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Development of an Automated Metabolite-based Liquid Chromatographic-High Resolution Tandem Mass Spectrometric Drug Screening Procedure for Urine and Blood Plasma

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1 GENERAL PART

1.1 Introduction

In analytical toxicology, a broad range of compounds have to be detected and identified in case of intoxications and poisonings [1]. The presence or absence of drugs, drugs of abuse, poisons, and/or their metabolites must be confirmed by unequivocal toxicological analyses [2]. As in most cases the administered drug or poison is not known, a so-called general unknown screening (GUS) is one of the major tasks in clinical and forensic toxicology, workplace drug testing, and doping control. Urine screening offered best prerequisites as drugs are excreted, more or less metabolized, in higher concentrations over a longer period of time in urine after concentration in the kidney compared to blood plasma [3]. Detection of metabolites provided several advantages. It allowed monitoring of drugs, which are excreted in completely metabolized form, and confirmed the body passage and thus the intake of a drug [2,4]. Furthermore, detection of various metabolites helps to confirm the taken drug and lowers the risk of false negative results if the ionization of the drug or one metabolite is completely suppressed e.g. by matrix [5]. Despite shorter detection windows, blood (plasma, serum) can be essential if urine cannot be provided because of e.g. drug-induced urinary retention or acute renal failure [3].

1.2 Screening Procedures

Immunoassays can be used for preliminary screening of a single drug or drug class [3]. Due to interferences with other drugs or biomolecules resulting in false-positive results, they should be confirmed by a second independent method [6]. Therefore, mass spectrometry (MS) was established in the field of clinical and forensic toxicology providing higher identification power, sensitivity and specificity [6]. Application of hyphenated mass spectrometric techniques such as gas chromatography-mass spectrometry (GC-MS) or liquid chromatography-mass spectrometry (LC-MS) in this field has been reviewed elsewhere [3,6]. In recent years, high resolution (HR) mass spectrometry, already established in proteomics, was introduced also in bioanalysis of small molecules [2,7-9].

1.2.1 Sample Workup

Sample workup procedures for GUS should be universal in order to assure reproducible recovery of a broad range of compounds with different physicochemical properties [10]. Apart from laborious workup approaches such as liquid-liquid extraction [11,12] or solid-phase extraction (SPE) [13,14], fast and simple dilute-and shoot approaches [15-17] and precipitation were used for comprehensive screening procedures [5]. Stahnke et al. described for the analysis of pesticides that a 25-40 fold dilution of samples could reduce the ion suppression to less than 20% [18]. Dilution or precipitation of a sample might result in reduced detectability [17]. Evaporation of the dilution/precipitation solvent might increase sensitivity by concentration as described by Wissenbach et al. [5,19], but could result in higher matrix effects [20].

Due to the high sensitivity of time-of-flight (TOF) and orbitrap (OT) mass spectrometers, simple and fast sample workup approaches should be feasible. Online SPE [21-24], micro-extraction [22,23], and on-line extraction by turbulent flow chromatography (TurboFlow) [23,25,26] represented fast and automated sample workup approaches. TurboFlow was first introduced in the late 1990s for online sampling in bioanalysis [26]. It was based on the principle of size exclusion chromatography to separate macromolecules such as proteins from smaller

molecules in complex biological fluids, such as blood plasma or urine [26]. The basis for this methodology was “turbulent flow” with flow rates above 1.2 mL/min using 0.5 mm I.D. columns packed with large particles of 30 – 50 µM resulting in low backpressures [25]. However, direct injection of undiluted biological samples might cause carry-over and autosampler blockage [26]. In order to prevent this, prior precipitation was still recommended to maximize column life. Most TurboFlow methods were applied for narrow target screening of e.g. beta-agonists, drugs of abuse, or the mushroom poisons α- and β-amanitin [27-29]. Recently, a new TurboFlow mode, the so-called pseudo quick elute mode, was developed for reliable detection of α- and β-amanitin as well as for fully validated quantification of α-amanitin [29]. It offered more options for method optimization and showed better practicability in routine and emergency analysis compared to previous methods. Mueller et al. developed the first screening approach for over 300 parent compounds and major phase I metabolites in urine after manual hydrolysis using TurboFlow [30].

1.2.2 Chromatography and Mass Spectrometry

1.2.2.1 Gas and Liquid Chromatography - Low Resolution Mass Spectrometry

GC-MS with its high separation power of GC and high selectivity of electron ionization MS in combination with comprehensive electron impact spectra based reference libraries and sophisticated search algorithms provided outstanding non-targeted screening results [3,10,31,32]. However, it required derivatization and the technique was limited to volatile and thermostable analytes.

LC-MS overcomes those disadvantages, thus several LC-MS, LC-MS/MS, or LC-MS^n screening procedures using library search were developed and reviewed elsewhere [2,10]. Different mass analyzers such as triple quadrupoles [33], ion traps [5,19,21,30], and hybrids of both techniques [34] were used. Wissenbach et al. [5,19] developed the first metabolite-based LC-MS^n urine drug screening procedure using data-dependent acquisition (DDA) and MS^2 and MS^3 reference spectra. The library consisted of data of more than 1,500 drugs and about 3,000 metabolites (including phase II metabolites) or artifacts recorded from reference standard solutions and authentic rat or human urine samples after administration or consumption of the corresponding drugs [35].

1.2.2.2 Liquid Chromatography-High Resolution Mass Spectrometry

Background

Instruments with increased high mass resolving power made it possible to calculate empirical formula of chemical compounds from accurate mass measurements [35,36]. The combined atomic masses (based on the most abundant isotope of each atom) of the empirical formula of an ion or molecule, for example 12.000000 + 4(1.007825) = 16.0313 Da for CH_4, allowed calculation of the exact molecular mass [36]. Already in early time of GC-MS, HRMS using double-focusing sector field mass analyzers were used for GUS in order to determine the accurate mass and the corresponding empirical formula of unknown compounds in blood or urine [2,37].

The principles of TOF- and OT-MS measuring exact masses were reviewed elsewhere in detail [36,38]. The principle of an OT-MS will shortly be explained here. In a multiple collision cell, higher-energy collisional dissociation (HCD) takes place [39]. Ions are trapped then radially about a central spindle electrode. Mass/charge values are measured from the frequency of harmonic ion oscillations along the axis of the electric field [39]. Ion frequencies are measured by acquisition of time-domain image current transients. Afterwards fast Fourier transforms are used for conversion to HR mass spectra. HR mass spectrometers provide several advantages such as high selectivity, sensitivity, and specificity in combination with the option to predict empirical formula of molecules or fragments formed during ionization due to accurate mass measurements [9].

Thus, they provide best prerequisites for its application for broad screening and library-assisted identification of drugs, poisons, and their metabolites. LC-HRMS screening procedures have been reviewed elsewhere in detail [9,40]. In the following, the application of HRMS for GUS will be described for TOF, sequential window acquisition of all theoretical fragment ion spectra (SWATH) technology, and then be focused on OT technology and adherence testing in cardiology.

Time-of-Flight-based Screening Procedures

For comprehensive screening by HRMS, mainly TOF analyzers were applied [11,13,41-43]. First approaches were based on accurate mass lists [44,45]. Polettini et al. [44] worked with an accurate mass list of over 50,000 compounds including metabolites taken from the PubChem Compound database. Comparing measured

accurate masses with lists of theoretical accurate masses (exact masses) provided only preliminary screening results. An important disadvantage of compound identification only by accurate mass is that there are several isomeric compounds, which cannot be differentiated by this approach and a following confirmation step is required. Liotta et al. [45] tried to improve isomer identification with a metabolomics approach by checking if isomeric compounds undergo major metabolic reactions. In the following, DDA based screening approaches with different identification criteria will be described. Compound identification by Lee et al. [11] was based on exact mass, retention time, nominal mass spectra followed by empirical formula analysis, and examination of the isotopic patterns. However, using two voltages for spectra recording increased the cycle time. De Castro et al. [13] described identification based on accurate mass, relative retention time, and collision induced dissociation (CID) spectra comparison with up to three qualifier ions. Broecker et al. [12] developed a screening approach for 2,500 compounds based on full scan (FS) and HR product ion spectra using a TOF instrument with a quadrupole in front (Q-TOF) in DDA mode with CID spectra recorded at three collision energies of 10, 30, 40 eV. DDA is the more established technique and has been successfully used for low-resolution (LR) [5,30], but also for HR instruments as described by Broecker et al. [12]. The precursor ions were identified in FS and then considered for MS/MS experiments [46]. The main criterion is signal intensity, only most abundant ions exceeding an intensity threshold are chosen for fragmentation experiments. In order to prevent generation of too many spectra per time frame and mixed spectra, only a limited number of possible precursor ions is introduced to MS/MS experiments. Therefore, dynamic exclusion and background subtraction were developed for DDA mode [47].

Besides, TOF screening procedures based on data-independent acquisition (DIA) were developed. Sundstrom et al. [42] developed an ultra-high-performance-liquid chromatography (UHPLC) HR-TOF-MS procedure for designer drugs and drugs of abuse in urine using all-ion fragmentation with a database of 277 compounds. Identification was based on accurate masses, retention time, and isotopic patterns and up to two fragment ions (FIs) as qualifier ions. Chindarkar et al. [48] presented a broad-spectrum UPLC-TOF-MS screening method for urine after dilution and enzymatic cleavage of conjugates with the identification criteria accurate mass precursor ion, retention time, and one FI match. They reported that certain drug

classes required high-energy collisions with the disadvantage of forming many low abundant FIs. Kinyua et al. [49] presented a qualitative screening procedure based on all-ion fragmentation mode on a Q-TOF-MS for new psychoactive substances and their metabolites with identification criteria accurate mass precursor ion and manual evaluation of FIs. Thoren et al. [50] compared the performance of a broad-spectrum Q-TOF unknown screening with a linear ion trap targeted drug screening, which were both operated in DDA mode. Q-TOF offered similar identification power compared to the linear ion trap.

In the described DDA and DIA based screening approaches different identification criteria such as accurate mass precursor ions and spectra comparison were described. However, spectra generation at two different conditions could increase cycle times and applying too high fragmentation energies might result in too many low abundant FIs. In addition, the lack of suitable software solutions for TOF data evaluation required manual data evaluation in some cases. In comparison to OT technology, TOF-MS provided higher acquisition frequencies and typically required internal calibration to correct the mass axis [51], which might provide some disadvantages such as interference with the ionization process of the sample. Nevertheless, comprehensive Q-TOF screening approaches could be an alternative for untargeted urine drug screening.

Sequential Window Acquisition of All Theoretical Fragment Ion Spectra based Screening Procedures

As already discussed by Maurer and Meyer [9], SWATH provides a promising alternative option for non-targeted HRMS Q-TOF screening in clinical and forensic toxicology. It is a DIA technique, where ions are allowed to pass quadrupole one in a defined medium window size of 20 or 25 Da across the scan range of interest [52,53]. Ions in each of those windows are transferred into the collision cell and product ions are generated under high collision energy. The product ions are then sequentially analyzed by the HR-TOF analyzer. In the following, different SWATH-based methods will be described.

Arnhard et al. [54] used SWATH in combination with Q-TOF for acquisition of HR-MS/MS spectra after precursor ion selection in 21 Da isolation windows. They showed that SWATH is a more sensitive and specific identification tool for compounds at lower concentration levels compared to DDA. But only semi-

automated data evaluation was possible confirming data processing to be a laborious and time-consuming process. Roemmelt et al. [55] investigated isolation windows of 20-35 Da for its suitability for systematic toxicological analysis. Although relative abundance of the fragments was concentration-dependent and influenced by co-fragmentation, SWATH detection rate was better than DDA, which failed to trigger about 10% of the analytes.

In theory, DIA allowed generation of mass spectral information for all precursor ions [46]. As described above, SWATH showed more identification power, sensitivity, and specificity compared to DDA. However, if multiple compounds were dissociated in one experiment, mixed spectra resulted and had to be interpreted. Sufficient deconvolution algorithms would be necessary for those screening procedures, but only semi-automated data evaluation was described yet.

Orbitrap-based Screening Procedures

As already discussed by Maurer and Meyer [9], only few OT-based methods were published up to now for comprehensive screening of drugs of abuse and pharmaceuticals in clinical and forensic toxicology.

Li et al. [56] developed a screening procedure for 65 drugs of abuse such as cannabinoids in human urine by using an LTQ-OT-MS. However, they used time consuming SPE and had to perform separate runs in positive and negative ionization mode due to slow switching times of the instrument. Identification was based on accurate mass, retention time, and exact masses of the FIs using ToxID software. Roche et al. [57] described a GUS procedure with a library of 616 mainly parent compounds in serum, urine, and whole blood samples after TurboFlow. The OT-MS was operated in positive and negative ionization mode with subsequent HCD fragmentation. Identification criteria were accurate masses, retention time, isotopic patterns, and the presence of specific FIs. Montesano et al. [58] developed a broad screening for new psychoactive substances on an OT with quadrupole precursor selection (Q-OT) instrument including cathinones and synthetic cannabinoids in blood plasma. They performed a FS for targeted screening with subsequent all ion fragmentation considering two specific product ions for data analysis of over 300 compounds by automated TraceFinder data evaluation software.

Novel Q-OT mass spectrometers offered long-term stability of external mass calibration, unique high resolving power, sub ppm mass accuracies over a wider

dynamic range compared to TOF instruments [51,59]. In contrast to TOF-based screening procedures, first automated data evaluation software packages were described. Therefore, Q-OT mass spectrometers should be appropriate for comprehensive toxicological screening of various drug classes and their metabolites [2].

Adherence Testing in Cardiology

Nonadherence of the patients to a prescribed drug therapy could be one reason for treatment-resistant hypertension. This is indicated by blood pressure levels > 140/90 mm Hg when three or more antihypertensive drugs of different classes including a diuretic were taken at maximal tolerated dose [60]. In hypertensive patients its prevalence ranges from 5 - 30% and is connected to an increased risk of cardiovascular and renal events [60-62]. Jung et al. [63] presented the first study on a targeted urine drug screening for patients with apparent resistant hypertension. Based on these screening results they confirmed adherence of patients and showed the utility of this approach. In the field of metabolomics, van der Hooft et al. [64] developed a urinary antihypertensive drug metabolite screening making use of molecular networking coupled to HRMS fragmentation. Their method offered possibilities for untargeted identification of drugs and their metabolites.

2 AIMS AND SCOPES

The aim of this dissertation was to develop a comprehensive, metabolite-based, automated LC-HR-MS/MS screening procedure by OT technology for drugs, poisons, and their metabolites, being relevant to clinical and forensic toxicology. As a complement to already existing GC- and LC-MS^n libraries [32,35], high resolution MS/MS spectra of drugs should be collected of reference standards in methanolic solution. Those of metabolites should be identified in urine samples of rats or humans after corresponding drug administration and in incubates of drugs with pooled human liver preparations.

The following should be conducted:

- Development of simple sample workup procedures and comparison with established ones
- Development of fast und sufficient universal UHPLC separation
- Development of a new HRMS detection method by Q-OT including study of conditions for mass spectra recording and reproducibility
- Validation of the method for a selection of drugs of various drug classes according to recommendations for qualitative methods [65,66] after different sample workups of urine and blood plasma
- HR-MS/MS drug spectra collection by using the above-mentioned reference standard solutions
- Identification of metabolites of drugs and poisons in urine or incubates of liver preparations by detailed HR MS/MS spectra and implementation of those into the new HRMS reference library
- Establishment of new, automated HR-MS/MS data evaluation with different detection and identification criteria
- Application of the screening method to particular drug classes
- Studies on the application of HRMS for blood plasma screening
- Evaluation of HRMS for differentiation of isobaric and isomeric compounds, and for GUS

3 PUBLICATIONS OF THE RESULTS

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

3.1 Orbitrap technology for comprehensive metabolite-based liquid chromatographic-high resolution-tandem mass spectrometric urine drug screening - exemplified for cardiovascular drugs [67] (DOI:10.1016/j.aca.2015.08.018)

3.2 LC-HR-MS/MS standard urine screening approach: Pros and cons of automated on-line extraction by turbulent flow chromatography versus dilute-and-shoot and comparison with established urine precipitation [68] (DOI: 10.1016/j.jchromb.2016.06.036)

3.3 Liquid chromatography-high resolution-tandem mass spectrometry using Orbitrap technology for comprehensive screening to detect drugs and their metabolites in blood plasma [69] (DOI: 10.1016/j.aca.2017.03.002)

4 DISCUSSION AND CONCLUSIONS

In the first part of this thesis, OT technology was successfully applied for development of a comprehensive metabolite-based LC-HR-MS/MS screening approach for urinalysis of drugs relevant in clinical and forensic toxicology [67]. Different new urine sample workup procedures using on-line extraction by TurboFlow and a dilute-and-shoot approach were developed and compared with already established urine precipitation [68]. The “pseudo quick elute mode” for TurboFlow, which has already been described for the analysis of amanitin [29], offered some advantages for urine drug screening such as less manual handling compared to precipitation. Only if a higher number of samples have to be analyzed per day, the reduction of total analysis time outweighed the limitations of TurboFlow such as additional costs for equipment, columns and maintenance. A broad range of drugs with different physicochemical properties could be detected and identified after common dosages after all sample workups with better identification limits for precipitation and TurboFlow. However, only some low-concentrated drugs and metabolites might not be identified after simple dilution. The screening approach was also suitable for qualitative blood plasma screening [69]. Due to lower drug concentrations in blood plasma and fewer metabolites compared to urine, targeted blood plasma screening with preferred MS/MS spectra acquisition was performed on 700 compounds and main metabolites in blood plasma. This was followed by DDA mode for identification of unknowns. Final identification criteria were the presence of accurate mass precursor ions, five most intense FIs, and HR-MS/MS library match. High amounts of both parent drugs and metabolites, covered by urine screening, in combination with blood plasma screening will ensure definite identification of compounds. The procedure was successfully applied for cardiovascular drugs to assess medication adherence of hypertension patients [70-72]. Thus, in the context of adherence testing, Linicus et al. [70] were able to demonstrate the relevance of excluding non-adherence to prescribed drugs by adherence screening before renal denervation. Ewen et al. [71,72] showed the usefulness of renal denervation to reduce blood pressure in patients with resistant hypertension and reported the corresponding mechanisms.

As mentioned above, HR-FS in combination with DDA mode allowed detection and identification of unknown drugs, their artifacts, and metabolites. Thus, new drugs appearing on the market such as novel psychoactive substances, which were neither added to the inclusion list nor to the current reference library, can be detected. Accurate mass measurement allows proposing the empirical formula of the precursor ion of the new compound and/or its metabolites. The combination with accurate masses/empirical formulas of the formed MS/MS fragments may help in identifying the compound. This was e.g. shown for elucidation of phase I and II metabolism of the novel psychoactive substance 4-methyl-*N*-ethcathinone by Helfer et al. [73]. In this case, isobaric compounds could be differentiated by HR-FS due to accurate mass measurements. As an example, Maurer and Meyer [9] described differentiation of the new psychoactive substances beta-keto-2CB ((2-amino-1-(4-bromo-2,5-dimethoxyphenyl)ethanone) and DOB (2,5-dimethoxy-4-bromamphetamine) showing in addition to the precursor ion three identical nominal mass FIs [74]. LC-HR-MS/MS allowed differentiation of the corresponding precursor ions and FIs by their accurate masses.

However, isomeric compounds cannot be differentiated in full scan mode by HR-MS, but by different fragmentation and/or chromatographic separation. The risk of overlooking co-eluting isomeric compounds due to common precursors and/or risk of complete ion suppression by the matrix could be minimized by inclusion of metabolites and by recording spectra in negative ionization mode. Some compounds showed better negative ionization properties, but some drugs such as thiazide diuretics were exclusively detected in this ionization mode due to their chemical structure.

Initial studies showed that HCD fragmentation at three collision energies provided a mixed spectrum with formation of enough FIs. However, less than five FIs could not be sufficient for differentiation of isomeric compounds based on different fragments. Due to a different search algorithm of the data evaluation software for FI matching compared to full HR-MS/MS library matching, FIs provided additional identification certainty. Isotopic patterns were no longer taken into account for final data evaluation because they were not completely detected although the most abundant isotope (accurate mass precursor ion) provided an MS/MS spectrum. Those findings confirmed that full HR-MS/MS library spectra provided highest identification power according to the European Commission Decision 2002/657/EC [75]. In cases of

doubt, retention times of the compounds can be considered. Despite the advantages of HRMS for GUS, the technique still showed some limitations [9]. The HR mass spectrometers are comparably expensive, require intensive maintenance, and the huge size of recorded data affords solutions regarding data storage and evaluation. Evaluating unknown HR data is laborious, time-consuming, and considerable mass spectrometry skills are required. In the author´s opinion, LR mass spectrometers fulfill the criteria for comprehensive drug screening at low purchase costs and acceptable identification power. However, high selectivity and identification power by HRMS can be mandatory for differentiation of isobaric compounds, co-eluting isomeric compounds, and tentative identification of unknown drugs and/or metabolites. Further studies investigating the transferability of the HR-MS/MS library to other HR instruments and the influence of different instrument types and settings on the quality of screening results are necessary.

5 SUMMARY

In the presented thesis, development of an automated and comprehensive metabolite-based LC-HR-MS/MS screening procedure using OT technology for detection of drugs and poisons relevant in clinical and forensic toxicology is described. Therefore, new urine sample workup procedures based on automated on-line extraction by TurboFlow and simple dilution were developed and compared with established urine precipitation. After universal UHPLC separation, drugs and metabolites were detected and identified by an OT mass spectrometer operated in FS mode with positive/negative switching and subsequent DDA mode. Based on these conditions, a new mass spectral reference library with over 5,000 HR-MS/MS spectra containing about 2,000 parent compounds and 3,000 metabolites was developed. The screening approach was successfully applied to blood plasma. A new automated HR-MS/MS data evaluation considering accurate mass precursor ions, five most intense FIs, and full HR-MS/MS library spectra was established. The new LC-HR-MS/MS screening approach complements the existing GC-MS and LC-MS^n screening procedures and is the basis for metabolism studies of various drug classes. It is successfully applied in daily routine analysis for GUS and adherence testing of hypertension patients. Analysis of thousands of patient samples showed robustness, selectivity, sensitivity, and specificity of the method.

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7 ABBREVIATIONS

CID	collision-induced dissociation
DDA	data-dependent acquisition
DIA	data-independent acquisition
FI	fragment ion
FS	full scan
GC	gas chromatography
GUS	general unknown screening
HCD	higher-energy collisional dissociation
HR	high resolution
LC	liquid chromatography
LR	low-resolution
MS	mass spectrometry
OT	orbitrap
SPE	solid-phase extraction
SWATH	sequential window acquisition of all theoretical fragment ion spectra
TOF	time-of-flight
TurboFlow	turbulent flow chromatography
UHPLC	ultra-high-performance-liquid chromatography

8 ZUSAMMENFASSUNG

Im Rahmen dieser Dissertation wird die Entwicklung eines automatisierten und umfangreichen, Metaboliten-basierten Screeningverfahrens mittels LC-HR-MS/MS-Technik unter Verwendung der Orbitrap-Technologie zum Nachweis von in der klinischen und forensischen Toxikologie relevanten Arznei- und Giftstoffen beschrieben. Hierzu wurden neuartige Probenaufarbeitungsverfahren für Urin basierend auf automatisierter Turbulent Flow On-line Extraktion und einfacher Verdünnung entwickelt und mit der bereits etablierten Proteinfällung verglichen. Unter Verwendung einer universellen UHPLC Trennung wurden die Muttersubstanzen und Metaboliten detektiert und identifiziert mittels eines Orbitrap Massenspektrometer, das im Full-Scan-Modus abwechselnd positiv oder negativ ionisierte Analyten erfasste und anschließend im datenabhängigen Erfassungsmodus betrieben wurde. Unter Anwendung dieser Bedingungen entstand eine neue Referenzspektrenbibliothek mit inzwischen über 5.000 hochaufgelösten Massenspektren, die rund 2.000 Muttersubstanzen und 3,000 Metaboliten umfasst. Das Verfahren wurde erfolgreich auf das Screening im Blutplasma übertragen. Eine neue automatisierte Auswertung, die die akkuraten Massen der Vorläuferionen, die der fünf intensivsten Fragmentionen und die vollständigen, hochaufgelösten MS/MS Bibliotheksspektren berücksichtigt, wurde etabliert. Das neue LC-HR-MS/MS Screeningverfahren ergänzt die bestehenden GC-MS und LC-MS^n Screeningverfahren und ist die Grundlage zur Aufklärung des Metabolismus von verschiedensten Subtanzklassen. Es wird erfolgreich in der täglichen Routineanalytik zum allgemeinen Screening auf unbekannte Substanzen und zur Überprüfung der Adhärenz von Bluthochdruckpatienten eingesetzt. Die Analyse tausender Patientenproben zeigte die Robustheit, Selektivität, Sensitivität und Spezifizität der Methode.