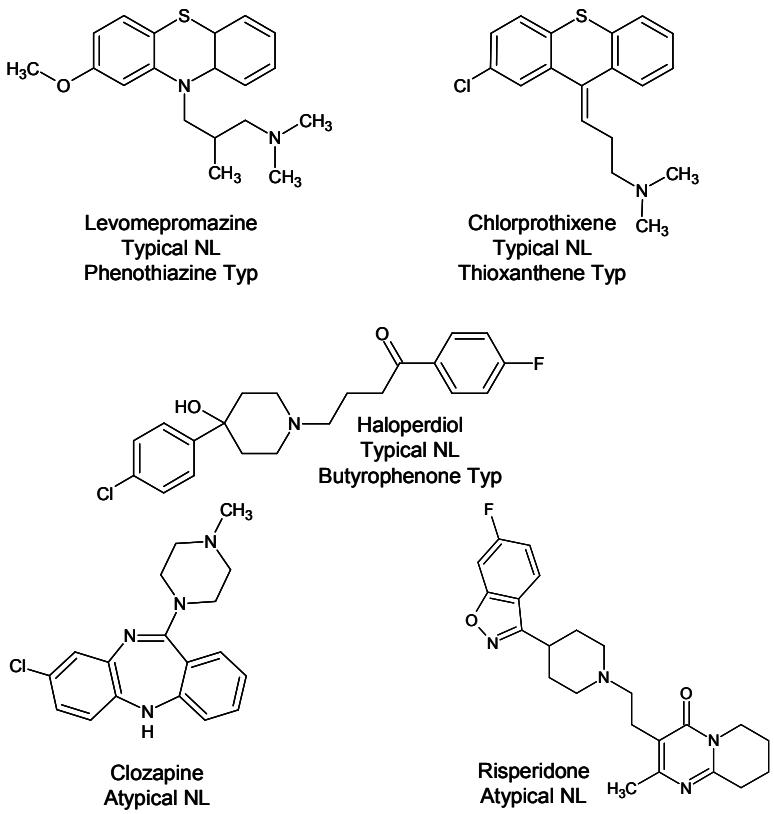


Fig. 2 Chemical structures of the different NLs

However, all the NLs may cause unpleasant side effects and severe poisoning after overdose.

1.2.3 Benzodiazepines

For the treatment of sleeplessness, anxiety, increased muscle tone, or epilepsy, BZs as well as the pharmacologically related Z-drugs, zaleplone, zolpidem, and zopiclone, are often assembled. The balance between activating and inhibiting neurotransmitter transmission is disturbed and has often moved to more activating synaptic response in the above mentioned diseases. To increase the inhibitory synaptic response the activity of the classical inhibiting neurotransmitter γ-aminobutyric acid (GABA) has to be increased. BZs and related Z-drugs act very selectively on $GABA_A$ receptors, amplifying the GABA affinity to its receptor site [44-46]. The frequency of the chloride channel opening is increased, resulting in an inhibitory synaptic response. The side-

effects of these agonists are similar; mainly dizziness, prolonged sleep, reduced ability to concentrate, leading e.g. to driving impairment [47]. Nevertheless, BZs can cause severe, even life-threatening, respiratory depression if taken in combination with other CNS-depressing substances, particularly alcohol. Furthermore, they have a high potential to produce tolerance and dependence. Examples of BZs and Z-drugs are given in Fig. 3.

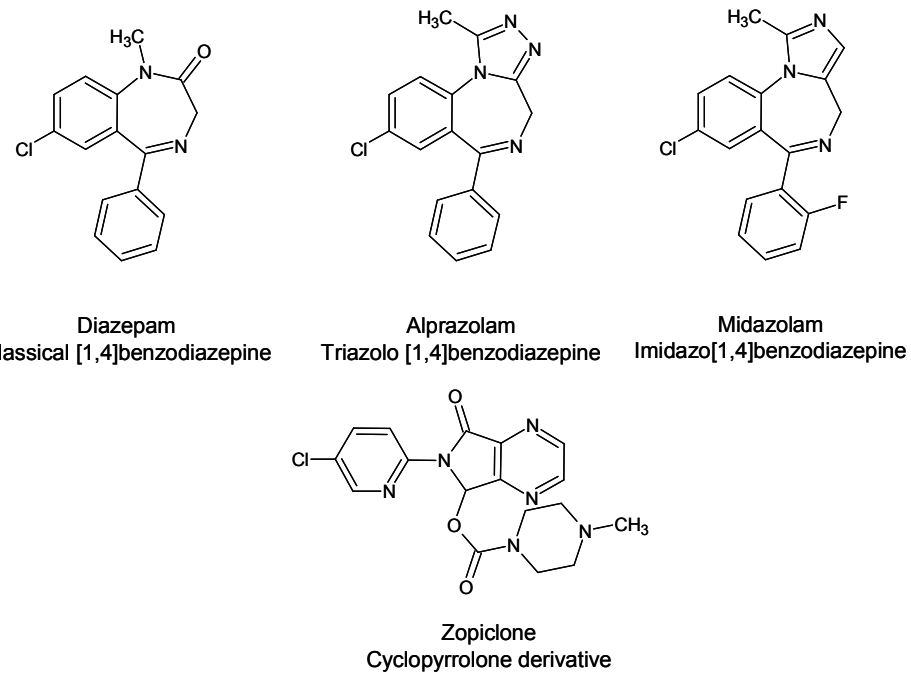
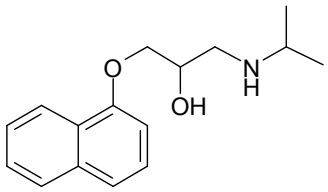


Fig. 3 Chemical structures of the different BZs and Z-drugs

1.2.4 Beta-Blockers

ß-Adrenoceptor antagonists, briefly beta-blockers, are used for treatment of hypertension, angina pectoris, cardiac insufficiency, and cardiac dysrhythmias. As all these diseases go along with a higher activation of the sympathico tonus, they act as antagonists of the ß-Adrenoceptors [44, 45]. Bradycardia, hypotension, bronchoconstriction, hypoglycemia, and fatigue are serious side-effects. In Fig 4, the typical structure of beta-blockers is depicted. Although there is no strong correlation between the plasma concentration of beta-blockers and their pharmacological and toxic effects [48], overdose of beta-blockers may lead to life-threatening situations

[49-51]. As they reduce heart rate and tremor, the World Anti Doping Agency prohibits the use of these drugs *In-Competition* in several sports e.g. archery, bobsleigh. dart, motorcycling, and shooting [52]. Therefore, analytical methods for screening, identification, and quantification are needed.

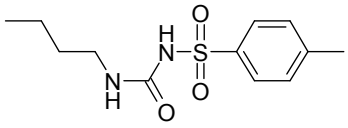


Propranolol
Phenoxypropanolamine derivative

Fig. 4 Typical structure of the beta-blockers

1.2.5 Oral Antidiabetics

Oral antidiabetics of the sulfonylurea-type are in use for treating diabetes mellitus type II. By blocking the adenosine triphosphate dependent potassium channel, they act as insulin sensitizer [44, 45]. Due to this mode of action, these drugs can lead to severe side effects such as hypoglycemia. As these drugs are not only used regularly, but are also abused in cases of Munchausen (by proxy) syndrome this can lead to serious poisonings.



Tolbutamide

Fig. 5 Typical structure of the sulfonylurea-type oral antidiabetics

1.2.6 Analytes measured in the context of brain death diagnosis

In this context, various analytes of different drug classes, BZs, anesthetics, narcotics, and opioids have to be monitored (see Fig. 6) to assure the certain absence of these drugs prior to organ explantation [53, 54]. As these drugs can suppress the cerebral activity, falsifying an electroencephalogram, this must be an important prerequisite in the determination of brain death diagnosis [54]. The studied BZs, diazepam, oxazepam, midazolam, and nordazepam, are agonists of the $GABA_A$ receptors and are widely used because of their anxiolytic, sedative, muscle relaxing, and anticonvulsive effects. Etomidate and ketamine, both used as anesthetics with a short half-life, act as $GABA_A$ receptor agonist or NDMA-receptor blocker, respectively. Whereas etomidate possesses no analgesic effect, ketamine possesses a strong analgesic action. The synthetic opioids, alfentanil, fentanyl, piritramide and sufentanil, as well as the opiate morphine are used intravenously to treat severe pain or as an adjunct to anesthesia. They are strong agonists on the μ-receptors and have an agonistic effect on the δ-receptors as well [44, 45].

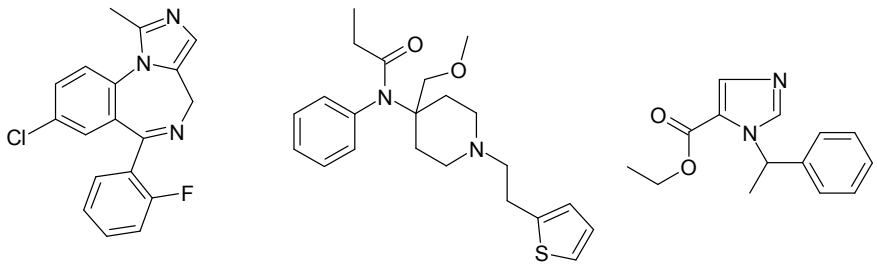


Fig. 6 Typical analytes of measured in the context of brain death diagnosis

2 AIMS AND SCOPES

Multi-analyte LC-MS methods have already been described, but only for a limited number of drugs [13, 30, 36, 38]. Therefore, a new multi-analyte procedure should be developed covering all relevant drugs of important drug classes.

As triple quadrupole LC-MS/MS devices combine high sensitivity with good identification and quantification power, this technology should be used for secure identification and reliable quantification of drugs in plasma. Today, ultra high performance liquid chromatography (UHPLC) is the gold standard in separation technology, as the number of theoretical plates is high and therefore high separation power is given to get sufficient separation results and to seprate matrix.

Therefore, the aims of this presented study were:

→ To develop a multi-analyte UHPLC-MS/MS method covering the most common drug classes involved in abuse and intoxication cases.

- → Antidepressants (35 analytes)
- → Neuroleptics (31 analytes)
- → Benzodiazepines and Z-drugs (28 analytes)
- → Beta-blockers (25 analytes)
- → Oral antidiabetics (10 analytes)
- → Analytes measured in the context of brain death diagnosis (11 analytes)

→ To develop this multi-analyte UHPLC-MS/MS method considering the following facts.

→ One chromatographic system should be used for separation.

→ One sample preparation should be found for extracting all analytes.

→ Testing for possible ion suppression/enhancement on the deuterated internal standards caused by the non labeled analytes.

→ Testing for possible ion suppression/enhancement on co-eluting analytes in the same drug class and between other drug classes.

→ To validate this multi-analyte method according to national and international accepted guidelines for the most important and biggest drug classes.

- → Antidepressants (35 analytes)
- → Neuroleptics (31 analytes)
- → Benzodiazepines and Z-drugs (28 analytes)

3 PUBLICATIONS OF THE RESULTS

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

3.1 FAST AND SIMPLE PROCEDURE FOR LIQUID-LIQUID EXTRACTION OF 136 ANALYTES FROM DIFFERENT DRUG CLASSES FOR DEVELOPMENT OF A LIQUID CHROMATOGRAPHIC-TANDEM MASS SPECTROMETRIC QUANTIFICATION METHOD IN HUMAN BLOOD PLASMA [55]

(DOI 10.1007/s00216-010-3820-7)

3.2 SYSTEMATIC INVESTIGATION OF ION SUPPRESSION AND ENHANCEMENT EFFECTS OF FOURTEEN STABLE-ISOTOPE-LABELED INTERNAL STANDARDS BY THEIR NATIVE ANALOGUES USING ATMOSPHERIC-PRESSURE CHEMICAL IONIZATION AND ELECTROSPRAY IONIZATION AND THE RELEVANCE FOR MULTI-ANALYTE LIQUID CHROMATOGRAPHIC/MASS SPECTROMETRIC PROCEDURES [56]

(DOI: 10.1002/RCM.4459)

3.3 ION SUPPRESSION AND ENHANCEMENT EFFECTS OF CO-ELUTING ANALYTES IN MULTI-ANALYTE APPROACHES: SYSTEMATIC INVESTIGATION USING ULTRA-HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY/MASS SPECTROMETRY WITH ATMOSPHERIC-PRESSURE CHEMICAL IONIZATION OR ELECTROSPRAY IONIZATION [57]

(DOI: 10.1002/RCM.4736)

3.4 FULL VALIDATION AND APPLICATION OF AN ULTRA HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC-TANDEM MASS SPECTROMETRIC PROCEDURE FOR TARGET SCREENING AND QUANTIFICATION OF 34 ANTIDEPRESSANTS IN HUMAN BLOOD PLASMA AS PART OF A COMPREHENSIVE MULTI-ANALYTE APPROACH [58]

(DOI 10.1007/s00216-011-4959-6)

3.5 ULTRA HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC-TANDEM MASS SPECTROMETRIC MULTI-ANALYTE PROCEDURE FOR TARGET SCREENING AND QUANTIFICATION IN HUMAN BLOOD PLASMA: VALIDATION AND APPLICATION FOR 31 NEUROLEPTICS, 28 BENZODIAZEPINES AND Z-DRUGS [59]

(DOI 10.1007/s00216-011-5187-9)

4 CONCLUSIONS

Systematic data of all steps in the development of a multi-analyte approach for fast target screening and quantification in plasma were shown in the respective papers [55-59]. Analytes of six of the most important drug classes in clinical toxicology were implemented into this procedure resulting in 136 analytes. After testing different column types and different mobile phase compositions, an UHPLC system was found to be sufficient for the separation of most of the analytes. For separating the analytes with different chemical structures, only one column and one gradient was used. In addition, the triple quadrupole technology in the time-schedule monitoring mode was shown to be sensitive enough in most of the cases. Furthermore, a fast and simple extraction procedure covering a wide range of different drug classes was developed. Recovery, matrix effects, and process efficiency tests were performed. The extraction procedure showed good results for 119 analytes and were comparable to extraction results of other multi-analyte methods. The recovery results, calculated without compensation by internal standardization were compared to those using internal standards. It could be shown that recovery results are more or less the same, but the reproducibility is better, as the internal standards compensate different variations of plasma.

Nevertheless, as there were still some co-eluting analytes, stable isotope labeled (SIL) internal standards (IS) and non labeled analytes and co-eluting analytes from the same and other drug classes, ion suppression and enhancement tests were performed. The ion suppression/enhancement tests on the SIL-IS caused by the non labeled analytes and of other co-eluting analytes showed severe effects especially using electrospray ionization technique. Therefore, the SIL-IS should be selected very carefully, especially if used for the quantification of analytes others than the non-labeled one. The presented studies showed the analytical fundamentals of such ion suppression and enhancement tests especially during method development and validation of multi-analyte procedures using LC-MS/MS technology. It was shown that these effects can influence the quantification results and lead to clinical or forensic misinterpretation. The cause of this ion suppression or enhancement could unfortunately not be found, but it can be concluded that a combination of different

effects, such as structure, molecular weight, concentration, and the chromatographic behavior of the analytes and the co-eluting analyte, should be responsible.

The UHPLC-MS/MS approach was finally validated for the drug classes of ADs, NLs, and, BZs with respect to selectivity, 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. Furthermore, accuracy and precision were obtained with full and one-point calibration. This approach allowed selective target screening as well as accurate and precise quantification of 28 ADs, 24 NLs, and 21 BZs.

The method fulfilled the requirements for a validated assay and has proved to be efficient in authentic cases and external quality control samples. Thus, it allows drug monitoring and confirmation of diagnosis of an overdose situation caused by ingestion of ADs, NLs, and BZs. Furthermore, the time- and cost-saving one-point calibration was shown to be applicable for many analytes for daily routine and especially for emergency cases. Nevertheless, analytes not fulfilling the validation criteria and/or having higher LLOQs than the lower therapeutic concentrations, seemed to have in common rather low therapeutic plasma concentrations and late retention times in the used chromatographic system. The low analyte concentrations and the higher amount of acetonitrile at the end of the chromatographic system result in narrow peaks. Because of the use of this method as multi-analyte approach, the amount of monitored transitions is high (>350) and it is not possible to get more than 10 data points of these peaks in the used settings. Although the number of data points is enough, the peaks could sometimes not be described as Gaussian curves because of fronting and tailing. Due to the high number of SRM transitions, narrow peaks could lead to a missing data point resulting in variations of the peak areas. E.g. using a more sophisticated sample processing, reducing the amount of transitions and/or changing the chromatographic system could solve this problem. As this method was set up for a multi-analyte approach changing these parameters will affect other analytes and is not reasonable in this particular case. It shows the limitations of broad multi-analyte procedures particularly using UHPLC after simple workup. This should always be in mind when using this method.

Regarding the fact that a lot of analytes with different chemical structures are implemented in this fast and simple work-up, compromises concerning recovery, precision, and LLOQ had to be made in some cases.

5 SUMMARY

In the presented studies, the development of an UHPLC-MS/MS approach for fast target screening and quantification in plasma was described. This approach covered 136 analytes out of the drug classes of antidepressants, neuroleptics, benzodiazepines, beta-blockers, oral antidiabetics, and analytes to be determined in the context of brain death diagnosis. Further, it was possible to find one chromatographic system with the same column and mobile phase compositions to separate almost all these analytes sufficiently. A fast and simple liquid-liquid extraction for all these analytes was developed. In addition, the presented studies showed that ion suppression and enhancement tests were essential, particularly for the selection of internal standards for UHPLC-MS/MS multi-analyte procedures and for combining co-eluting analytes during method validation. Furthermore, this approach was fully validated for the most important drug classes of antidepressants, neuroleptics, and benzodiazepines. This approach was valid for 28 antidepressants, 24 neuroleptics, and 21 benzodiazepines. In addition, the applicability was shown successfully using authentic human blood samples and external quality control samples. The cost and time saving one-point calibration was shown to be sufficient for more than half of the analytes.

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7 ZUSAMMENFASSUNG

Im Rahmen dieser Dissertation wurde die Entwicklung einer UHPLC-MS//MS Methode für schnelles Target-Screening und Quantifizierung im Plasma beschrieben. Die Methode umfasste 136 Arzneistoffe aus den Stoffgruppen der Antidepressiva, Neuroleptika, Benzodiazepine, Betablocker, orale Antidiabetika und Arzneistoffe, die im Rahmen der Hirntoddiagnostik untersucht werden müssen. Weiterhin war es möglich, ein chromatographisches System, unter Verwendung derselben Säule und Fließmittelzusammensetzung, für die Trennung aller Arzneistoffe zu finden. Eine schnelle und einfache Flüssig-Flüssig-Extraktion wurde für alle Arzneistoffe entwickelt. Die Notwendigkeit von Untersuchungen zur Ionenunterdrückung und Ionenerhöhung konnte ebenfalls in dieser Arbeit belegt werden. Diese Untersuchungen waren für die Wahl des internen Standards für LC-MS/MS Methoden und für die Zusammensetzung von co-chromatographierenden Stoffen wichtig. Weiterhin wurde diese Methode für die wichtigsten Stoffgruppen nämlich die Antidepressiva, Neuroleptika und Benzodiazepine voll validiert. Es konnte gezeigt werden, dass die Methode für 28 Antidepressiva, 24 Neuroleptika und 21 Benzodiazepine valide war. Die Anwendbarkeit wurde mittels humaner authentischer Plasmaproben und mittels externen Qualitätskontrollproben gezeigt. Die kosten- und zeitsparende Ein-Punkt-Kalibrierung erwies sich für über die Hälfte der Arzneistoffe als akzeptabel.