Development of a Screening System for the Determination of Compounds in Urine by Automated On-line Extraction HPLC-DAD for Toxicological Analysis

Dissertation

zur Erlangung des akademischen Grades
doctor rerum naturalium (Dr. rer. nat.)

Vorgelegt der
Naturwissenschaftlichen Fakultät I / Biowissenschaften
der Martin-Luther-Universität Halle-Wittenberg

von Lena Schönberg
geb. am 08.09.1976 in Tauberbischofsheim

Gutachter:
1. Prof. Dr. Ch. Kloft
2. PD. Dr. D. Lampe
3. Prof. Dr. P. Imming

Halle (Saale), 28.01.2008

urn:nbn:de:gbv:3-000013146
[http://nbn-resolving.de/urn/resolver.pl?urn=nbn%3Ade%3Agbv%3A3-000013146]

Abstract

Screening for a wide range of xenobiotics in biological samples is an important task for clinical toxicological and forensic laboratories. The aim of this thesis was to develop a fully automated on-line extraction HPLC-DAD screening method for the determination of compounds in urine with access to a commercially available UV spectra library with approximately 2600 reference spectra for compound identification. The method should allow simple analysis of those compounds that are difficult to detect in plasma due to short half-lives in blood (e.g. alkaloids) and therefore usually require work- and/or cost-intensive analytical methods. Furthermore, the method should be used for drugs of abuse (DOA) confirmation screening in urine. This objective was pursued by employing weak cation-exchange on-line material for the automated extraction of basic compounds from urine with subsequent isocratic separation on two coupled strong cation-exchange columns under acidic mobile phase conditions compatible with a commercially available UV spectra library. Efficient extraction and sufficient separation of the basic target analytes was achieved as was demonstrated by the successful analysis of spiked urine and authentic clinical samples. Parallel analysis with an existing automated urine screening system (Remedi^{TM}-HS) showed that the developed method can be used alternatively for the investigated field of application, but offers advantages such as the possibility to set-up further methods and the use of common laboratory material. The developed screening method was successfully validated following international guidelines and effectively applied to clinical toxicological routine use and DOA confirmation screening.

In a second step, a laboratory internal toxicological screening method for plasma analysis was established in the same system taking advantage of column switching valves. The effective set-up of the method was successfully confirmed by a performance test for accuracy control, within-laboratory system-to-system precision and analysis of clinical plasma samples.

Furthermore, an automated method for the determination of neutral, weakly acidic and weakly basic compounds in urine was developed to supplement the urine screening method for basic compounds. The method was based on polymer on-line extraction and separation on C8 material and showed to be useful for the analysis of toxicologically relevant benzodiazepines as was underlined by reliable analysis of clinical samples and by the obtained validation data. Weakly acidic barbiturates were only identified in high concentrations ($c \geq 1$ µg/mL).

Besides the commercial UV library, metabolite spectra obtained from clinical sample analysis were stored in a specific library for each method. The developed system provides a simple solution for broad screening of compounds in urine and plasma based on common laboratory equipment and material. All three established methods should be regarded as complementary methods in order to achieve maximum compound identification within the scope of systematic toxicological analysis and DOA confirmation screening.

Zusammenfassung

Die ungerichtete Suchanalyse nach vergiftungsrelevanten Wirkstoffen in biologischen Proben ist eine der zentralen Aufgaben in klinisch toxikologischen und forensischen Laboratorien. Ziel der vorliegenden Arbeit war es, eine vollautomatische On-line Extraktion-HPLC-DAD Methode für die Bestimmung von Substanzen im Urin zu entwickeln, die aufgrund ihrer geringen Halbwertszeit nur in einem sehr geringen Zeitfenster im Plasma bestimmt werden können (wie z.B. Alkaloide). Des Weiteren sollte die Methode im Rahmen der Suchtstoffanalytik eingesetzt werden. Durch den Einsatz eines schwachen Kationenaustauschermaterials für die automatische Extraktion und die isokratische Trennung auf zwei gekoppelten starken Kationenaustauschersäulen wurde eine effiziente Extraktion und Trennung der Fremdstoffe erzielt. Die analytische Trennung erfolgte unter sauren Bedingungen, um den Zugang zu einer kommerziell erhältlichen, pH-abhängigen Spektrenbibliothek mit derzeit ca. 2600 Einträgen für die Substanzidentifizierung zu ermöglichen. Die Anwendbarkeit der Methode wurde durch die Analyse von repräsentativen Testlösungen sowie anhand authentischer Proben erfolgreich überprüft. Durch Paralleluntersuchungen mit einem existierenden automatischen Urinuntersuchungsverfahren auf HPLC-Basis (Remedi^{TM}-HS) wurde gezeigt, dass die entwickelte Methode für die untersuchte Fragestellung alternativ eingesetzt werden kann und darüber hinaus Vorteile wie z. B. die Erstellung weiterer Methoden und die Verwendung üblicher Laborausstattung bietet. Die entwickelte Methode wurde erfolgreich entsprechend internationaler Richtlinien validiert und in die toxikologische Routine sowie die Suchtstoffanalytik eingeführt.

Zusätzlich wurde eine im Labor bereits etablierte toxikologische Suchmethode für die Fremdstoffbestimmung im Plasma durch den Einsatz von Säulenschaltventilen in das System integriert. Diese Methode wurde durch die Bestimmung der System-zu-System-Präzision mit einem Referenzsystem sowohl durch die Analyse von Kontrollmaterial als auch von klinischen Proben erfolgreich überprüft. Eine dritte Methode wurde für die Bestimmung von neutralen, schwach sauren und schwach basischen Substanzen im Urin entwickelt. Die Extraktion erfolgte auf einem Polymermaterial und die anschließende Trennung auf einer Umkehrphase. Diese Methode eignete sich insbesondere für die Bestimmung von Benzodiazepinen, wie anhand der erzielten Validierungsdaten und Probenuntersuchungen gezeigt werden konnte. Schwach saure Barbiturate werden nur in hohen Konzentrationen ($c \geq 1$ µg/mL) mit dieser Methode nachgewiesen.

Neben der kommerziellen Spektrenbibliothek wurden Spektren identifizierter Metabolite aus realen Proben in methodenspezifischen Bibliotheken gespeichert. Das entwickelte System ermöglicht die einfache Suchanalyse nach Fremdstoffen im Urin und Plasma, wobei die drei Methoden als komplementäre Methoden bei der systematisch toxikologischen Analyse und der Suchtstoffanalytik eingesetzt werden können, um eine maximale Anzahl von Fremdstoffen zu identifizieren.

Table of Contents

Abstract ii
Zusammenfassung iii
Table of Contents iv
List of Abbreviations ixx

1 Introduction 1

1.1 Systematic Toxicological Analysis 1

1.1.1 Sample Preparation 2
1.1.1.1 Liquid-Liquid Extraction 2
1.1.1.2 Solid Phase Extraction 3
1.1.2 Analytical Procedure: General Approach 5
1.1.2.1 Immunoassays 5
1.1.2.2 Chromatographic Methods 5
1.1.3 Compound Identification 7
1.1.4 Choice of Specimen 8

1.2 Aim 10

2 Materials and Methods 12

2.1 Materials 12

2.1.1 Buffer and Solutions 12
2.1.2 Consumables 16
2.1.3 Equipment 16
2.1.4 Samples and Sample Preparation 18

2.2 Screening Method for Basic Compounds in Urine 19

2.2.1 Circuit Diagram 19
2.2.2 Analytical Procedure 20
2.2.2.1 HPLC Separation and Detection 20
2.2.2.2 Method Development and Optimisation 21
2.2.3 On-line Extraction Procedure 22
2.2.3.1 Extraction Method 22
2.2.3.2 Extraction Method Development and Optimisation 23
2.2.4 Validation 25
2.2.4.1 Selectivity/Specificity 25
2.2.4.2 Stability 26

2.2.4.3 Extraction Recovery 26
2.2.4.4 Precision 27
2.2.4.5 Carry-Over Experiments 27
2.2.4.6 Linearity 27
2.2.4.7 Limit of Detection 27
2.2.4.8 Batch-to-Batch Reproducibility 28
2.2.4.9 Calibration for Semi-Quantitative Determination 28
2.2.5 Method Modifications for Critical Compounds 28
2.2.5.1 Benzoylecgonine Method 28
2.2.5.2 Method for Late Eluting Compounds 29
2.2.6 Analysis of Authentic Samples and Comparison with the Remedi^{TM}-HS 29
2.2.6.1 Sample Pre-Treatment Remedi^{TM}-HS 30
2.2.6.2 Extraction and Analytical Procedure Remedi^{TM}-HS 30

2.3 Screening Method for Plasma 30

2.3.1 Analytical Procedure 30
2.3.2 Validation 31

2.4 Screening Method for Neutral, Weakly Acidic and Weakly Basic Compounds in Urine 32

2.4.1 Analytical Procedure 32
2.4.2 Method Development and Optimisation 32
2.4.3 Validation 33
2.4.4 Analysis of Authentic Benzodiazepine Positive Samples 33

2.5 Strategies for Systematic Toxicological Analysis with the New Analytical Screening System 33

2.5.1 Samples Spiked with Reference Standards 33
2.5.2 Authentic Toxicological Samples 34

3 Results 35

3.1 Circuit Diagram 35

3.2 Screening Method for Basic Compounds in Urine 35

3.2.1 Analytical Separation 35
3.2.2 On-line Extraction 40
3.2.3 Final Analytical Procedure 43
3.2.4 Compound Identification 45
3.2.5 Validation 46

3.2.5.1 Selectivity/Specificity 46
3.2.5.2 Stability 47
3.2.5.3 Recovery 49
3.2.5.4 Precision 50
3.2.5.5 Carry-Over Experiments 50
3.2.5.6 Linearity 50
3.2.5.7 Limit of Detection 50
3.2.5.8 Batch-to-Batch Reproducibility 51
3.2.5.9 Calibration for Semi-Quantitative Determination 52
3.2.6 Method Modifications for Critical Compounds 55
3.2.6.1 Benzoylecgonine Method 55
3.2.6.2 Method for Late Eluting Compounds 56
3.2.7 Analysis of Authentic Urine Samples and Comparison with the Remedi^{TM}-HS 57
3.2.7.1 STA 57
3.2.7.2 DOA Confirmation Analysis 59
3.2.8 Routine Use 62
3.2.8.1 Case 1 62
3.2.8.2 Case 2 63
3.2.8.3 DOA Cases 64

3.3 Screening Method for Plasma 67

3.3.1 Validation 67
3.3.2 Compound Identification 68
3.3.3 Analysis of Clinical Plasma Samples 69

3.4 Screening Method for Neutral, Weakly Acidic and Weakly Basic Compounds in Urine 71

3.4.1 Analytical Separation 71
3.4.2 On-line Extraction 72
3.4.3 Glucuronide Hydrolysis 74
3.4.4 Final Procedure 75
3.4.5 Compound Identification 75
3.4.6 Validation 75
3.4.6.1 Selectivity/Specificity 76
3.4.6.2 Stability 76
3.4.6.3 Recovery 77
3.4.6.4 Precision 77
3.4.6.5 Carry-Over Experiments 78

3.4.6.6 Linearity 78
3.4.6.7 Limit of Detection 78
3.4.6.8 Batch-to-Batch Reproducibility 78
3.4.7 Analysis of Authentic Benzodiazepine Samples 79
3.4.8 Routine Use 79
3.5 Systematic Toxicological Analysis with the Analytical System 81
3.5.1 Samples Spiked with Reference Standards 81
3.5.2 Authentic Toxicological Samples 81
3.5.3 Quality Assessment Schemes 84
4 Discussion 86
4.1 Screening Method for Basic Compounds in Urine 86
4.1.1 Analytical Separation 86
4.1.2 On-line Extraction 88
4.1.3 Compound Identification 89
4.1.4 Validation 90
4.1.5 Method Modifications for Critical Compounds 91
4.1.6 Analysis of Authentic Samples and Comparison with the Remedi^{TM}-HS 92
4.2 Screening Method for Plasma 93
4.2.1 Validation 94
4.2.2 Analysis of Clinical Samples 94
4.3 Screening Method for Neutral, Weakly Acidic and Weakly Basic Compounds in Urine 95
4.3.1 Analytical Separation 96
4.3.2 On-line Extraction 96
4.3.3 Compound Identification 97
4.3.4 Validation 97
4.4 Routine Use 98
5 Conclusions and Future Perspectives 101
6 Bibliography 104
7 Appendix 112
7.1 Performance Control Samples 112
7.2 Remedi^{TM}-HS 115
7.3 HPLC Equipment Reference System 116

7.4 Libraries 117
7.5 Stability Investigations 125
7.6 Validation Data 127
7.7 Example Chromatograms 129
7.8 Evaluation of Benzodiazepine Positive Samples 134
7.9 Negative List 136
7.10 Comparison of Column Shelf-Lives: Developed System versus Remedi^{TM}-HS 137
Curriculum Vitae 138
Publications 139
Acknowledgements 142

List of Abbreviations

AC	Analytical column
ACN	Acetonitrile
APCI	Atmospheric pressure chemical ionisation
AU	Absorption unit
α	Separation factor (resolution)
6-AM	6-Monoacetylmorphine
b	Slope
BBGes	Berliner Betrieb für Zentrale Gesundheitliche Aufgaben
BEC	Benzoylecgonine
BV	Bed volumes
C	Concentration
C_{calc}	Calculated concentration
CEDIA	Cloned enzyme donor immunoassay
CV	Coefficient of variation
DAD	Diode array detection
DOA	Drugs of abuse
EC	Extraction column
EDDP	2-Ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidene
EI	Electron impact ionisation
EIA	Enzyme immunoassay
ELISA	Enzyme linked immunosorbent assay
ESI	Electro spray ionisation
FID	Flame ionisation detector
FDA	Food and Drug Administration
FLD	Fluorescence detector
GC	Gas chromatography
Glc	Glucuronide
GTFCh	Gesellschaft für Toxikologische und Forensische Chemie
HPLC	High performance liquid chromatography
I	Ionic strength
I.S.	Internal standard
κ	Capacity factor
λ	Wavelength
LC	Liquid chromatography
LEC	Late eluting compounds

LLE	Liquid/liquid-extraction
LLOD	Lower limit of detection
LOD	Limit of detection
m	Mass
MDA	3,4-Methylene-dioxy-amphetamine
MDMA	3,4-Methylene-dioxy-methamphetamine
MPPH	5-(p-Methylphenyl)-5-phenylhydantoin
MS	Mass spectrometry
PA	Peak area
PC	Pre-(guard)cartridge
PCS	Performance control sample
PCS 1	Reference standards diluted with 0.01 M phosphate buffer pH 6
PCS 2	Reference standards diluted with a mixture of urine and 0.01 M phosphate buffer pH 6 (2/1, v/v)
PCS-BARB 1	Barbiturates diluted with 0.01 M phosphate buffer pH 6
PCS-BARB 2	Barbiturates diluted with a mixture of urine and 0.01 M phosphate buffer pH 6 (2/1, v/v)
PCS-BDP 1	Benzodiazepines diluted with 0.01 M phosphate buffer pH 6
PCS-BDP 2	Benzodiazepines diluted with a mixture of urine and 0.01 M phosphate buffer pH 6 (2/1, v/v)
PCS-PA	PCS plasma analysis, reference standards diluted with mobile phase 2
Peek	Polyetherketone
pH	The negative decade logarithm of the H_3O^+ concentration
pK_a	The negative decade logarithm of the acid dissociation constant K in the law of mass action
Q	Quotient
R	Recovery
R^2	Coefficient of determination
RAM	Restricted access material
RE	Relative error
RIA	Radio immunoassay
RP	Reversed phase
RT	Retention time
RRT	Relative retention time
RSD	Relative standard deviation (equivalent to coefficient of variation)
SD	Standard deviation
SI	Similarity index

SCX	Strong cation-exchange
S/N	Signal-to-noise ratio
SPE	Solid phase extraction
SPSS™	Statistical Package for the social sciences (Statistic software)
STA	Systematic toxicological analysis
SU	Spiked urine
TCA	Tricyclic antidepressants
TDM	Therapeutic drug monitoring
t_0	Time of an unretained peak
TOF	Time of flight
ULOD	Upper limit of detection
UV	Ultraviolet
V	Valve
WCX	Weak cation-exchange
$\overline{x}$	Arithmetic mean
z	Charge

1 Introduction

1.1 Systematic Toxicological Analysis

Acute intoxication represents an emergency case. Fast and reliable diagnosis is very important for the prognosis and further treatment. In toxicological laboratories systematic toxicological analysis (STA, general unknown screening), a rational chemical-analytical approach [1], is performed to identify the toxic agents. Today, this strategy usually involves immunoassays and chromatographic methods ideally coupled to specific detectors such as mass spectrometers (MS) or ultraviolet-diode array detectors (UV-DAD) following acidic and basic extraction of biological samples [2].

Altogether 2210 acute intoxications were investigated within the scope of STA at the Institute of Toxicology-Clinical Toxicology and Poison Control Centre Berlin (Berliner Betrieb für Zentrale Gesundheitliche Aufgaben, BBGes, Berlin) in 2005 [3]. Most frequently abused substances (accidentally or in suicide attempts) were ethanol, tranquilizers, antidepressants or anticonvulsants [3]. In approximately 10% of the cases, illegal drugs were found to be the intoxication cause. To a smaller extent but not less serious, intoxications with alkaloids occurred [3].

Although STA procedures follow the aim to allow the simultaneous detection of as many potentially xenobiotics as possible in bio samples [4], not all compounds can be analysed with one analytical method and some analytes require specialised analytical methods. This can be due to very low concentrations or to their analytical properties. In Fig. 1-1 an example strategy for STA is shown, which demonstrates the combined use of different analytical methods, sample extraction at different pH levels and investigation of different sample matrices (plasma, urine) in order to detect as many toxic agents as possible.

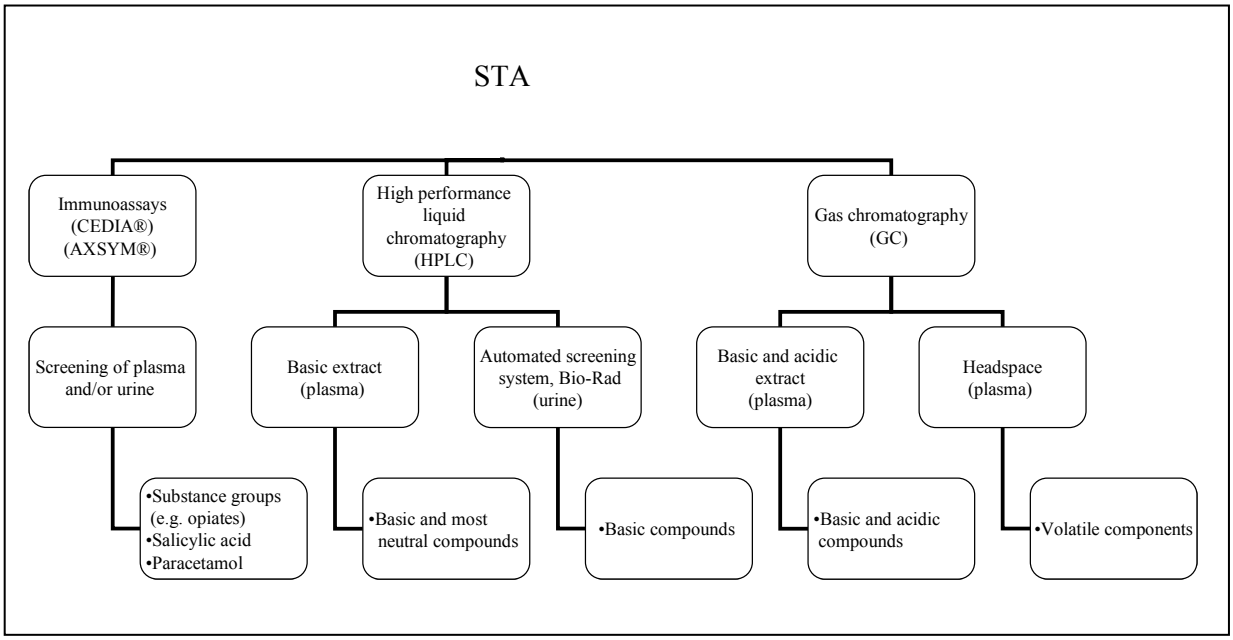


Fig. 1-1 *Example strategy for systematic toxicological analysis (STA)*

A number of established STA methods have been described and reviewed in the literature [5-23] and is available to toxicologists. General information about screening methods in toxicology is e.g. reviewed in literature 5-8, 13, 17, databases [9, 11, 22] and screening methods for specific compound groups [10, 18-21, 23] or broad spectrum screening [12-14, 16] in either serum or urine are described. In each instance, according to de Zeeuw [24], STA can be divided in three steps: sample preparation, analytical procedure and compound identification, which will be described in the following sections. Main emphasis will be put on the techniques used in this thesis, such as solid phase extraction (SPE), high performance liquid chromatography (HPLC) and diode array detection (DAD).

1.1.1 Sample Preparation

The analysis of biological fluids places high demands on sample clean-up prior to analysis [25]. Sample preparation is carried out in order to isolate the analytes from interferences, to concentrate them into a sufficient concentration and to recover them into an appropriate form for analysis [26]. A traditional technique for this purpose is liquid-liquid extraction (LLE). A study showed that the time spent in sample preparation is approximately up to ten times higher than in the actual measurement [27]. Given the time factor for rapid analysis of a broad spectrum of compounds in intoxication cases [28] and the aim to lower time- and cost-intensive work steps, interest in automation is increasing [29]. SPE is an effective alternative to LLE for sample pre-treatment and clean-up and offers advantages such as high efficiency, selectivity, recovery and not at least automation options [30].

1.1.1.1 Liquid-Liquid Extraction

In liquid-liquid extraction (LLE) usually a distribution of the solute occurs between two immiscible liquids, whereas the bio sample presents the aqueous and the extraction solvent the organic phase. Extraction has to be carried out at a pH level where the target analyte is uncharged to transfer it from the aqueous to the organic phase. Most frequently used extraction solvents are ethyl acetate, chlorbutane, chloroform, toluene, dichloromethane, butyl acetate and diethyl ether [5]. Following extraction, the organic extract is evaporated to dryness and the residue is then redissolved in an appropriate phase for analytical separation [5].

Although LLE proved to be suitable for sample extraction in many cases it has major drawbacks such as matrix interferences, emulsion formation and use of large volumes of hazardous solvents [7]. Moreover, LLE is difficult to automate and therefore a rather work-intensive and time consuming method.

1.1.1.2 Solid Phase Extraction

SPE is defined as follows: "A solid phase extraction consists of bringing a liquid or gaseous test portion/solution in contact with a solid phase, or (ad)sorbent, whereby the analyte is selectively adsorbed on the surface of the solid phase. The solid phase is then separated from the solution and other solvents (liquids or gases) are added. The first such solvent is usually a wash to remove possible adsorbed matrix components; eventually an eluting solvent is brought into contact with the sorbent to selectively desorb the analyte" [31].

SPE can be used either as off-line extraction technique where the eluate is collected and then prepared for further analysis or as on-line extraction technique where the target analytes are directly eluted from the extraction material into the analytical columns with the mobile phase used for the analytical separation. In each instance, SPE is based on four work steps: conditioning, sample loading (adsorption), washing and elution. In the first step, the columns are equilibrated in order to allow optimum retention of the target analytes in the following 2nd step (sample loading). In the 3rd step (wash) interfering matrix components are selectively removed and in the 4th step (elution) the target analytes are eluted from the extraction column and are returned to a liquid phase that is suitable for analytical measurement.

Extraction materials used for SPE are similar to those employed for liquid chromatography except for the particle size [31]. The common particle size for SPE ranges from 30-60 µm [32]. The wide range of SPE materials is divided into three groups according to the interaction mechanisms: 1. reversed phase or non polar phase material, 2. normal phase or polar phase material, 3. ion-exchange material [29, 33]. Depending on the nature of the extraction sorbent, the target analytes have to be uncharged when the sorbent is non polar (reversed phase, polymer material) and charged when a polar or ion-exchange sorbent is used to guarantee retention. The interaction mechanisms are summarised in Table 1-1.

Table 1-1 *Interaction mechanisms in SPE [27]*

Interaction	Interaction mechanisms	Bond energy (kJ/mol)
Non polar (reversed phase, polymer)	Van der Waals forces	4-20
Polar (normal phase, silica phase)	Hydrogen bond	20-40
	Dipolar interaction	10-40
	Induced dipolar interaction	8-25
Ion-exchange (anion-/cation-exchange)	Ion-exchange interaction	200-1100
Covalent bond	Covalent bond	410-3400

For on-line extraction, polymer, weak ion-exchange, strong ion-exchange and mixed mode columns are used. Polymer extraction material is usually based on polystyrene-divinylbenzene;

functional groups of weak ion-exchangers are either carboxylic acids (weak cation-exchanger) or amines (weak anion-exchanger) and for strong ion-exchangers sulfonic acids (strong cation-exchanger) or modified amines (strong anion-exchanger), respectively. Table 1-2 gives a survey of the SPE working steps in accordance to the used extraction material. In literature, the basic principles of SPE for the analysis of biological samples have been described [38-41] and the use for STA has been reviewed [7]. Screening methods using off-line SPE [16, 19-21] or on-line SPE [12-15, 34-37] have been reported and method development and automating SPE is e.g. summarised in [42, 43].

Table 1-2 *SPE working steps in accordance to the used extraction material*

Work step	Non-polar (reversed phase, polymer)	Polar (normal phase, silica phase)	Ion-exchange (anion-/cation-exchange)
Conditioning	Solvation with polar organic solvents	Solvation with polar organic solvents	Solvation with polar organic solvents
	Equilibration with a solvent similar to the sample/matrix	Equilibration with a solvent similar to the sample/matrix	Equilibration with a solvent similar to the sample/matrix
	Ionic strength (I) < 30 mM	I < 30 mM	I < 30 mM
	Flow rate 1-3 mL/min	Flow rate 2-4 mL/min	Flow rate 2-4 mL/min
	≥ 2 Bed volumes (BV)	≥ 2 BV	≥ 2 BV
	pH = 1-14, analyte uncharged	$pK_{a\ (analyte)}$ ± 2 pH units	$pK_{a\ (analyte)}$ ± 2 pH units
Loading	Flow rate 1-3 mL/min	Flow rate ≤ 2 mL/min	Flow rate ≤ 2 mL/min
	Sample dilution with water or buffer, fraction of water-miscible organic solvents < 5%	Sample dilution with low ionic strength buffers	Sample dilution with low ionic strength buffers
	pH = 1-14, analyte uncharged	$pK_{a\ (analyte)}$ ± 2 pH units, analyte charged oppositely to extraction phase	$pK_{a\ (analyte)}$ ± 2 pH units, analyte charged oppositely to extraction phase
Washing	Aqueous solvents containing small fraction of organic solvents (5-50%)	Aqueous or organic solvents, solvents with a medium I (50-100 mM) increase the elution of interfering matrix components	Aqueous or organic solvents, solvents with a medium I (50-100 mM) increase the elution of interfering matrix components
Elution	Organic solvents containing water, acids or bases	Mixture of acidic or basic buffer solutions (I > 200 mM) plus organic solvents	Polar organic solvents plus acids or bases
		$pK_{a\ (analyte)}$ ± 2 pH units	$pK_{a\ (analyte)}$ ± 2 pH units

1.1.2 Analytical Procedure: General Approach

The success of STA, following the aim to identify as many compounds in biological matrices as possible, largely depends on the quality of the used method [1]. As mentioned before, not all possible xenobiotics can be analysed and identified in a single analytical run. Therefore, STA usually involves more than one analytical method as is shown in Fig. 1-1 or e.g. described in [44]. The main used methods for STA can be divided in two groups: immunological and chromatographic techniques [45]. In general, the knowledge of the sensitivity of a used method and sufficient selectivity, the ability of an analytical method to differentiate the analyte in the presence of other components in the sample [96], plays an important role for STA to avoid false negative or false positive results.

1.1.2.1 Immunoassays

Immunoassays are used to monitor single drugs or substance groups such as amphetamines, benzodiazepines, barbiturates or opiates and therefore are an important tool for STA and for high-throughput screening for drugs of abuse (DOA).

The principle of immuno-chemical methods is an antigen-antibody-reaction. In most cases competitive immunoassays are used, where the analyte of the sample competes with a marked antigen reagent for antibody binding. Either the amount of free or bound antigen can be determined in the sample [46]. Depending on the used antigen marker, enzyme immunoassays (EIA, enzyme linked immuno sorbent assay (ELISA), cloned enzyme donor immunoassay (CEDIA)), fluorescence polarisation immunoassays (FPIA) and radio immunoassays (RIA), are available [45].

For urine control analysis of DOA immunoassays are of high importance as they allow fast and automated preliminary screening to distinguish between positive and negative samples. In order to avoid false positive results, a cut-off value has to be exceeded. For legal validity, the positive result must be confirmed by a chromatographic method [2]. The confirmation method has to be at least as sensitive as the immunological screening and must offer high sensitivity and selectivity in the result.

1.1.2.2 Chromatographic Methods

Chromatographic techniques are based on the distribution of the analyte between a mobile and a stationary phase. The analytes are separated due to their chemical properties resulting in different distribution coefficients between the two phases and therefore different movement rates [47].

Most frequently used chromatographic methods today are gas chromatography (GC) and HPLC coupled to MS and UV (DAD) detectors. Other hyphenated techniques are GC coupled to nitrogen

phosphorus detectors (NPD), flame ionisation detectors (FID) or HPLC coupled to fluorescence detectors (FL).

Gas chromatography-mass spectrometry (GC-MS) is most often based on fused silica capillaries as stationary phase, for the mobile phase an inert gas (nitrogen, hydrogen or helium) is used [4, 48]. Prerequisite for applying GC analysis are analytes that are volatile or that can be derivatised into volatile compounds. Thus, this technique requires necessarily laborious and time-consuming sample preparation and is unsuitable for the direct analysis of polar compounds [8, 23, 51]. On the other hand GC offers high separation power and high selectivity when coupled to MS detectors. Therefore GC-MS is accepted as "gold standard" for DOA confirmation screening [4]. Beside this application GC methods are used for toxicological screening within the scope of STA [47-49].

In many toxicological laboratories HPLC is adopted for STA because a number of drugs, chemical poisons and their metabolites are either too polar, non-volatile or thermally instable to be analysed by GC within an acceptable time required for sample preparation [51]. HPLC is a versatile technique that allows the analysis of compounds over a wide range of polarity, molecular mass and thermal stability. In addition, the mobile phase is compatible with aqueous biological samples that simplifies sample preparation and offers automation options. Reversed phase columns are the most frequently used HPLC materials for STA [8, 23]. The potential of HPLC-UV (DAD) screening methods for toxicology has frequently been described [1, 8, 16, 18, 23, 52, 53].

Remedi^{TM}-HS

An automated HPLC-UV screening system developed in the 1980s is represented by the Remedi^{TM}-HS (Bio-Rad, Munich, Germany), a drug-profiling system based on multicolumn extraction of urine samples and high performance liquid chromatography with UV detection. It is coupled to a computer and allows automated extraction, separation and identification. Approximately 900 spectra are established in a stored spectra library for compound identification. The Remedi^{TM}-HS is used for screening of basic drugs such as alkaloids and other DOA in urine within confirmation screening or as additional STA method if urine is available in intoxication cases. Its major draw backs are its lack of extracting neutral and acidic compounds like barbiturates and benzodiazepines [10, 54], the relatively high costs for analysis and the work intensive maintenance due to relatively low column life time and dated computer hard- and software. In addition, all solvents and reagents are company-dependent and the method cannot be modified by the operator. The development and experience with the Remedi^{TM}-HS [55-63] as well as its use for plasma analysis [64] and the coupling to a tandem mass spectrometer [65] have been described.

For the analysis of benzodiazepines Bio-Rad provides a second automated tool, or more precise a second reagent kit and extra columns [66-68]. A separate spectra library of approximately 33 benzodiazepine spectra including 2 internal standards is available.

At the end of 2008 the Remedi^{TM}-HS will be taken out of service and therefore alternative and more sophisticated analytical on-line methods will be required.

Liquid chromatography-mass spectrometry (LC-MS (-MS)) combines the advantages of HPLC with the selectivity and sensitivity of mass detectors. Therefore this technique gains more and more popularity and a mass spectra library for toxicological screening is in preparation [69]. Prerequisite for the use of LC-MS is a volatile mobile phase. The suitability of LC-MS in clinical toxicology has been discussed [4] and reviewed [17, 70, 71]. SPE carried out on reversed phase, polymer or cation-exchange material and subsequent LC-MS screening has been described [34, 68-71]. However, until now, LC-MS had only limited deployment for STA screening purposes due to a lack of yet available libraries [8] and high costs. Therefore, within the scope of this thesis a HPLC-DAD system was preferred for sample analysis.

1.1.3 Compound Identification

Besides the analytical separation, the strength of every method depends on the capacity for compound identification [18, 76].

With HPLC-UV, the identification is based on the comparison of the UV absorption spectrum of a compound with the spectrum of a spectra library. Interactions of UV radiation with a conjugated system of π-electrons and unbound electron pairs ($\pi \rightarrow \pi^*$, $n \rightarrow \pi^*$ transitions) are responsible for the absorption spectrum in the UV wavelength range [77]. The part of a molecule that absorbs UV radiation is called chromophore. As the involvement of σ-electrons is limited to compounds with sulphur, iodine or bromide atoms, the absorption spectrum mainly originates from the conjugated system of π-electrons and unbound electrons (chromophore) and not from the complete molecule [78]. The fact that metabolites and their parent compounds as well as some classes of drugs may have similar UV spectra due to the same chromophore is advantageous for toxicological analysis because information about the parent compound or yet not investigated substances related to certain classes of drugs can be obtained [23, 78, 79]. In Fig. 1-2 an example is given by the spectrum of benzoylecgonine and its parent drug cocaine. However, this also means, that a second parameter for secure compound identification, such as retention time (RT) is necessary. As it is unusual that absolute RTs remain constant over a period of time due to gradual changes of the stationary or mobile phase, relative retention time or retention index can be used to scale the RT of a substance to the RT of a reference substance. Moreover, matrix substances that unavoidably are coextracted should be recognised so that they may be included in the database in order to evaluate the influence of the biological matrix [76].

Since the introduction of multiwavelength detectors (DAD) in the early 1980s, which record the absorbance of compounds over a range of wavelengths and in combination of retention parameters,

HPLC-DAD represents a reliable analytical method [79]. For this purpose different UV spectrum databases were established [11, 16, 18, 23, 81].

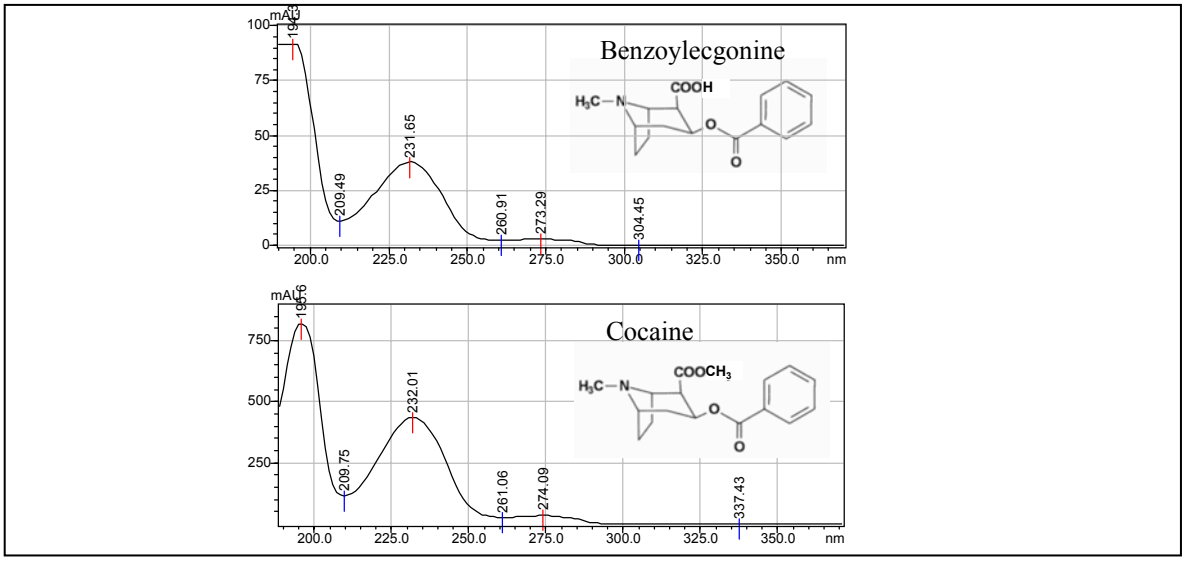


Fig. 1-2 *Spectra of benzoylecgonine and its parent drug cocaine*

In mass spectrometry molecules are ionised into negative or positive ions in an ion source. Most common techniques in practice are electron impact ionisation (EI) as well as electrospray ionisation (ESI) and atmospheric pressure chemical ionisation (APCI). The ions are then separated in a magnetic or electric field according to their mass/charge relation (m/z) and a characteristic mass spectrum with the masses and relative intensities is registered at the detector. The height and/or area of a signal is proportional to the amount of the molecule of interest [76]. In time-of-flight (TOF) mass spectrometers the pace of the ions in the analyser is additionally taken into account, taking advantage of the fact that lighter ions reach the detector faster than heavier ions. Another technique to increase the sensitivity of mass detection is the use of tandem mass spectrometry (MS-MS), where besides the parent ion characteristic fragments are detectable. Compared to DAD-systems, MS detectors represent a more sensitive and selective detection method because of the high substance specificity and the low amount of substance needed.

Identification power of a method could be increased by combination of UV and mass selective detectors. But still, any analytical method will only be able to identify an unknown substance whose reference data is included in the database [77].

1.1.4 Choice of Specimen

The choice of specimen is mainly determined by the clinical situation. Usually blood/plasma/serum and/or urine are investigated. Whereas the sample collection in case of urine is not invasive and therefore easier to obtain, in emergency cases blood/plasma/serum is most often accessible.

Blood/plasma/serum analysis allows the determination of the actual active xenobiotic concentration and calculation of pharmaco- and toxicokinetic data which are helpful for assessment of clinical diagnosis and further treatment. Thus, this matrix is very important for clinical toxicology.

Urine analysis on the other hand offers the advantage of a larger time window of detection compared to blood due to higher cumulative amounts of renally excreted compounds. Therefore urine allows the detection of compounds which have a short half-life in blood, such as alkaloids, and is the matrix of choice for DOA screening. Quantitation in urine requires a reliable reference parameter such as creatinine because the water content of urine may vary and 24 h urine is practically seldom available.

In both cases, dealing with biological samples, matrix specific compounds have to be removed prior to analysis. In Table 1-3 the main compounds found in plasma and in Table 1-4 the main compounds found in twenty-four-hour urine that may interfere with the analysis procedure are summarised.

Table 1-3 *Main compounds in plasma [82]*

Substance	**Concentration (g/L)**
Glucose	0.600-1.000
Lactic acid	0.090-0.160
Pyruvic acid	0.005-0.017
Compounds with urinary excretion	
Urea	0.200-0.600
Uric acid ♂ / ♀	0.034-0.070 / 0.024-0.060
Creatinine	0.004-0.012
Creatine	0.002-0.005
Lipids (total)	**3.050-8.800**
Cholesterol	1.000-2.500
Phospholipids	1.250-2.300
Triglycerides	0.500-2.000
Free fatty acids	0.080-0.120
Bilirubin	0.002-0.010
Ions	
Chloride	0.0362
Sodium	0.0327
Potassium	0.0020
Sulphate	0.0005
Phosphate (calculated as P)	0.0010
Bicarbonate	0.0165
Calcium	0.0010
Magnesia	0.0002
Proteins	0.7200

Table 1-4 *Main compounds in 24 h urine [83, 84]*

Substance	Excreted amount (g)
Urea	~ 20
Creatinine	1.2-1.8
Amino acids	~ 0.8
Uric acid	~ 0.5
Protein (total)	< 0.2
Hippuric acid	0.1-1.0
D-Glucose	~ 0.07
Oxalic acid	< 0.03
Ions	
Chloride	6.00-9.00
Sodium	3.00-6.00
Potassium	1.00-5.00
Sulphate	1.80-3.50
Phosphate (calculated as P)	0.70-1.50
Ammonia nitrogen	0.40-1.00
Calcium	0.05-0.40
Magnesia	0.05-0.15

1.2 Aim

STA based on GC, HPLC and immunological methods is usually performed in plasma/serum and urine. However, some compounds such as atropine (D,L-hyoscyamine), psilocin and scopolamine with short half-lives in blood, are difficult to detect with common STA screening methods and require specialised analytical methods. The aim of this work was to develop a chromatographic screening method for toxicological analysis in urine which focused on these basic compounds, taking advantage of the larger time window of detection in urine compared to blood. Furthermore, as urine presents the matrix of choice for drugs of abuse analysis, it should be proved if the method is suitable for this field of application.

A HPLC-DAD system was chosen to access a commercially available spectra library with approximately 2600 spectra [22] and to allow the identification of toxicologically relevant metabolites by comparing their spectra to those of the parent compound. In addition, chromatographic data of all investigated compounds should be stored in an additional library for spectra and relative retention time (RRT) comparison (method-specific library). To hold sample pre-treatment and costs to a minimum, the intention was to characterise the developed method by fully automated on-line extraction and common HPLC equipment. With the developed method specialised methods such as the Remedi^{TM}-HS (analysis of alkaloids) and GC-MS (several cases of DOA confirmation screening) should be replaced. The deployment of column switching valves should offer the opportunity to integrate further analytical methods, such as a routine HPLC-DAD screening method for the analysis of compounds in plasma (see Fig. 1-1) and a screening method

for the determination of neutral, weakly acidic and weakly basic compounds in urine in order to complete the analytical system.

The research objectives were addressing the following tasks:

I. Development of a screening method for the determination of basic compounds in urine
   - Development and optimisation of the analytical procedure, such as selection of suitable separation material, an appropriate mobile phase and investigation of flow rates
   - Development and optimisation of the on-line extraction procedure, such as selection of a suitable extraction material, investigation of loading and wash solvents, optimisation of flow rates and volumes
   - Selection of an internal standard (I.S.) for RRT calculation
   - Set-up of a method-specific spectra library for compound identification
   - Validation of the developed method
   - Feasibility of the method with the analysis of real toxicological specimens
   - Comparison of the method to an existing urine screening system (Remedi^{TM}-HS)
   - Suitability to toxicological routine use

II. Establishment of a known toxicological screening method for the determination of xenobiotics in plasma
   - Set-up of the method in the analytical system
   - Control check of the set-up with an accuracy control test and parallel analysis with a reference system in routine use
   - Analysis of authentic plasma samples

III. Development of a screening method for the determination of neutral, weakly acidic and weakly basic compounds in urine
   - Development and optimisation of the analytical procedure, such as selection of suitable separation material, an appropriate mobile phase and investigation of flow rates
   - Development and optimisation of the on-line extraction procedure, such as selection of a suitable extraction material, investigation of loading and wash solvents, optimisation of flow rates and volumes
   - Selection of an I.S. for RRT calculation
   - Set-up of a method-specific spectra library for compound identification
   - Validation of the developed method
   - Feasibility of the method with the analysis of real toxicological specimens
   - Suitability to toxicological routine use

2 Materials and Methods

2.1 Materials

Acetonitrile (ultra gradient, HPLC grade)	J. T. Baker, Deventer, Netherlands
Ammonium carbonate (analytical-reagent grade)	Fluka, Neu-Ulm, Germany
Ammonium hydroxide (25% (v/v), picograde)	VWR, Darmstadt, Germany
Dichloromethane (analytical-reagent grade)	VWR, Darmstadt, Germany
N, N-Dimethyloctylamine (purum ≥ 96%)	Fluka, Neu-Ulm, Germany
Disodium hydrogenphosphate dehydrate (picograde)	VWR, Darmstadt, Germany
Acetic acid (glacial)	VWR, Darmstadt, Germany
Glucuronidase reagent from E.coli, 140 units/mg	Roche, Mannheim, Germany
Hexane (picograde)	Promochem, Wesel, Germany
Hydrochloric acid (26% (v/v), analytical-reagent grade)	VWR, Darmstadt, Germany
Methanol (analytical-reagent grade)	VWR, Darmstadt, Germany
5-(p-Methylphenyl)-5-phenylhydantoin (MPPH)	VWR, Darmstadt, Germany
Neostigmine bromide	Promochem, Wesel, Germany
Phosphoric acid (85% (v/v), analytical-reagent grade)	VWR, Darmstadt, Germany
Potassium dihydrogenphosphate (picograde)	VWR, Darmstadt, Germany
Potassium hydroxide (picograde)	VWR, Darmstadt, Germany
Potassium tetraborate tetrahydrate (analytical-reagent grade)	Fluka, Neu-Ulm, Germany
Reference standards and solutions	Promochem, Wesel, Germany
Tetramethylammonium chloride (picograde)	Fluka, Neu-Ulm, Germany
Tris-(hydroxymethylene)-amino-methane (tris-buffer)	VWR, Darmstadt, Germany
Water (osmosis purification)	MembraPure, Bodenheim, Germany

2.1.1 Buffer and Solutions

Borate buffer pH 8

Borate solution A:

Potassium tetraborate tetrahydrate	19.1 g
Water	ad 1000.0 mL

Borate solution A	55.9 mL
0.1 N HCl	44.1 mL

0.05 M Phosphate buffer pH 2.3

Potassium dihydrogenphosphate	6.7 g
Water	ad 1000.0 mL

(pH adjustment with phosphoric acid to pH 2.3)

0.1 M Phosphate buffer pH 6

Potassium dihydrogenphosphate	13.6 g
Water	ad 1000.0 mL

(pH adjustment with 1.0 M potassium hydroxide to pH 6)

0.01 M Phosphate buffer pH 6 (loading buffer)

0.1 M Phosphate buffer pH 6	100.0 mL
Water	ad 1000.0 mL

Phosphate buffer pH 7

Potassium dihydrogenphosphate	6.8 g
Disodium hydrogenphosphate	11.2 g
Water	ad 1000.0 mL

(pH adjustment with 1.0 M potassium hydroxide to pH 7)

0.2 M Tris-buffer pH 9.1

Tris-(hydroxymethylene)-aminomethane	12.1 g
Water	ad 500.0 mL

(pH adjustment with 0.1 M hydrochloric acid to pH 9.1)

Ammonium carbonate buffer pH 10

Ammonium carbonate	4.8 g
Water	ad 1000.0 mL

(pH adjustment with 25% ammonium hydroxide to pH 10)

Mobile phase 1 (screening method for basic compounds)

0.05 M Phosphate buffer pH 2.3	685.0 mL
Acetonitrile/water (90/10, v/v)	315.0 mL

Mobile phase 2 (screening method for plasma)

0.05 M Phosphate buffer pH 2.3	640.0 mL
Acetonitrile/water (90/10, v/v)	360.0 mL

Mobile phase 1 with amine modifiers

N,N-Dimethyloctylamine	30.2 mg
Tetramethylammonium chloride	30.5 mg
Mobile phase 1	ad 100.0 mL

1.0 M Acetic acid

Acetic acid (glacial)	5.8 mL
Water	ad 100.0 mL

0.01 M Acetic acid

Acetic acid (glacial)	58.0 µL
Water	ad 100.0 mL

0.1 M Hydrochloric acid

Hydrochloric acid (26% (v/v))	12.5 mL
Water	ad 1000.0 mL

1.0 M Potassium hydroxide

Potassium hydroxide	5.6 g
Water	ad 100.0 mL

Following preparation, all buffers and solutions were filtrated (membrane filter, cellulose acetate, pore diameter 0.22 µm).

Stock solutions

Stock solutions were prepared by diluting commercial methanol reference standard solutions of the compounds to 1 µg/mL and 0.1 µg/mL with mobile phase 1 (direct injection) or 0.01 M phosphate buffer pH 6 (on-line extraction).

Performance control sample screening method for basic compounds in urine

A performance control sample (PCS) stock solution was prepared by diluting commercial methanol reference standard solutions of codeine, the methadone metabolite 2-ethyliden-1,5-dimethyl-3,3-diphenylpyrrolidene (EDDP), 3,4-methylene-dioxy-amphetamine (MDA), morphine, scopolamine (each 1 mg/mL) to 1 µg/mL and the I.S. neostigmine bromide to 5 µg/mL with 0.01 M phosphate buffer pH 6 (PCS 1) and with a mixture of urine and 0.01 M phosphate buffer pH 6 (2/1, v/v) (PCS 2), respectively. The PCS 1 and 2 were stored frozen (-18 ± 3 °C) in aliquots of 1.5 mL.

Performance control sample urine analysis benzodiazepines

A performance control sample stock solution for benzodiazepines (PCS-BDP) was prepared by diluting commercial methanol reference standard solutions of bromazepam, demoxepam, nordiazepam, oxazepam, temazepam (each 1.0 mg/mL) to 1.0 µg/mL and the I.S. 5-(p-methylphenyl)-5-phenylhydantoin (MPPH) to 5 µg/mL with 0.01 M phosphate buffer pH 6 (PCS-BDP 1) and with a mixture of urine and 0.01 M phosphate buffer pH 6 (2/1, v/v) (PCS-BDP 2), respectively. The PCS-BDP 1 and PCS-BDP 2 were stored frozen (-18 ± 3 °C) in aliquots of 1.5 mL.

Performance control sample urine analysis barbiturates

A performance control sample stock solution for barbiturates was prepared by diluting commercial methanol reference standard solutions of cyclobarbital, crotylbarbital, methohexital, phenobarbital, pentobarbital and the I.S. MPPH (each 10 mg/mL) to 10.0 µg/mL with 0.01 M phosphate buffer pH 6 (PCS-BARB 1) and with a mixture of urine and 0.01 M phosphate buffer pH 6 (2/1, v/v) (PCS-BARB 2), respectively. The PCS-BARB 1 and PCS-BARB 2 were stored frozen (-18 ± 3 °C) in aliquots of 1.5 mL.

Performance control sample toxicological screening method for plasma

A performance control sample stock solution for plasma analysis (PCS-PA) was prepared by diluting commercial methanol reference standard solutions of dipyridamol, MPPH and diazepam to 0.1 mg/mL with mobile phase 2. The PCS-PA was stored at 5-8 °C.

The chemical structure and pK_a values of all PCS are shown in Table 7-1 to 7-4, chapter 7.1 in the appendix.

Internal standard solution screening method for basic compounds

The I.S. solution was prepared by diluting neostigmine bromide standard solution (1 mg/mL) to the concentration of 15 µg/mL with 0.01 M phosphate buffer pH 6. The I.S. solution was stored at 5-8 °C.

Internal standard solution urine analysis benzodiazepines and barbiturates

The I.S. solution was prepared by diluting MPPH standard solution (0.5 mg/mL) to the concentration of 5.0 µg/mL with 0.01 M phosphate buffer pH 6. The I.S. solution was stored at 5-8 °C.

Internal standard solution toxicological screening method for plasma

The I.S. solution was prepared by dissolving 50.0 mg MPPH in a 100.0 mL volume of methanol. The I.S. solution was stored at 5-8 °C.

Extraction solution plasma analysis

The extraction solution for LLE of plasma samples was prepared by diluting 0.5 mL MPPH I.S. solution (plasma analysis) to the concentration of 2.5 µg/mL with dichloromethane. The I.S. solution was stored at 5-8 °C.

2.1.2 Consumables

Eppendorf cups, 1.5 mL and 2.0 mL	Eppendorf, Hamburg, Germany
Membrane filter (0.22 µm, cellulose acetate)	Millipore, Eschborn, Germany
Monovettes	Sarstedt, Nümbrecht, Germany
Peek capillary 1/16`` AD x 0.13 mm	Chromatographie Service, Langerwehe, Germany
Peek capillary 1/16`` AD x 0.25 mm	Chromatographie Service, Langerwehe, Germany
Pipette tips	Eppendorf, Hamburg, Germany
Screw-Top vial, 2 mL XL	Laubscher LABS, Rixheim, France
Screw cap, XL	Laubscher LABS, Rixheim, France
Vial inlays	Kunz & Müller, Berlin, Germany
Urine sampling cups	Lamprecht, Berlin, Germany

2.1.3 Equipment

Centrifuge 5415D	Eppendorf, Hamburg, Germany
IKA® Vortex Genius 3	IKA, Staufen, Germany
Turbo Vap® LV Evaporator	Zymark, Idstein, Germany
Syva Micro Trak® EIA Dry-bath	Dade Behring, Schwalbach, Germany
Eppendorf MixMate	Eppendorf, Hamburg, Germany
Pipettes	Eppendorf, Hamburg, Germany
Dispenser piccolo, 500 µL	Vitlab, Grossostheim, Germany
HPLC-DAD System	Shimadzu, Duisburg, Germany
Ternary pump system (LC-10 ADVP)	
Gradient unit (FCV-10 ALVP)	
Solvent degasser (DGU-14 A)	
Auto sampler (SIL-10 AF)	
Sample loop (2 mL)	
Option box VP	

High pressure valves (FCV-12 AH)	
Oven (CTO-10 ACVP)	
Column switching valves (FCV-14 AH)	
DAD system (SPD-M10 AVP)	
System controller (SCL-10 AVP)	
Shimadzu LC Solution™ software 1.21	
UV spectra of toxic compounds [22]	
Rheodyne two-position valve, 6-port	Techlab GmbH, Erkerode, Germany
Computer and monitor	Acer, Ahrensberg, Germany
Remedi™-HS including reagents and columns	Bio-Rad, Munich, Germany
Olympus AU 640	Olympus Deutschland GmbH, Hamburg, Germany
CEDIA DAU® reagents and immunoassays	Microgenics, Passau, Germany, part of Fisher Scientific, Schwerte, Germany

Analytical columns (length x diameter, particle size)

Ionosphere™ (250 x 4.6 mm, 5 µm)	Varian, Darmstadt, Germany
Luna C8™ (30 x 3.0 mm, 5 µm)	Phenomenex, Aschaffenburg, Germany
Luna C18™ (30 x 3.0 mm, 5 µm)	Phenomenex, Aschaffenburg, Germany
Luna Polar™ (30 x 3.0 mm, 5 µm)	Phenomenex, Aschaffenburg, Germany
LunaSCX™ (150 x 4.6 mm, 5 µm)	Phenomenex, Aschaffenburg, Germany
LunaSCX™ (250 x 4.6 mm, 5 µm)	Phenomenex, Aschaffenburg, Germany
Metasil™ (250 x 4.6 mm, 5 µm)	Varian, Darmstadt, Germany
Nucleosil™ 100 C8 (200 x 4.0 mm, 5 µm)	VDS Optilab, Berlin, Germany
Nucleosil™ 100 C8 (50 x 4.0 mm, 5 µm)	VDS Optilab, Berlin, Germany
Synergy 4 µ Hydro RP™ (250 x 4.6 mm, 4 µm)	Phenomenex, Aschaffenburg, Germany
Synergy 4 µ Polar- RP™ (250 x 4.6 mm, 4 µm)	Phenomenex, Aschaffenburg, Germany

Extraction columns (length x diameter, particle size)

Bond Elut CBA™ (20 x 2.1 mm, 40/120 µm)	Varian, Darmstadt, Germany
Nexus™ (20 x 2.1 mm, 70 µm)	Varian, Darmstadt, Germany
Oasis HLB™ (15 x 2.0 mm, 25 µm)	Waters, Eschborn, Germany
Oasis MCX™ (15 x 2.0 mm, 30 µm)	Waters, Eschborn, Germany
StrataX™ (20 x 2.0 mm, 35 µm)	Phenomenex, Aschaffenburg, Germany
StrataX-C™ (20 x 2.0 mm, 35 µm)	Phenomenex, Aschaffenburg, Germany
StrataX-CW™ (20 x 2.0 mm, 35 µm)	Phenomenex, Aschaffenburg, Germany

Pre-(guard)cartridges (length x diameter, particle size)

Security guard cartridge C8 (4.0 x 2.0 mm, 5 µm)	Phenomenex, Aschaffenburg, Germany
Security guard cartridge C18 (4.0 x 2.0 mm, 5 µm)	Phenomenex, Aschaffenburg, Germany
Security guard cartridge C18 wide pore (4.0 x 2.0 mm, 5 µm, pore size 300Å)	Phenomenex, Aschaffenburg, Germany
Security guard cartridge NH_2 (4.0 x 2.0 mm, 5 µm)	Phenomenex, Aschaffenburg, Germany
Security guard cartridge SCX (4.0 x 3.0 mm, 5 µm)	Phenomenex, Aschaffenburg, Germany

2.1.4 Samples and Sample Preparation

Biological samples

Biological samples sent to the Institute of Toxicology–Clinical Toxicology and Poison Control Centre Berlin from hospital emergency rooms, psychiatric units and substance abuse clinics, were used.

Urine samples

The urine samples were centrifuged for 5 min at 15 000 x g in monovettes and 1.0 mL of the supernatant was transferred to a 2.0-mL polypropylene cup, diluted with 500 µL I.S. solution, vortexed and centrifuged again for 5 min at 15 000 x g. The samples were placed into the auto sampler. The injection volume was 1.0 mL.

Performance control sample urine analysis

A total of 1.5 mL of the PCS 1 or PCS 2 was transferred into a 2.0-mL polypropylene cup, centrifuged at 15 000 x g for 5 min and placed into the auto sampler. The injection volume was 1.0 mL.

Glucuronide hydrolysis

A 1.0-mL volume of urine was adjusted to pH 6 with phosphate buffer, when necessary, before 0.1 mL of glucuronidase reagent was added. Hydrolysis was carried out at 45° C for 1.5 h following the method of Grieshaber et al. [85]. The hydrolysed sample was centrifuged for 5 min at 15 000 x g and 1.0 mL was transferred to a new 2.0-mL polypropylene cup, diluted with 500 µL I.S. solution, vortexed and centrifuged again for 5 min at 15 000 x g.

Plasma samples

A total of 0.5 mL plasma/serum, 0.1 mL tris-buffer (pH 9.1) and 0.4 mL extraction solution were mixed in a 1.5-mL polypropylene cup for 2 min. The sample was centrifuged for 2 min at 15 000 x g and 0.2 mL of the organic phase was transferred to a second 1.5-mL polypropylene cup

and evaporated to dryness under a stream of nitrogen at 40 ± 3 °C. The residue was redissolved in 100.0 µL mobile phase 2 and vortexed for 15 s. The sample was placed into the auto sampler. The injection volume was 50 µL.

Performance control sample plasma analysis

A total of 0.1 mL of the PCS-PA was transferred into a vial inlay and the vial was placed into the auto sampler. The injection volume was 50 µL.

2.2 Screening Method for Basic Compounds in Urine

2.2.1 Circuit Diagram

Prior to the method development, the HPLC-DAD equipment was set-up and a circuit diagram was designed in order to direct various liquids through the extraction and analytical columns. The system set-up followed the aim to keep the dead time as low as possible; the circuit diagram had to fulfil six major demands:

1. Option to forward and back flush the extraction column
2. Option to elute undesired fractions or interferences to waste
3. Elution of the target analytes from the extraction column into the analytical column
4. Elution from the extraction column in the forward or back flush mode
5. Multiple analytical column set-up
6. Direct injection on the analytical columns (without on-line extraction)

To meet the above demands, two types of valves were used: two two-position switching valves (Fig. 2-1 (a)) and two column switching valves (Fig. 2-1 (b)). The two-position switching valves could be programmed in position 0 and 1, the column switching valves allowed the parallel set-up of up to six different analytical columns.

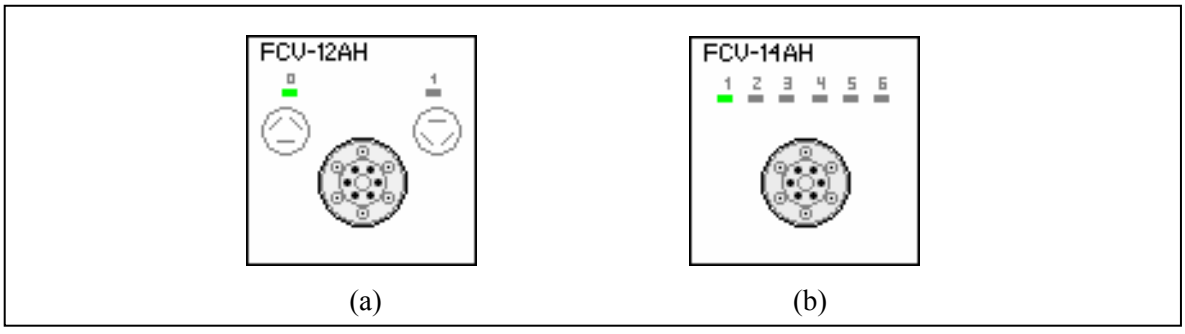


Fig. 2-1 *Switching valves: two-position switching valve (a), column switching valve (b)*

Three HPLC pumps (A, B, C) were connected to the two-position valves by capillary tubes. Pump A was also connected to a gradient unit, capable to mix four different solvents; pump B and pump C were connected by a T-piece. Pump A should be used to load, wash (forward flush) and subsequently flush and equilibrate the extraction column. Pump C should deliver mobile phase for the elution of the target analytes and the analytical separation, pump B should be employed for the back flush wash of the extraction column. In Fig. 2-2 the schematic drawing of the four basic positions: a) loading and wash (forward), b) back flush mode, c) elution, d) direct injection on the analytical columns without on-line extraction is shown. The circuit diagram will be explained in more detail in the results section 3.2.3 in context with the final optimised method.

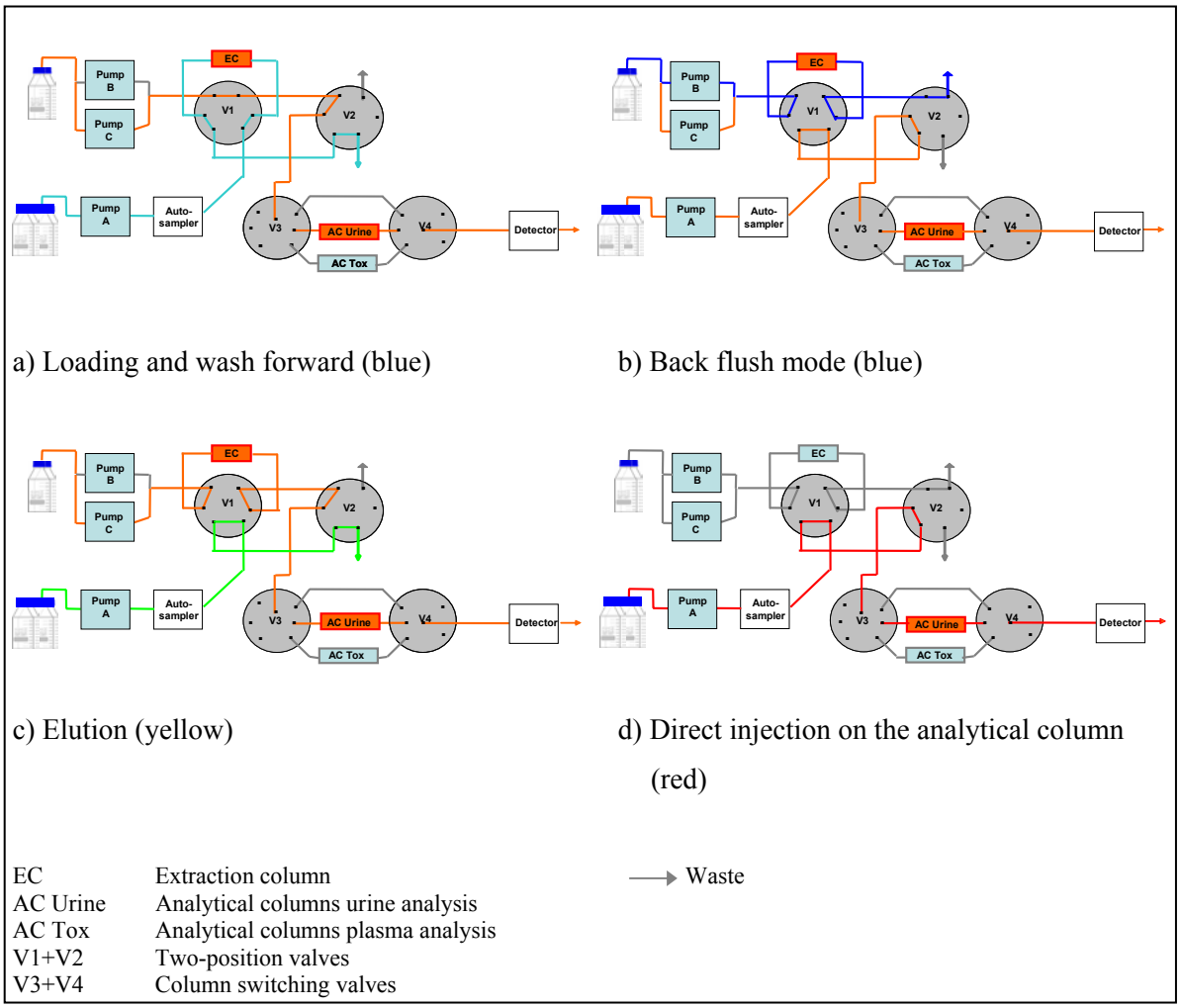


Fig. 2-2 *Basic positions of the circuit diagram: a) Loading and wash forward, b) back flush mode, c) elution, d) direct injection on the analytical column without on-line extraction*

2.2.2 Analytical Procedure

2.2.2.1 HPLC Separation and Detection

The principles of HPLC separation and DAD are thoroughly explained in the literature [77, 86-88]. HPLC separation was performed on two coupled strong cation-exchange (SCX) columns

(2 x LunaSCX™ 150 x 4.6 mm, 5 µm, including a SCX 4.0 x 3.0 mm pre-(guard)cartridge) at a flow rate of 1.2 mL/min under isocratic conditions. The mobile phase consisted of a mixture of 0.05 M dihydrogenphosphate buffer pH 2.3 (68.5%) and acetonitrile/water (90/10, v/v) (31.5%). The oven temperature was 40 °C and the wavelengths were set at 205/235 nm. The injection volume was 1.0 mL. To allow secure compound identification by spectra and RRT comparison the spectra of all investigated analytes were stored in a spectra library using the library editor. A commercially available library of approximately 2600 spectra [22] was used as backup spectra library. Criteria for positive identification was a 99.9% agreement between the obtained and the library spectrum expressed in a similarity index (SI) > 0.999 and a maximum deviation of the RRT of ± 5%. All data were processed using the Shimadzu LC Solution™ software 1.21.

2.2.2.2 Method Development and Optimisation

The mobile phase for the used spectra library consisted of 0.05 M dihydrogenphosphate buffer pH 2.3 and acetonitrile/water (90/10, v/v). If a commercial spectra library is used, it will be very important to use the same mobile phase solvents that were used for the library set-up, as UV spectra show pH dependency [53]. Therefore, only the column material and the buffer/organic solvent ratio could be varied and not the mobile phase solvents. Within the scope of the analytical separation development, various column materials (reversed phase, embedded polar phase, cation-exchange material), different column lengths and the coupling of analytical columns were investigated. The analytical method development and optimisation followed the aim to separate analytes from a wide range of chemical structures and polarities and to allow the separation of polar analytes from the injection peak. In Table 2-1 a survey of all investigated analytical columns is given. The percentage of 0.05 M dihydrogenphosphate buffer and acetonitrile/water (90/10, v/v) as well as the flow rates for each column were optimised. A mixture of seven reference standards (6-acetylmorphine (6-AM), codeine, EDDP, morphine, psilocin, scopolamine and tilidine) diluted to 0.1 µg/mL in 0.05 M dihydrogenphosphate buffer pH 2.3 was used for the evaluation of the analytical separation. The capacity (κ) and separation (α) factors for each column and flow rate (0.7, 0.8, 0.9, 1.0, 1.1, 1.2 mL/min) were calculated using the equations Eq. 1 and Eq. 2. Optimum κ values range from 1-5, α should be ≥ 1.1 to achieve sufficient resolution [87]. The mixture was injected directly onto the analytical columns; the injection peak was used as a dead time marker for the κ value determinations.

$$\kappa = (RT\text{-}t_0/t_0) \qquad \text{Eq. 1}$$

κ *Capacity factor*
RT *Retention time*
t_0 *Dead time (time of an unretained peak)*

$$\alpha = (\kappa_{n+1}/\kappa_n) \qquad \text{Eq. 2}$$

α *Separation factor*
κ *Capacity factor*

Separation of target analytes for this method was evaluated under optimised conditions, injecting buffer solution spiked with reference standards e. g. atropine/scopolamine, MDA/3,4-methylene-dioxy-methamphetamine (MDMA), amphetamine/methamphetamine, 6-AM/morphine/methadone.

Table 2-1 *Investigated HPLC columns*

Column type	Material	Particle size [µm]	Pore size [Å]	Surface [m^2/g]	Length x diameter [mm]	PH range
Cation-exchange						
Ionospher™	*	5	125	165	250 x 4.6	2.0- 5.0
Luna SCX™	*	5	100	400	150 x 4.6	2.0- 7.0
Luna SCX™	*	5	100	400	250 x 4.6	2.0- 7.0
Metasil™	*	5	80	200	250 x 4.6	2.0- 8.0
Embedded polar phase						
Synergi Hydro-RP™	**	4	80	475	250 x 4.6	1.5- 7.0
Synergi Polar-RP™	***	4	80	475	250 x 4.6	1.5- 7.0
Reversed phase						
Luna C8™	C8	5	100	400	30 x 3.0	1.5-10.0
Luna C18™	C18	5	100	400	30 x 3.0	1.5-10.0
Nucleosil™ 100 C8	C8	5	100	350	200 x 4.0	1.5-10.0
Nucleosil™ 100 C8	C8	5	100	350	50 x 4.0	1.5-10.0

* *Strong acid ion-exchange material (functional cation-exchange group: sulfonic acid)*
** *C18 reversed phase material with polar endcapping*
*** *Ether-linked phenyl phase with polar endcapping*

In order to pursue better separation results, the influence of the amine modifiers N,N-dimethyloctylamine ($N(CH_3)_2C_8H_{11}$) and tetramethylammonium chloride ($N(CH_3)_4Cl$) on the separation, peak shape or symmetry was studied.

2.2.3 On-line Extraction Procedure

2.2.3.1 Extraction Method

The system set-up described in section 2.2.1 was used for the on-line extraction. The samples were applied to the extraction column (StrataX-CW™, 20 x 2.1 mm, 35 µm) with 0.01 M phosphate buffer pH 6.0 followed by a wash step with acetonitrile/water (90/10, v/v). Solvents were transported by pump A, each step took 2.5 min and the flow rate was set to 2 mL/min. During the

loading and wash step, the analytical columns were conditioned with mobile phase (pump C, flow-rate = 1.2 mL/min). The extraction column was then washed with water transported by pump B in the back flush mode (1 min; flow rate = 3 mL/min). The following elution into the analytical columns with mobile phase 1 was again in the back flush mode using pump C.
The time required for the on-line extraction was 6 min. The final extraction method together with the according circuit diagram is described in more detail in the results section (3.2.3).

2.2.3.2 Extraction Method Development and Optimisation

The on-line column to be employed should allow the extraction of a wide range of analytes and effectively work in combination with the separation column and the mobile phase optimised. Polymer (electroneutral), weak cation-exchange (carboxylic acid functions, WCX) and strong cation-exchange (benzenesulphonic acid functions, SCX) sorbents were investigated for on-line extraction. In Table 2-2 the studied extraction materials are summarised.

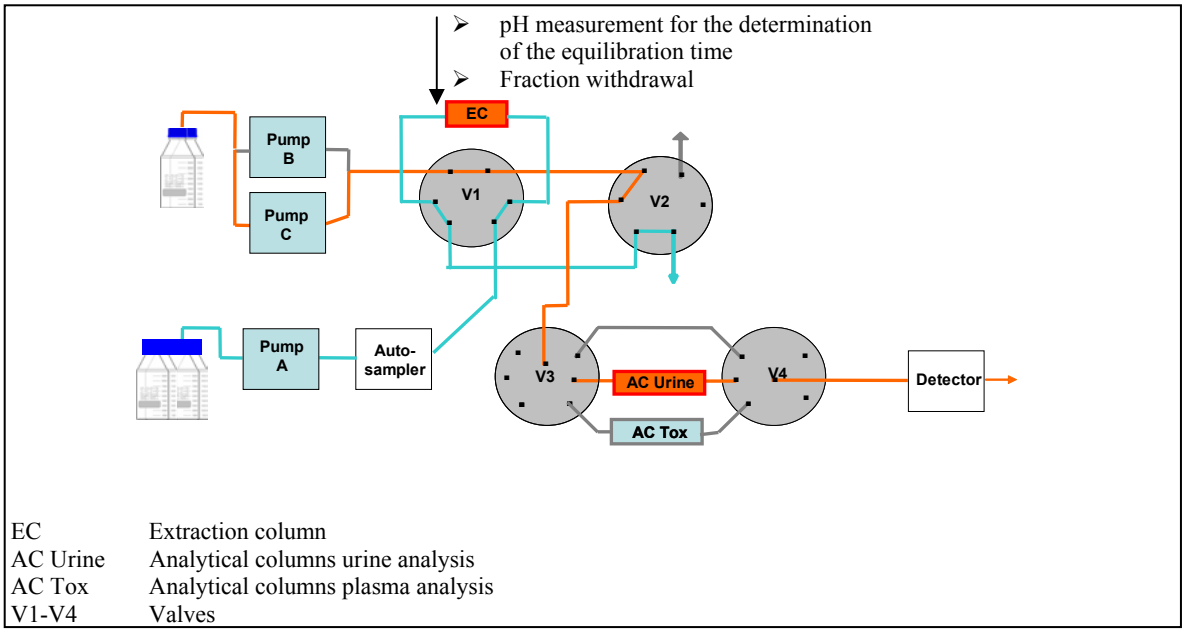
Table 2-2 *Investigated extraction materials*

Material	Particle size (µm)	Pore size (Å)	Surface (m^2/g)	Length x diameter (mm)	Extraction capacity (meq/g)
1. Polymer material					
Polystyrene-divinylbenzene	35	85	800	20 x 2.0	-
Styrenedivinylbenzene/ methacrylate copolymer	70	100/400	575	20 x 2.1	-
N-vinylpyrrolidone/divinyl-benzene copolymer	25	80	810	15 x 2.0	-
2. Weak cation-exchange material					
Polymer based carboxylic acid functions	35	85	800	20 x 2.0	Approx. 1.00
Silica based carboxylic acid function	40/120	60	Not given	20 x 2.1	Approx. 0.35
3. Strong cation-exchange material					
Polymer based benzenesulphonic acid functions	35	85	800	20 x 2.0	Not given
Polymer based benzenesulphonic acid functions	30	78	792	15 x 2.0	Approx. 0.81

Depending on the nature of the extraction sorbent, the target analytes had to be uncharged when the sorbent was apolar (electroneutral) and charged when a polar sorbent (WCX, SCX) was used to guarantee retention. Therefore, borate buffer pH 8, tris-buffer pH 9.1 and ammonium carbonate buffer pH 10 were used to load the analytes onto polymer (electroneutral) material, and phosphate buffer pH 6.0 for the loading onto the cation-exchangers, respectively.

The loading, wash, back flush and elution steps were optimised by the determination of the optimal solvents and solvent composition, volumes and flow rates for each working step. Water, acetic acid, phosphate buffer pH 2.3, pH 6 and pH 7, borate buffer pH 8, tris-buffer pH 9.1 and ammonium carbonate buffer pH 10 were investigated as loading and wash buffers. The molarity of the buffer solutions (0.01 M, 0.05 M, 0.1 M, 0.2 M) and the percentage of acetonitrile/water (90/10, v/v) were varied and flow rates ranging from 0.5-5.0 mL/min examined. The time required to equilibrate and adjust the extraction column to a certain pH, switching from an acidic to a basic solvent, was determined by pH measurement behind the extraction column (Fig. 2-3).

The compound break-through in the loading and wash steps was investigated by collecting fractions behind the extraction column (Fig. 2-3). The organic fractions were evaporated to dryness under a stream of nitrogen at 40 ± 3 °C. The residue was redissolved in 1.0 mL of mobile phase 1, vortexed for 15 s and reanalysed by direct injection on the analytical columns. The aqueous fractions were directly injected into the analytical columns.



EC	Extraction column
AC Urine	Analytical columns urine analysis
AC Tox	Analytical columns plasma analysis
V1-V4	Valves

Fig. 2-3 *Circuit diagram, loading and wash position (forward)*

The elution with the mobile phase was determined by directly connecting the extraction column to the analytical column and direct injection of PCS 1 (n = 6) on the three coupled columns (Fig. 2-4). The peak areas of the PCS 1 analytes were compared to those obtained from the direct analysis of the PCS 1 (n = 6) without the coupled extraction column.

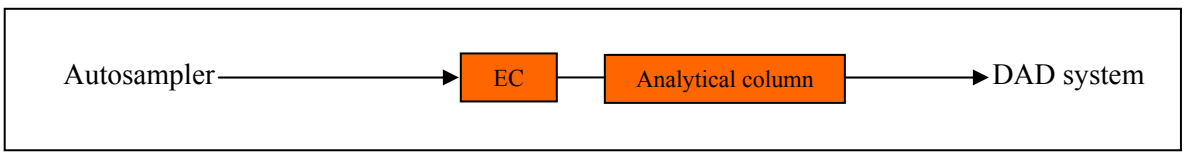


Fig. 2-4 *Direct connection of the extraction column (EC) with the analytical column*

The extraction of buffer spiked with reference standards, blank and spiked blank urine samples was carried out, evaluating the extraction efficiency and the absence of interferences for each investigated column.

In order to increase the life-time of the extraction columns, the filter effect of a C8, C18, C18 wide pore, NH_2 and SCX pre-(guard)cartridge was studied by connecting the respective pre-(guard)cartridge to the extraction column prior to analysis. The influence on the life-time of the extraction column by measuring the pressure was determined. The pore size of the investigated pre-(guard)cartridges was 100 Å and 300 Å for the wide pore cartridge, respectively.

2.2.4 Validation

With the following investigations the suitability of the developed method for its intended use should be demonstrated and documented (method validation).

Despite their frequent use, only few strategies for the validation of qualitative analytical methods are described in the literature [89]. Selectivity and the lower limit of detection are the most important validation parameters for the validation of qualitative methods according to different organizations [90-94]. The comparison with described methods of the same field of application [16, 19, 21, 34, 55, 95] led to the addition of the validation parameters recovery, precision, linearity and the performance of carry-over experiments. Main emphasis was put on the ruggedness of the method; especially the batch-to-batch reproducibility of the extraction and analytical columns should be given, to allow the simple column exchange by the operator. The PCS 1 (buffer matrix) and PCS 2 (urine matrix) consisting of six different analytes were used for the assay validation which represented the following groups of interest: alkaloids (scopolamine), amphetamine-derivatives (MDA) opiates (codeine, morphine), opioids (EDDP) and neostigmine bromide (I.S.). The determination of the above mentioned parameters followed the Food and Drug Administration (FDA) guidelines for biomedical method validation [96] which was originally developed for the validation of quantitative methods.

2.2.4.1 Selectivity/Specificity

To demonstrate the selectivity of the analytical procedure, six blank urine samples obtained from six healthy volunteers were analysed (each n = 3). The absence of interfering peaks (signal-to-noise ratio (S/N) > 3) at the RT of the analytes of the PCS 1 and PCS 2 in the chromatogram was verified. In addition, the secure identification of multiple drug cases was ensured by the analysis of spiked urine samples with possible opiates, alkaloids, amphetamine-derivatives, tilidine and tilidine metabolites.

2.2.4.2 Stability

The stability of the PCS 1 and PCS 2 was assessed by dividing the freshly prepared performance control sample in three aliquots; the first was frozen immediately, the second was stored in the fridge at 5-8 °C and the third was kept in the dark at room temperature. Three samples of the freshly prepared performance control sample were analysed immediately as reference samples, samples of each aliquot were analysed after 3, 7, 14, 21, 28 days (n = 3).

Long-term stability evaluations over 67 days (n = 3) were carried out using the PCS 1, but scopolamine was replaced by tilidine. The samples were portioned in 1.5-mL cups and stored frozen. Prior to analysis, the samples were thawed and immediately analysed.

Psilocin stability

Psilocin is the dephosphorylated pharmacologically-active fraction of psilocybin, which represents the main psychoactive compound of Psilocybe mushrooms. Psilocin is usually present in smaller amounts in the so called “magic mushrooms”, but it is formed as an in vivo metabolite and therefore is available for analysis in biological fluids [97]. Psilocin is partially excreted in urine in the glucuronide conjugated form [98], thus glucuronide hydrolysis prior to psilocin analysis is recommended [85, 99-101].

As the weak stability of psilocin is described in the literature [97], the UV and temperature influence on the psilocin stability should be examined with the following investigations. A number of 16 blank urine samples was prepared and spiked with 5 µg/mL psilocin reference standard. To 8 samples 0.1 mL glucuronidase reagent was added. Four sample aliquots each were wrapped in aluminium foil to protect them from UV radiation. The samples containing glucuronidase reagent were hydrolysed at 45 °C. After 0, 4, 8 and 24 h, a UV-protected sample with and without hydrolysis and a non-protected sample with and without hydrolysis were extracted and analysed.

In a second experiment, blank urine samples spiked with 5 µg/mL psilocin reference standard were stored at 5-8 °C (fridge) and < -15 °C (freezer). Samples of each aliquot were analysed after 0, 1, 7, 28 and 42 days (n = 3). The recoveries (R, %) for both stability experiments were calculated with the following equation:

$$R, \% = \text{Peak area}_{(n\ h\ or\ m\ days)} / \text{Peak area}_{(0\ h\ or\ 0\ days)} \times 100 \qquad \text{Eq. 3}$$

n 0, 4, 8, 24 h
m 0, 1, 7, 28, 42 days

2.2.4.3 Extraction Recovery

The extraction recovery was calculated from the average peak areas (arithmetic mean) of the extracted PCS 1 (buffer matrix, n = 6) and PCS 2 (urine matrix, n = 6) in relation to the average peak areas (arithmetic mean) of the direct injection of PCS 1 into the analytical columns (n = 6) at

three concentrations (1.0 (level 1), 5.0 (level 2), 15.0 (level 3) µg/mL). The following equation (Eq. 4) was used for the calculation:

$$\text{Recovery, \%} = \text{Peak area}_{\text{(PCS 1 or 2, on-line extraction)}} / \text{Peak area}_{\text{(PCS 1, direct injection)}} \times 100 \quad \text{Eq. 4}$$

The influence of any matrix components on the recovery was determined by comparison of the recoveries of PCS 1 and PCS 2 at level 1-3.

2.2.4.4 Precision

Within-day precision of the system was assessed by the direct injection of the PCS 1 (buffer matrix, n = 6) and calculation of the standard deviation (SD) and the relative standard deviation (RSD) of the six replicates, respectively. Within-day precision of the method was determined by the extraction and analysis of the PCS 2 (urine matrix, n = 6) and calculation of the SD and the RSD of the six replicates. For each parameter three concentrations (1.0, 5.0, 15.0 µg/mL, level 1-3) were investigated. Between-day precision was determined by the analysis of PCS 2 on six different days (n = 3).

2.2.4.5 Carry-Over Experiments

For the determination of the concentration that led to a carry-over, blank urine samples were injected after each sample of the following concentrations 0.25, 0.50, 1.00, 2.00, 5.00, 10.00, 15.00, and 20.00 µg/mL. In each instance, the chromatograms obtained from the blank samples were tested for appearing peaks.

2.2.4.6 Linearity

Linearity was evaluated extracting and analysing matrix samples spiked with the PCS 2 analytes at the following concentrations: 0.10, 0.20, 0.25, 0.50, 1.00, 2.00, 5.00, 10.00, 15.00, and 20.00 µg/mL (n = 6).

2.2.4.7 Limit of Detection

The lower limit of detection (LLOD) of the system was assessed by comparing the chromatograms of empty matrix with those obtained from spiked blank urine samples (PCS 2) near the expected LLOD (c = 0.25, 0.20, 0.10 µg/mL (n = 6)). The LLOD was regarded as the lowest concentration of the performance control sample analytes yielding a signal-to-noise (S/N) ratio of at least 3.0.

2.2.4.8 Batch-to-Batch Reproducibility

For the evaluation of the batch-to-batch reproducibility of the extraction column and the analytical columns, the PCS 2 was analysed using three different batches of extraction columns (each n = 3) and three different batches of analytical columns (each n = 6). The SD and RSD of the replicates were calculated for within- and between-reproducibility (peak area and RRT).

2.2.4.9 Calibration for Semi-Quantitative Determination

For a selection of analytes a 7-point calibration (0.1, 0.2, 0.5, 1.0, 5.0, 10.0, 15.0 µg/mL, n = 6) for semi-quantitative analysis was carried out. Within-day precision (n = 6), between-day precision (on 6 different days) and linearity by correlation coefficient, intercept and slope were calculated using Microsoft Office Excel™ 2003. A Grubbs-test was performed to determine any outliers.
Semi quantitative concentration calculation was carried out using equation Eq. 5, accuracy by relative error (RE, %) was calculated using Eq. 6. For the determination of both latter parameters the PCS was exemplarily used.

$$c = (\text{Peak area analyte} / \text{Peak area I.S.-intercept}) / \text{slope} \quad \text{Eq. 5}$$

$$RE, \% = 100 \times (c_{(calculated)} - c_{(nominal)}) / c_{(nominal)} \quad \text{Eq. 6}$$

2.2.5 Method Modifications for Critical Compounds

The developed method was modified for the analysis of compounds which had a low recovery due to early elution in the acetonitrile/water wash step (benzoylecgonine (BEC)) or could not be analysed on the strong cation-exchange columns within an acceptable time range due to strong interactions with the HPLC material (late eluting compounds (LEC): aripiprazol, cetirizine, clozapine, olanzapine, opipramol, pipamperone, quetiapine, risperidone, ziprasidone).

2.2.5.1 Benzoylecgonine Method

The BEC method was established in order to increase the BEC detection signal. The second wash step with acetonitrile/water (90/10, v/v) was reduced (0.5 min) and the loading and wash step with 0.01 M phosphate buffer was extended to 4.5 min in comparison to the earlier described extraction method. After 4 min of elution with mobile phase, the extraction cartridge was separated and washed with acetonitrile/water (90/10, v/v) while parallel analytical separation was carried out on the analytical columns. The total run time was 41 min.

2.2.5.2 Method for Late Eluting Compounds

For compounds with very late RTs (> 8 h) on the deployed cation-exchange HPLC material, the elution into coupled C8 columns (Nucleosil™ 100 C8, 200 x 4.0 mm, 5 µm + Nucleosil™ 100 C8, 50 x 4.0 mm, 5 µm) was programmed. This method was used when samples of acute intoxications were screened negative in plasma and in urine (developed on-line extraction method).

2.2.6 Analysis of Authentic Samples and Comparison with the Remedi™-HS

Four-hundred-five urine samples were analysed by the Remedi™-HS and asservated during the method development. In order to prove the applicability of the developed method and compare the results to an automated HPLC reference method, the samples were analysed in both systems before introducing the developed method to the routine use.

Samples used in cases of DOA confirmation analysis were previously screened positive by immunological pre-screening [102] and confirmed with GC-MS [103]. HPLC confirmation with the developed method and the Remedi™-HS was carried out when the positive immunological results equalled or exceeded the in-house cut-off values (ng/mL) for the HPLC methods shown in Table 2-3.

Table 2-3 *Immunological cut-off values*

Substance	Immunological cut-off value (CEDIA DAU®)* (ng/mL)	Immunological in-house cut-off value for HPLC analysis* (ng/mL)
Amphetamines	≥ 1000	≥ 1000
Cocaine	≥ 300	≥ 1000, (300 BEC method)
Opiates	≥ 300	≥ 1000
6-AM	≥ 10	> 20
EDDP	≥ 100	≥ 100

* *Immunological pre-screening was carried out using the associated CEDIA DAU® kits (Microgenics, Passau, Germany) and following the manufacturer's instructions [102].*

Besides confirmation screening of DOA, acute intoxications were investigated in cases where additional urine samples were available. The comparison of the results was evaluated with the SPSS™ software 12.0 (SPSS, Stanford, California).

2.2.6.1 Sample Pre-Treatment Remedi^{TM}-HS

The sample preparation followed the instructions of the manufacturer for the Remedi^{TM}-HS analysis. The urine samples were centrifuged for 5 min at 15 000 x g and 1.0 mL was transferred to a 1.5-mL polypropylene cup, diluted with 200 µL I.S. solution (BioRad), vortexed and centrifuged again for 5 min at 15 000 x g. The samples were placed into the auto sampler. The injection volume was 1.0 mL.

2.2.6.2 Extraction and Analytical Procedure Remedi^{TM}-HS

Following sample preparation, the sample was applied to the first column (purification column) with application buffer. On this column on-line extraction was carried out retaining basic, neutral and weakly acidic compounds. Hydrophilic endogenous compounds, salts and glucuronides were not retained and passed through that column. On the 2nd column (extraction column) endogenous organic acids were retained. The target analytes were eluted into a 3rd and 4th column (separation 1 cartridge and separation 2 cartridge) for separation by an exchange and transfer buffer. The separation 1 cartridge was a reversed-phase cartridge that separated weakly basic compounds; the separation 2 cartridge (silica material) separated basic compounds by cation-exchange. Separation was carried out under isocratic conditions. A 5th cartridge (mobile phase saturator cartridge) was used to saturate the mobile phase with silica, protecting the separation cartridges from dissolution. Identification of the compounds was performed by spectra comparison and chromatographic data. For the RT calculation two internal standards (N-ethyl-nordiazepam and chlorpheniramine) were used. Time required for analysis including extraction was approximately 20 min. A detailed description of the system is given by Binder et al. [55]. In 7.2 in the appendix a picture of the Remedi^{TM}-HS (Fig. 7-1) and of its circuit diagram (Fig. 7-2) are shown.

2.3 Screening Method for Plasma

The toxicological screening method for substances in plasma or serum followed a laboratory internal method and has been described in the literature [104]. The method has been part of the routinely performed STA at the laboratory of the Institute of Toxicology-Clinical Toxicology and Poison Control Centre Berlin since 1988 and was established and examined with the analysis of an accuracy control test in the described analytical system in order to allow screening of plasma.

2.3.1 Analytical Procedure

Following LLE (2.1.4), the samples were brought onto the coupled Nucleosil^{TM} 100 C8 columns, where isocratic analytical separation was carried out at a flow rate of 0.7 mL/min. The mobile

phase consisted of 64% 0.05 M phosphate buffer pH 2.3 and 36% acetonitrile/water (90/10, v/v). The oven temperature was 40 °C and the detection wavelength was set at 210 nm.

Prior to analysis, the column switching valves were switched in-line with the required analytical column and an equilibration gradient was run (0.05 M phosphate buffer pH 2.3: increase from 20% to 64% linear in 10 min, plus 10 min isocratic under the mobile phase conditions). The time required for analysis was 50 min. The schematic set-up for the analytical system using column switching is shown in Fig. 2-5.

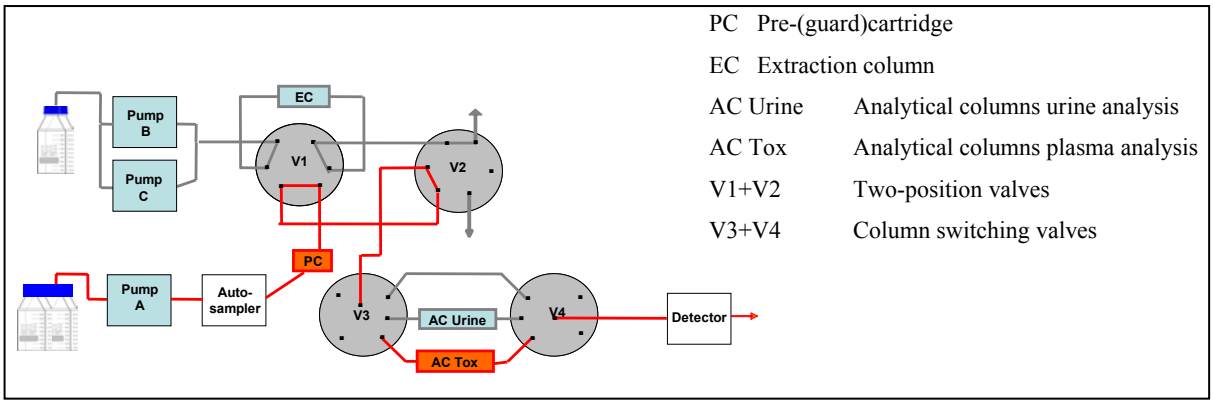


Fig. 2-5 *Circuit diagram of the toxicological screening method in plasma (red)*

2.3.2 Validation

The set-up of the method in the analytical system was checked by a suggested performance test for accuracy control [22] consisting of histamine (0.1 mg/mL) for the determination of the time of an unretained peak, caffeine (0.1 mg/mL) for accuracy measurement of the auto sampler, MPPH (0.1 mg/mL) for reproducibility and precision evaluation of the RRTs and benzene (1.0 mg/mL) was used to control the resolution, precision and reproducibility of the UV spectra measurement (vibration bands between 240 and 270 nm). The latter parameters were determined by analysis of six consecutive samples (n = 6). The injection volume was 10 µL.

Between-day precision was assessed with the analysis of the PCS on six different days. The accuracy of the method was assessed by calculation of the mean percentage deviation (RE, %) of measured concentrations of the PCS-PA from their nominal concentration (2.2.4.9, Eq. 6).

The precision from HPLC-system-to-HPLC-system was investigated by analysis of the PCS-PA in the developed system and in an HPLC-UV reference system from Shimadzu used for routine plasma analysis at the Institute of Toxicology-Clinical Toxicology and Poison Control Centre Berlin (7.3, appendix), each n = 6. Accuracy from HPLC-system-to-HPLC-system was determined by calculation of the RE using Eq. 7. The injection volume was 50 µL for evaluation of the latter described validation parameters.

$$RE, \% = 100 \times (c_{(developed\ system)} - c_{(reference\ system\ in\ routine\ use)}) / c_{(reference\ system\ in\ routine\ use)} \quad \text{Eq. 7}$$

In addition, the analysis of real toxicological specimens in both systems was evaluated and compared.

2.4 Screening Method for Neutral, Weakly Acidic and Weakly Basic Compounds in Urine

2.4.1 Analytical Procedure

Since neutral, weakly acidic and weakly basic compounds cannot be extracted via the weak cation-exchange material, an on-line extraction method for neutral, weakly acidic and weakly basic compounds was developed. The samples were loaded on apolar extraction material (StrataX^{TM} 20 x 2.0, 35 µm) with loading buffer consisting of 90% 0.01 M phosphate buffer pH 6.0 and 10% acetonitrile/water (90/10, v/v). A subsequent wash step in the forward (80% 0.01 M phosphate buffer pH 6.0/20% acetonitrile/water (90/10, v/v)) and back flush mode (water) was performed. Flow rates, loading/wash time and the use of the three pumps were adapted from the on-line extraction method for basic compounds described in section 2.2.3.1. Analytical separation was carried out on coupled Nucleosil^{TM} 100 C8 columns at a flow rate of 0.7 mL/min under isocratic conditions. The detection wavelength was set to 205 nm and time for analysis including on-line extraction was 50 min.

For integration of this method in the analytical system, a fifth valve was established in order to allow automatic switching between two on-line extraction columns (see Fig. 2-6).

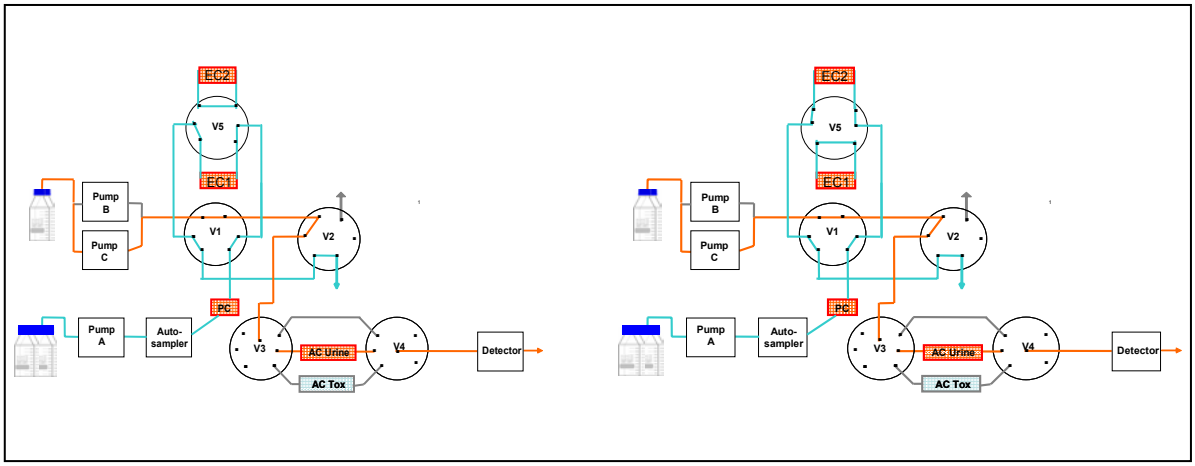


Fig. 2-6 *Circuit diagram including a 5th valve, EC 1: 1st extraction column, EC 2: 2nd extraction column, PC: pre-(guard)cartridge, AC: analytical columns, V1, V2, V5: two-position valves, V3, V4: column switching valves*

2.4.2 Method Development and Optimisation

For the development of the method the consistence of the loading and wash solvents was optimised and the sample break-through examined (2.2.3.2, Fig. 2-3). Different polymer (electroneutral)

extraction materials were investigated (2.2.3.2, Table 2-2), the analytical columns for the on-line extraction method (2 x LunaSCX 150 x 4.6 mm) and the screening method in plasma (Nucleosil™ 100 C8, 200 x 4.0 mm + Nucleosil™ 100 C8, 50 x 4.0 mm) were studied in order to pursue optimum separation of benzodiazepines and barbiturates in particular.

In order to increase the extractable amount of benzodiazepines in the urine sample, glucuronide hydrolysis was carried out as described in section 2.1.4. Optimisation of the required hydrolysis time was performed by hydrolysing the same benzodiazepine-positive samples for 30, 60, 120, 150 and 180 min. The glucuronide cleavage was evaluated by the peak area of the benzodiazepine peaks in the corresponding chromatogram (n = 3). Furthermore glucuronide hydrolysis with HCl at 80 °C for 30 min was investigated and the results compared.

2.4.3 Validation

The validation of the method was carried out with the exemplary use of PCS-BDP 1 and 2 and PCS-BARB 1 and 2. The following parameters were determined as described in section 2.2.4: selectivity, stability, recovery, precision, LOD, carry-over experiments, linearity and reproducibility were carried out. Linearity was investigated over the range of the following concentrations: 0.05, 0.10, 0.50, 1.00, 5.00, 10.00, 20.00 µg/mL (n = 6). The dependency of the pH on the extraction behaviour of MPPH (I.S.) was studied by calculating the recovery of spiked MPPH samples (c = 5 µg/mL) adjusted to pH 4.5, 6.0, 7.0 and 9.0 (each n = 3) compared to the direct injection (n = 3).

2.4.4 Analysis of Authentic Benzodiazepine Positive Samples

The applicability of the method was investigated with real toxicological samples. For this purpose urine samples that were previously positively pre-screened by immunological analysis (CEDIA DAU®, cut-off for benzodiazepines 200 ng/mL) were used.

2.5 Strategies for Systematic Toxicological Analysis with the New Analytical Screening System

2.5.1 Samples Spiked with Reference Standards

Frequently prescribed and/or toxicologically relevant compounds (c = 1 µg/mL) were analysed with the three described methods in order to establish a systematic structure of the developed analytical system and determine the method choice in dependence on the sample type. The analytical data for each compound (RT, RRT, UV spectrum) were stored in a separate library for

each method, which was programmed using the Library editor of the LC Solution™ software. The set-up of the separate spectra libraries for each method, in addition to the commercial library, was used to allow compound identification by comparison of retention and RRTs.

2.5.2 Authentic Toxicological Samples

Within the scope of the analysis of acute intoxications at the Institute of Toxicology-Clinical Toxicology and Poison Control Centre Berlin, authentic toxicological samples were analysed following the analysis strategy presented in Fig. 1-1, 1.1. Over a time period of six weeks all samples where plasma and urine were sent for analysis were asservated and subsequently reanalysed with the developed system. Urine samples were investigated with the two on-line extraction methods, plasma samples were analysed following LLE with the toxicological screening method in plasma, respectively. The switching between the different methods using different analytical columns was handled by programming an equilibration sequence prior to sample analysis. The injection volume, method parameters and method depending report formats were stored in a template for batch-analysis in order to simplify the routine use and to avoid mistakes by switching from one method to the other. Following sample analysis, a wash sequence was run before the corresponding method was set to the standby mode.

In Fig. 2-7 a schematic drawing of all used methods in accordance to the investigated sample matrix and the spectra libraries used for compound identification is shown.

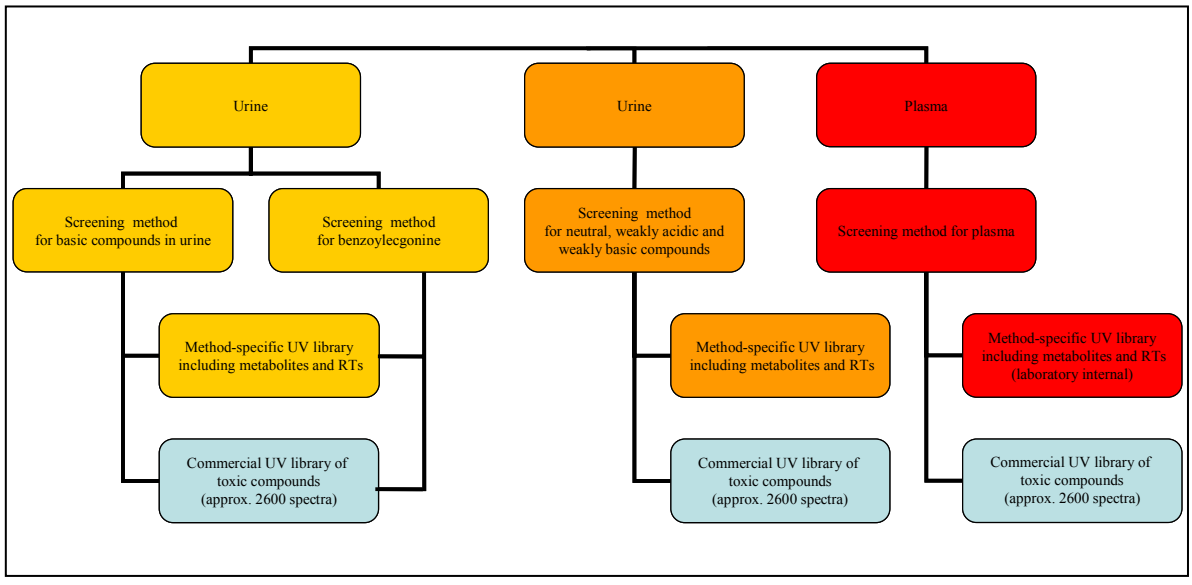


Fig. 2-7 *Schematic drawing of the methods used for STA*

3 Results

3.1 Circuit Diagram

In order to allow automated on-line extraction with subsequent chromatographic separation, a circuit diagram was designed. The set-up of the circuit diagram met the demands given in the materials and methods section (2.2.1) such as options to forward and back flush the extraction column, options to elute undesired fractions, interferences to waste, elution of the target analytes from the extraction column into the analytical columns, elution from the extraction column in the forward or back flush mode, multiple analytical column set-up and direct injection on the analytical columns (without on-line extraction).
The circuit diagram will be described in more detail in 3.2.3 in context with the final analytical procedure.

3.2 Screening Method for Basic Compounds in Urine

3.2.1 Analytical Separation

Isocratic elution was chosen for the separation of the target analytes to pursue stable analytical conditions without baseline disturbances. As the composition of the mobile phase for the analytical separation was restricted to the mobile phase of the used commercial spectra library due to pH dependency of the recorded spectra, only the column material could be varied in order to gain optimum compound separation. The mobile phase for the used spectra library consisted of 0.05 M dihydrogenphosphate buffer pH 2.3 and acetonitrile/water (90/10, v/v). In this acidic mobile phase the target compounds, such as alkaloids, opiates, and amphetamines were protonated. This led to an early elution of the polar compounds on reversed phase material (Nucleosil^{TM} 100 C8, 200 x 4.0 mm, 5 µm + Nucleosil^{TM} 100 C8, 50 x 4.0 mm, 5 µm) and resulted in an unsuccessful separation from the injection peak. On the other hand, less polar compounds like the methadone metabolite EDDP showed RTs > 30 min. The same effect appeared for embedded polar phase material (Synergy 4 µ Hydro-RP^{TM}, 250 x 4.6 mm, Synergy Polar-RP^{TM}, 250 x 4.6 mm) under the required conditions. Thus, SCX material was tested. This approach showed good results concerning the retention and separation of the analytes from the injection peak due to the strong ionic interactions between the polar analytes and the stationary phase.
Investigations on different SCX columns (2.2.2.2) with a mixture of seven relevant reference standards for this application (6-AM, codeine, EDDP, morphine, psilocin, scopolamine, tilidine) and the optimisation of the buffer/organic solvent ratio and flow rates was carried out for each column. The resolution could be slightly improved by coupling a short reversed phase column (Luna C8^{TM} or Luna C18^{TM} 30 x 3.0 mm) to the investigated SCX columns.

It could be shown that under optimised conditions a 250 mm SCX material was required for sufficient separation as on the 150 mm SCX material (LunaSCX™) two out of seven substances were overlaid and low resolution was gained. Only on one column (Ionosphere™, 250 x 4.6 mm, analysis time 40 min) all substances were separated. On the other two tested columns two substances (LunaSCX™, 250 x 4.6 mm, analysis time 25 min) and three substances (Metasil™, 250 x 4.6 mm, analysis time 52 min) were not separated. The results are summarised in Table 3-1 and Table 3-2.

Table 3-1 *Optimised analytical conditions for each investigated SCX column*

Parameter	**Ionosphere™ (250 x 4.6 mm) + C18 (30 x 3.0 mm)**	**LunaSCX™ (150 x 4.6 mm) + C8 (30 x 3.0 mm)**	**LunaSCX™ (250 x 4.6 mm) + C18 (30 x 3.0 mm)**	**Metasil™ (250 x 4.6 mm) + C8 (30 x 3.0 mm)**
Buffer/organic solvent ratio (v/v)	55/45	71/29	68/32	63/37
Flow rates (mL/min)	1.0	1.0	1.0	0.8
Time for analysis (min)	40	22	25	52

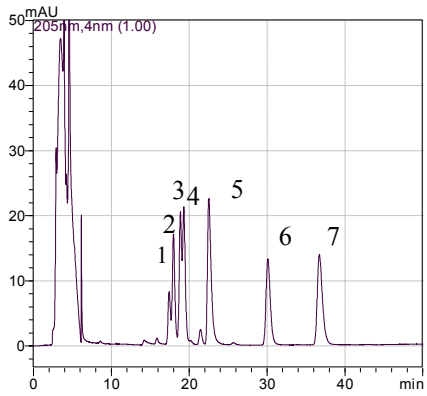
Table 3-2 *Analytical separation results on investigated SCX columns*

Substance	**Ionosphere™ (250 x 4.6 mm) + C18 (30 x 3.0 mm)**	**LunaSCX™ (150 x 4.6 mm) + C8 (30 x 3.0 mm)**	**LunaSCX™ (250 x 4.6 mm) + C18 (30 x 3.0 mm)**	**Metasil™ (250 x 4.6 mm) + C8 (30 x 3.0 mm)**
6-AM	+	-*	-*	-*
Codeine	+	+	+	+
EDDP	+	+	+	+
Morphine	+	+	+	+
Psilocin	+	+	+	-*
Scopolamine	+	-*	-*	-*
Tilidine	+	+	+	+

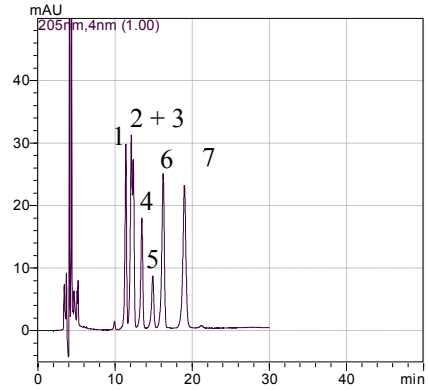
\+ *separated*
-* *not separated substances*

Criteria for the evaluation and column choice were the peak shape, capacity and separation factors. Calculated dead time for the direct injection was 2.8 min. Depending on latter parameters the 250 mm long LunaSCX™ column showed best results. Compared to the other SCX columns it showed good separation of most substances, a good peak shape, better resolution than the 150 mm material and results within a reasonable time range. On the Ionosphere™ column all substances

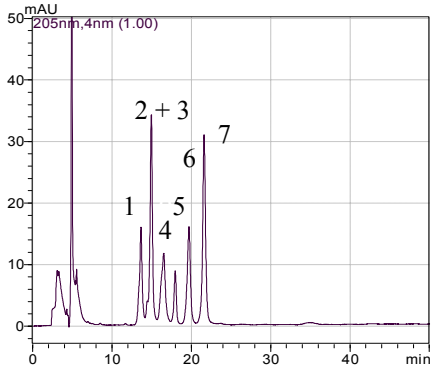
were separated, but time for analysis was too long and correspondingly all capacity factors were ≥ 5.0. The use of the 150 mm LunaSCX™ and Metasil™ column led to poor separation. In addition, on the Metasil™ column strong peak tailing was observed. Example chromatograms of each column under optimised conditions are shown in Fig. 3-1. In Table 3-3 the capacity and separation factors are summarised and in Fig. 3-2 a graphic comparison of the capacity values is given.



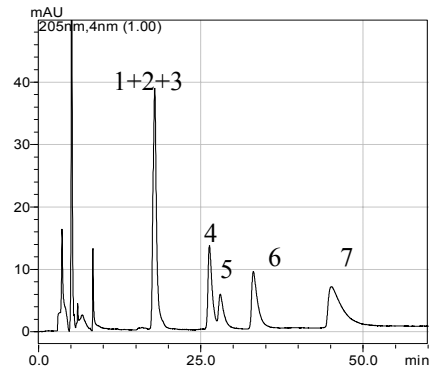
Ionosphere™ + C8
1: Tilidine, 2: Scopolamine 3: 6-AM, 4: Psilocin, 5: EDDP, 6: Morphine, 7: Codeine



LunaSCX™ 150 mm + C8
1: Psilocin, 2: Scopolamine, 3: 6-AM 4: Morphine, 5: Tilidine, 6: Codeine 7: EDDP



LunaSCX™ 250 mm + C18
1: Psilocin, 2: Scopolamine, 3: 6-AM 4: Morphine, 5: Tilidine, 6: Codeine 7: EDDP



Metasil™ + C8,
1: Psilocin, 2: Scopolamine, 3: 6-AM, 4: Morphine 5: Tilidine, 6: Codeine, 7: EDDP

Fig. 3-1 *Example chromatograms of investigated SCX columns*

Table 3-3 *Capacity (κ) and separation (α) factors for investigated column combinations*

Substance number (elution order*)	Ionosphere™ (250 x 4.6 mm) + C18 (30 x 3.0 mm)		LunaSCX™ (150 x 4.6 mm) + C8 (30 x 3.0 mm)		LunaSCX™ (250 x 4.6 mm) + C18 (30 x 3.0 mm)		Metasil™ (250 x 4.6 mm) + C8 (30 x 3.0 mm)	
	κ	**α**	**κ**	**α**	**κ**	**α**	**κ**	**α**
1	5.0	1.1	3.4	1.1	4.3	1.1	4.6	1.0
2	5.5	1.0	3.8	1.0	4.7	1.0	4.6	1.0
3	5.6	1.1	3.8	1.1	4.7	1.2	4.6	1.7
4	6.4	1.1	4.3	1.1	5.5	1.1	7.8	1.2
5	6.7	1.4	4.8	1.1	6.2	1.1	9.4	1.2
6	9.4	1.2	5.3	1.1	6.7	1.3	11.7	1.1
7	11.6		6.0		8.7		13.3	

** Elution order is shown in Fig. 3-1 for each column*

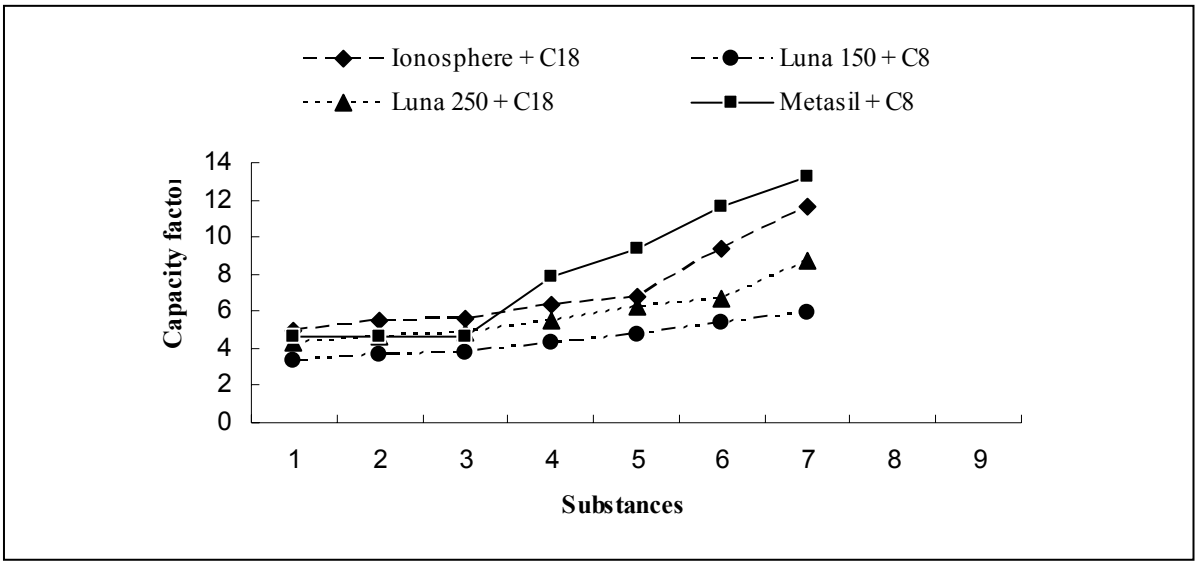


Fig. 3-2 *Comparison of capacity factors for investigated columns under optimised conditions*

As illustrated in Table 3-3 and Fig. 3-2 the following results were achieved: Although the Ionosphere™ column showed best separation, capacity factors were high and thus a long analysis time would be required. Moreover, resolution of the first 4 peaks was $\alpha \leq 1.1$. On the Metasil™ column coelution of the first three peaks occurred, whereas the following 4 peaks were eluted and separated over a time range of 25 min. This can be seen in Fig. 3-2 where the graph of the latter column shows a plateau in the first part (coelution) followed by the widest distribution of capacity factors over the graphic in comparison to the other investigated columns. On the LunaSCX™ columns separation ($\alpha \geq 1.1$), except for the separation of scopolamine and 6-AM, within an appropriate time range was achieved. As can be seen from the capacity and separation factors in

Table 3-3, the resolution of peak 3 and 4 as well as 6 and 7 was improved on the 250 mm material. Unfortunately, separation of scopolamine and 6-AM (peak 2 and 3) was still not possible under the optimised conditions.

In order to improve resolution and pursue better separation of pharmacological and toxicological relevant analytes, the coupled reversed phase column was replaced by a second LunaSCX^{TM} column. Different SCX column lengths were investigated (150 x 4.6 mm + 150 x 4.6 mm and 150 x 4.6 mm + 250 x 4.6 mm). With the coupling of two 150 mm LunaSCX^{TM} columns, improved resolution of the seven reference standards ($\alpha \geq 1.1$) within a reasonable time for analysis (30 min) was achieved under optimised buffer/organic solvent ratio (68.5/31.5, v/v) and flow rate (1.2 mL/min) conditions. On the 150 x 4.6 mm + 250 x 4.6 mm columns in comparison, RTs were increased (+ 2 min) but no better peak separation was gained as can be seen in Fig. 3-3, where example chromatograms of both coupled columns are shown. Coupling with the Ionosphere^{TM} column resulted in even longer RTs and decreased peak shape. Consequently, the coupled 150 mm LunaSCX^{TM} columns were chosen for the analytical separation.

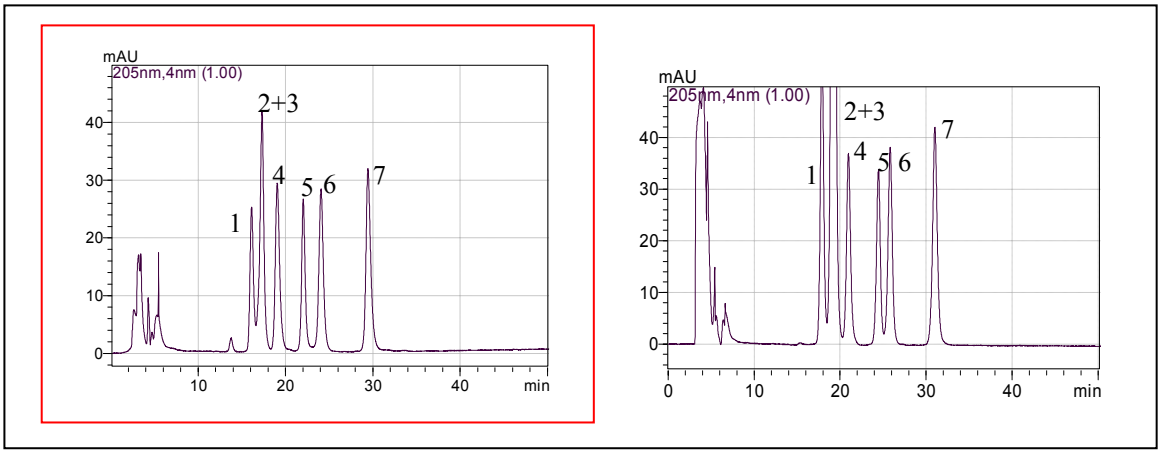


Fig. 3-3 *Chromatogram of the direct injection onto the finally chosen columns for the analytical separation (2 x LunaSCX^{TM} 150 x 4.6 mm, flow rate 1.2 mL/min, left, red frame) and of the 150 + 250 mm LunaSCX^{TM} columns (right) under the same conditions. Peak identification: 1: Psilocin, 2: Scopolamine, 3: 6-AM, 4: Morphine, 5: Tilidine, 6: Codeine, 7: EDDP*

Surprisingly, scopolamine and 6-AM were still not separated. However, as the combination of these latter two compounds is not to be expected in routine toxicology samples, further focus was put on the separation of compounds, which in practice will occur together.

Therefore, in a following step the separation of target analytes for this method, which will appear together (e.g. atropine/scopolamine, MDA/MDMA, amphetamine/methamphetamine, 6-acetyl-morphine/morphine/methadone) was successfully proved.

The oven temperature was set to 40 °C, as the toxicological screening method for plasma [22, 102] (2.3), which should be established in the same system, was run at this temperature. The injection volume was raised to 1.0 mL.

In summary, HPLC separation was performed on the selected two coupled strong cation-exchange columns (2 x LunaSCX™ 150 x 4.6 mm, 5 µm, including a SCX 4.0 x 3.0 mm pre-(guard)cartridge) at a flow rate of 1.2 mL/min under isocratic conditions. The mobile phase was optimised to 68.5% 0.05 M dihydrogenphosphate buffer pH 2.3 and 31.5% acetonitrile/water (90/10, v/v). Under these conditions separation of substances from a wide range of chemical structures and separation of polar substances like psilocin or scopolamine from the injection peak was achieved.

In order to increase resolution, the influence of amine modifiers was investigated. In general, amine modifiers are used as competitive bases for the analysis of polar compounds because they reduce peak tailing caused by acidic silanol groups of the stationary phase. On the other hand the use of amine modifiers can lead to unwanted side products (salt and water) which may absorb in the lower UV range.

The addition of N,N-dimethyloctylamine, ($N(CH_3)_2C_8H_{11}$) and tetramethylammonium chloride ($N(CH_3)_4Cl$) reduced the analysis time by 1 min, led to a pH increase of the mobile phase to 2.43 and resulted in slightly better peak shapes. However, these advantages were too small to justify the required preparation time, the use of toxic chemicals, the possibility of unwanted side products and the lower quality of UV spectra due to the increased pH of the mobile phase.

3.2.2 On-line Extraction

Polymer (electroneutral), weak cation-exchange (carboxylic acid functions, WCX) and strong cation-exchange (propylbenzene sulfonic groups, SCX) sorbents were investigated for on-line extraction as described in 2.2.3.2.

Using on-line extraction, the analytes were directly eluted by the mobile phase from the extraction into the analytical columns. Hence, the extraction column had to fulfil three major criteria: selective extraction of the analytes, elution of possible interfering matrix compounds in a wash step, and elution of the target analytes with the mobile phase 1.

The use of polymer (electroneutral) materials allowed the elution with the acidic mobile phase, which converted the retained analytes to cations and thus eluted them. However, this led to an early elution (break-through) of the analytes during the wash with an organic wash solvent (acetonitrile/water, 90/10, v/v).

Due to the $pK_a = 1$ of the sulfonic acid groups, SCX material is charged at nearly every pH. Hence, elution of the analytes required a high percentage of organic solvents and a pH adjustment to $pH \geq 10$ to neutralise and then elute the analytes. The latter described elution conditions were not compatible with the use of the commercial spectra library and therefore SCX material could not be used although all target analytes were easily retained (= extracted) on the sulfonic acid functional groups. Elution of the analytes from the SCX material with mobile phase 1 was not possible.

Only the polymer based WCX sorbent (StrataX-CW™, exchange capacity = approx. 1 meq/g) met the requirements of the method concerning sample clean-up and elution with the mobile phase. The samples were loaded onto the extraction column with phosphate buffer pH 6 (loading buffer), so the analytes and the functional carboxylic acids of the WCX were converted to an opposite charge and therefore pursued selective extraction of the analytes from the urine matrix. It could be demonstrated, that when using WCX material, the loading and sample diluting buffer should be of low ionic strength to increase recovery. Therefore 0.01 M phosphate buffer pH 6 was used for this purpose. Due to the high exchange capacity of the WCX used, a wash step with a high organic fraction (acetonitrile/water, 90/10, v/v, first wash solvent) was possible without any decrease in recovery. When a silica based WCX sorbent with a mean exchange capacity of 0.35 meq/g (Bond Elut CBA™) was evaluated under identical conditions, the analytes were eluted from the extraction column during the wash step. This might have been caused by the lower exchange capacity compared to the StrataX-CW™ material, which means fewer options to bind the target analytes by covalent binding.

Table 3-4 gives a survey of the investigated extraction sorbents and their extraction behaviour concerning the requirements of the developed method such as break-through and elution.

Table 3-4 *Data of investigated extraction columns*

Material	Column name	Break-through	Elution
1. Polymer (electroneutral)			
Polystyrene-divinylbenzene	StrataX™	yes	yes
Styrenedivinyl-benzene/methacrylate copolymer	Nexus™	yes	yes
N-vinyl-pyrrolidone/divinyl-benzene copolymer	Oasis HLB™	yes	yes
2. Weak cation-exchanger (WCX)			
Polymer based carboxylic acid functions	StrataX-CW™	no	yes
Silica based carboxylic acid function	Bond Elut CBA™	yes	yes
3. Strong cation-exchanger (SCX)			
Polymer based benzene-sulphonic acid functions	StrataX-C™	no	no
Benzene-sulphonic acid functions	Oasis MCX™	no	no

It can be seen that all polymer materials allowed elution with the mobile phase 1 but that the target analytes were washed from the extraction column during the acetonitirile/water wash step. On the SCX material on the other hand, the target analytes were retained in the acetonitirile/water wash step but were not eluted with the mobile phase 1. Only the polymer based WCX material (StrataX-CW™) allowed both, retention of all target analytes during the wash step and elution with the mobile phase 1.

The time required to equilibrate and adjust the extraction column to a certain pH was 4 min at a flow rate of 1 mL/min. This was due to the dead volume of the gradient unit enabling pump A to transport four different solvents. When switching from one solvent to another, the first solvent had to be fully displaced by the second solvent. Therefore, when switching from the loading buffer to the first wash solvent, 4 min at a flow rate of 1 mL/min were required until the extraction column was equilibrated with the latter solvent. Subsequently, the extraction column could be washed with the first wash solvent for 1 min without substance break-through. In order to shorten time for the extraction procedure, the flow rate was raised to 2 mL/min, which resulted in a total wash time of 2.5 min.

Before eluting the analytes, the front-head of the extraction column, where matrix particulates usually accumulate [55], was washed with water (second wash solvent) to avoid contamination of the analytical columns. Using water instead of the first wash solvent, the flow through the extraction column was reversed without any decrease in recovery. With the combination of a more apolar and a polar wash solvent as described, a good clean-up by removing interferences prior to elution was achieved. Finally, the mobile phase 1 (pH 2.3) was neutralising the carboxylic function of the weak cation-exchanger thus eluting the analytes.

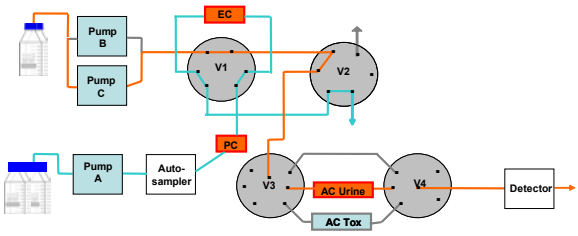
In conclusion, for the application only the StrataX-CW^{TM} column permitted the wash step with the first wash solvent and the elution of the analytes with the mobile phase 1.

In order to increase the shelf-life of the extraction column, which had to be renewed every 50th injection due to clogging of the front head, different pre-(guard)cartridges were investigated to protect the extraction column (2.1.3). Of all investigated pre-(guard)cartridges, the C 18 material (pore size 100 Å) showed best results concerning the filter effect and column clogging. The life-time of the extraction column could be increased from 50 to 300 injections taking advantage of the filter effect of the C 18 pre-(guard)cartridge, which had to be replaced every 50 injections. The C18 widepore column (pore size 300 Å) in comparison had to be replaced every 90 injections, but did not efficiently protect the extraction column from contamination.

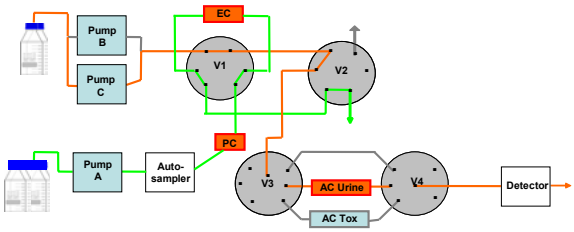
Because of the wash and elution step in the back flush mode, the C18 pre-(guard)cartridge, 100 Å was placed in front of the extraction column and also before the switching valves so eluting interferences from the pre-(guard)cartridge onto the analytical column was avoided (for schematic set-up, see Fig. 3-4).

3.2.3 Final Analytical Procedure

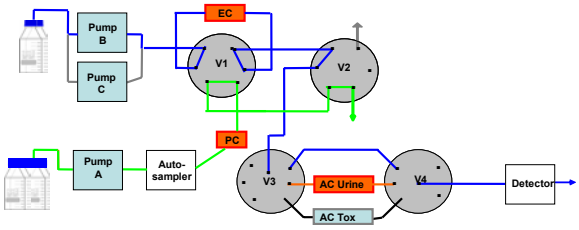
For the final optimised procedure samples were prepared as described in 2.1.4 and placed into the auto sampler. The sampling needle was raised 0.6 cm above the bottom of the 2.0-mL polypropylene cup to avoid sucking up possible precipitate. The sample aliquot of 1 mL was brought onto the extraction column with loading buffer. The positively charged analytes were retained at the activated weak cation-exchange function; the matrix was eluted into the waste. The loading buffer and the following first wash solvent were transported by pump A, which was connected to a gradient unit, enabled to mix up to 4 solvents. The latter two steps took 2.5 min each; the flow rate was 2 mL/min. During the first 4.5 min of the on-line extraction, the analytical columns were conditioned with mobile phase via pump C. Pump C was connected to pump B by a T-piece. Thus, before switching to the back flush mode, the first two valves were activated in order to elute mobile phase from the capillaries to avoid an early elution of the analytes. After 5 min the first two valves were switched again to perform the second wash step with water in the back flush mode (pump B). In that step, the analytes were still retained at the cation-exchange function while the front end of the extraction column, where matrix particulates accumulate, was washed to avoid contamination of the analytical columns. The flow rate was raised to 3 mL/min, which reduced the time required for that step to 1 min. At that time the condition of the analytical columns was maintained by pump A. The following elution was achieved by neutralising the carboxylic functions of the cation-exchange material with mobile phase 1. The isocratic elution step was performed in the back flush mode to avoid peak broadening and separation of the analytes on the extraction column. The flow rate of the mobile phase, maintained by pump C, was 1.2 mL/min. During the analytical separation the flow rate of pump A was lowered to 0.5 mL/min until 5 min before the end of analysis, the switching valves were activated again in order to separate the extraction column from the analytical columns. This approach permitted another wash step of the extraction column with the first wash solvent and the equilibration with loading buffer for the next analysis. The final running time of the method including on-line extraction and analytical separation was 41 min. In Fig. 3-4 the circuit diagram for each working step is shown and the final procedure is summarised in Table 3-5.



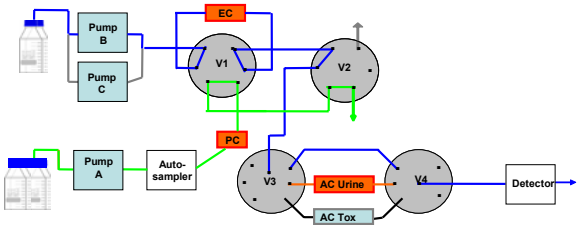
Loading (light blue)



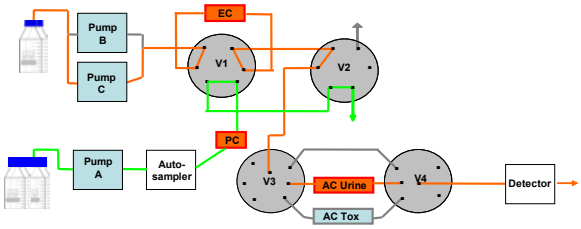
1st wash (green)



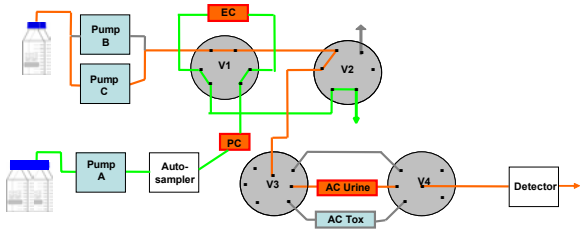
Preparation of the 2nd wash (blue)



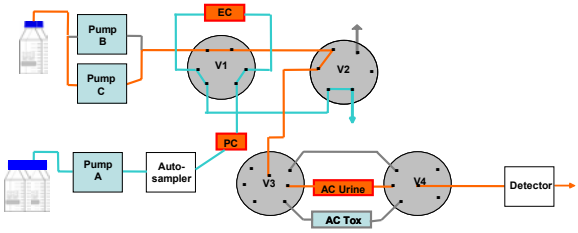
2nd wash (blue)



Elution and analytical separation (yellow)



Analytical separation (yellow) wash of the extraction column (green)



Analytical separation (yellow), equilibration of the extraction column (light blue)

PC:	Pre-(guard)cartridge
EC:	Extraction column
AC:	Analytical columns
AC Urine	2 x LunaSCX™ 150 x 4.6 mm
AC Tox	Coupled Nucleosil ™ 100 C8 columns
V1, V2:	Two-position valves
V3, V4:	Column switching valves
	Loading buffer (0.01 M phosphate buffer pH 6)
	1st wash solvent (acetonitrile/water (90/10, v/v))
	2nd wash solvent (water)
	Mobile phase 1
	Not in use

Fig. 3-4 *Circuit diagram for each working step*

Table 3-5 *Event table of the final procedure*

Working step	Time (min)	Pump A		Pump B		Pump C	
		Flow rate (mL/min)	Solvent*	Flow rate (mL/min)	Solvent*	Flow rate (mL/min)	Solvent
Conditioning, loading	0.0- 2.5	2.0	1	0	-	1.2	4
1st Wash	2.5- 5.0	2.0	2	0	-	1.2	4
Preparation for the 2nd wash	4.5- 5.0	2.0	2	3.0	3	1.2	4
2nd Wash	5.0- 6.0	1.2	4	3.0	3	0	-
Elution and analytical separation	6.0-35.0	0.5	4	0	-	1.2	4
Analytical separation, wash of the extraction column	35.0-37.5	2.0	2	0	-	1.2	4
Analytical separation, conditioning of the extraction column	37.5-41.0	2.0	1	0	-	1.2	4

**1 = loading buffer (0.01 M phosphate buffer pH 6), 2 = first wash solvent (acetonitrile/water (90/10, v/v), 3 = second wash solvent (water), 4 = mobile phase1*

3.2.4 Compound Identification

An additional, method-specific library, which included extractable, reliably identifiable analytes and provided spectra and RRT of basic drugs and their metabolites (7.4, appendix) was set-up and continuously expanded. Neostigmine bromide, a quaternary drug, with renal excretion < 5% as unchanged drug [97], was used as I.S.. A relatively high similarity (≥ 0.999) was chosen in order to allow secure determination between compounds with similar spectra like 6-AM and morphine besides the RT. The detection wavelengths were set at 205 and 235 nm. The choice of the wavelengths led to an additional identification hint of amphetamine derivatives, which showed good absorption at 205 nm and almost no absorption at 235 nm. The same effect applied for the I.S.. To monitor the developed system the PCS was used, an example chromatogram is shown in Fig. 3-5.

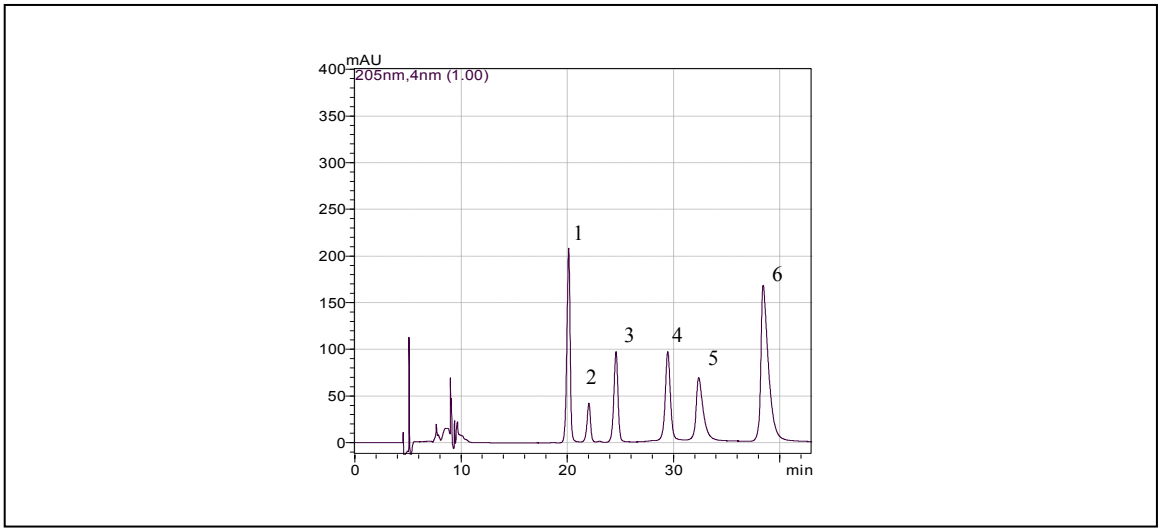


Fig. 3-5 *Example chromatogram of the on-line extraction and analysis of PCS (Peak identification: 1: MDA, 2: Scopolamine, 3: Morphine, 4: Codeine, 5: EDDP, 6: I.S.*

3.2.5 Validation

The purpose of developing qualitative screening methods is to identify a broad spectrum of analytes. Therefore, extraction and analysis conditions always present a compromise for the different analytes [89]. Regarding validation of the system the PCS 1 (buffer matrix) and PCS 2 (urine matrix) consisting of six analytes from different chemical classes and pK_a values were used for evaluation following the FDA guideline for bioanalytical method validation [96].

3.2.5.1 Selectivity/Specificity

For the assessment of selectivity/specificity the absence of interfering peaks at the RT of the analytes (S/N > 3) analysing urine samples obtained from six healthy volunteers was verified. In addition, secure identification of multiple drug cases was evaluated with the successful separation and identification of possible opiates, alkaloids, amphetamine-derivatives, tilidine and tilidine metabolites. In Fig. 3-6 example chromatograms of a blank urine sample and a blank urine sample spiked with I.S. are shown. During the first six minutes of the analysis, on-line extraction was performed, which resulted in visible switching peaks in the chromatogram (2-10 min). Within the following separation no interfering matrix peaks with a S/N > 3 were detected.

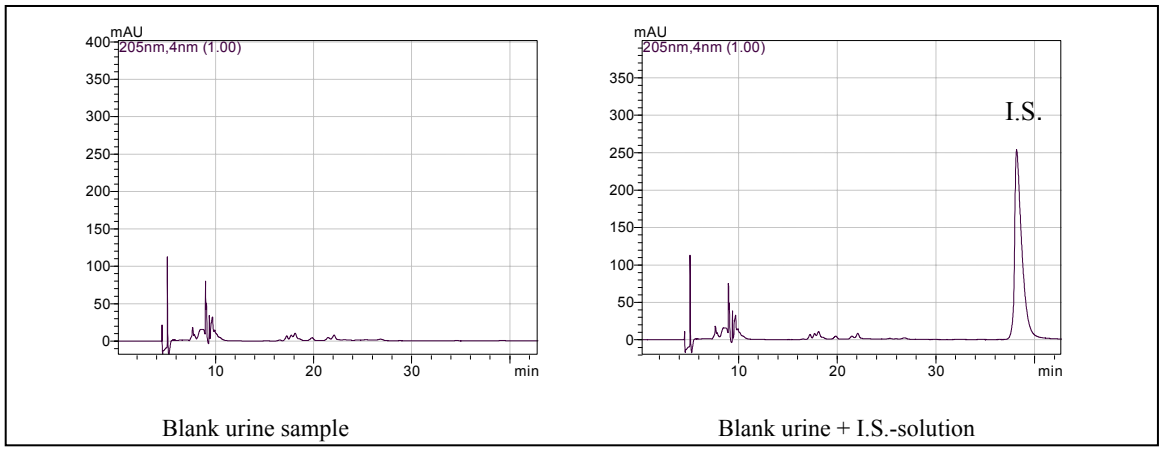


Fig. 3-6 *Example chromatograms of a blank urine sample and a blank urine sample spiked with I.S. (c = 5 µg/mL)*

3.2.5.2 Stability

Stock solutions and PCS 2 (urine matrix) stored in the freezer showed stability over the investigated time range of 28 days. Mean recovery ranged from 93-104% compared to the freshly prepared PCS 2 (each n = 3). It was found that storing PCS 2 at room temperature or in the fridge, respectively, led to strong degradation of scopolamine (-80%-points in recovery) due to an increase in the pH of the urine sample over time because of formation of ammonia [82]. This result corresponded with the described pH optimum of pH 3 for the scopolamine stability described in the literature [105]. Calculated mean recoveries for frozen PCS 1 (buffer matrix) ranged from 98-99% over the investigated time period of 28 days. The stability results are summarised in Table 7-5 - 7-9, 7.5 in the appendix.

As the PCS should be used to monitor the analytical system, such as control the analytical separation and extraction performance, it was decided to only use PCS 1 samples (0.01 M phosphate buffer pH 6 spiked with PCS analytes). In comparison to PCS 2, PCS 1 samples remained pH stable and therefore analyte concentrations were not affected by pH changes or degradation when thawed. Therefore, changes in recovery (concentration) corresponded and will only correspond to the extraction column performance. Moreover, scopolamine was replaced by tilidine, because tilidine showed better stability at pH 6. Although tilidine is a prodrug and therefore is excreted in urine to less than 0.1% [106], it was chosen as it has the same basicity as scopolamine (pK_a 7.6) and an appropriate RT for the distribution over the chromatogram. The heroin metabolite 6-AM was added to the new PCS 1* sample in order to allow secure distinction between normorphine/morphine and 6-AM by RT. In Fig. 3-7 an example chromatogram of the new PCS 1* sample is shown.

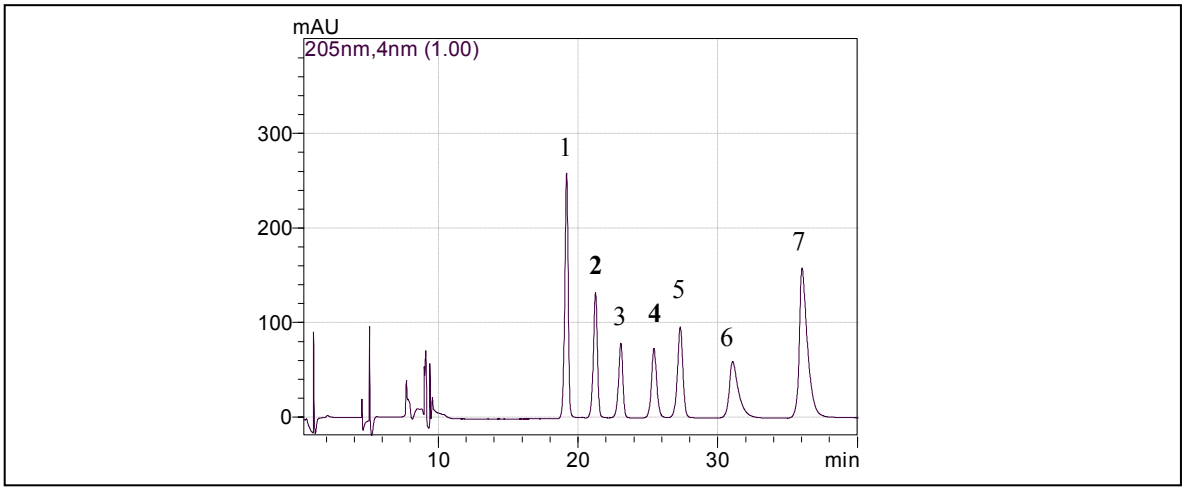


Fig. 3-7 *Chromatogram of the new PCS 1* (Peak identification: 1: MDA,* ***2: 6-AM****, 3: Morphine,* ***4: Tilidine****, 5: Codeine, 6: EDDP, 7: I.S.*

Long-term stability investigations over a time period of 76 days (n = 3) resulted in the following recovery and RSD values: 6-AM 96%, 2.3% RSD, codeine 99%, 8.6% RSD, EDDP 98%, 1.7% RSD, MDA 98%, 0.9% RSD, morphine 98%, 4.8% RSD, tilidine 96%, 4.8% RSD, I.S. 95%, 10.3% RSD. The RSD values of the new PCS 1* sample were < 15% over the investigated time range and therefore stability was accepted. The detailed results are given in Table 7-10, 7.5 in the appendix.

Psilocin stability

The stability of psilocin was investigated with the aim to avoid psilocin degradation during sample hydrolysis and storage. In a first experiment (2.2.4.8) the psilocin stability during glucuronide hydrolysis at 45 °C was investigated. It was shown, that psilocin was stable during the first four hours (SD of the mean recovery ± 5%). As a result, glucuronide hydrolysis following the method of Grieshaber et al. [85] with a hydrolysis time of 1.5 h was carried out without psilocin degradation. After 4 h of hydrolysis, psilocin degradation was observed. As can be seen in Table 3-6, UV influence played an important role for the stability of psilocin than temperature. The better stability results of the samples that were hydrolysed without UV protection compared to those without hydrolysis and without UV protection were explained by the partly UV protection from the heating block (dry-bath) used for hydrolysis.

Table 3-6 *Psilocin stability (n = 3)*

	Psilocin stability (mean recovery, % ± SD)			
Time (h)	**plus UV**	**without UV**	**plus hydrolysis plus UV**	**plus hydrolysis without UV**
0	100 ± 5.3	100 ± 5.3	100 ± 5.3	100 ± 5.3
4	100 ± 3.4	99 ± 4.2	100 ± 4.5	100 ± 5.5
8	97 ± 6.4	100 ± 5.6	92 ± 3.5	100 ± 3.1
24	74 ± 4.3	100 ± 5.3	84 ± 2.8	91 ± 4.9

In a second experiment storage stability of psilocin was investigated by comparing the stability of frozen samples (-18 ± 3 °C) to those stored in the fridge (5-8 °C). Frozen psilocin samples showed stability over the investigated time period of 42 days (RSD of the peak area = 9.8%, n = 3). Psilocin samples stored in the fridge showed low stability: after seven days of storage only 50% of psilocin was detected and on day 42, psilocin was not detected at all. The results are summarised in Table 3-7 and Fig. 3-8. In conclusion, psilocin samples were and should be immediately prepared, analysed and then stored in the freezer. Reanalysis should preferably be performed within 28 days.

Table 3-7 *Psilocin stability during storage (n = 3)*

Time (days)	**Psilocin recovery$_{(fridge)}$, % ± SD**	**Psilocin recovery$_{(freezer)}$, % ± SD**
1	100.0 ± 5.5	100.0 ± 5.5
7	49.0 ± 3.2	99.9 ± 2.4
21	9.9 ± 7.4	86.0 ± 3.5
28	4.2 ± 3.3	92.3 ± 2.9
42	0 ± 0	79.4 ± 4.4

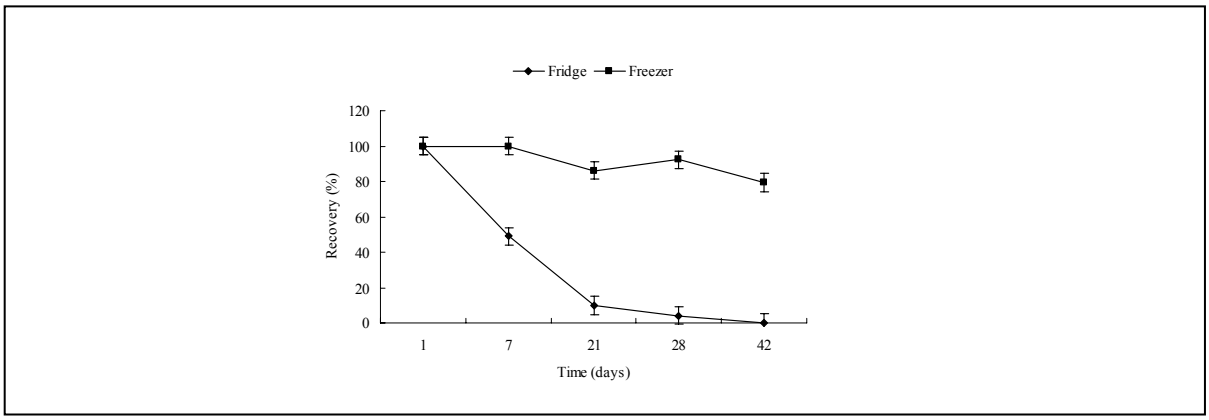


Fig. 3-8 *Psilocin stability during sample storage (n =3)*

3.2.5.3 Recovery

Recovery calculated from the average peak areas (arithmetic mean) of the extraction of the PCS 1 (n = 6) and PCS 2 (n = 6) at three concentrations in relation to the average peak areas (arithmetic mean) of the direct injection of PCS 1 (n = 6) was ≥ 73-97% for the analysed analytes. Matrix

influence on the recovery was ≤ 3%. The validation data is summarised in Table 7-12, 7.6 in the appendix.

3.2.5.4 Precision

Precision of the system was ≤ 0.8% (arithmetic mean level 1-3) for the peak area of all PCS 1 analytes (n = 6). The results for the within-day precision of the method (PCS 2, n = 6) ranged from 0.8-7.2% (arithmetic mean level 1-3) for the peak area and was 0.2% (arithmetic mean level 1-3) for the RRT. Results for between-day precision were ≤ 8.0%. In total, the acceptance criteria for precision for bioanalytical method validation of the FDA guidelines [96] were met.

3.2.5.5 Carry-Over Experiments

Carry-over experiments, injecting blank urine samples after each sample of the following concentrations 0.25, 0.5, 1.0, 2.0, 5.0, 10.0, 15.0, and 20.0 µg/mL showed a carry-over effect at concentrations > 15.0 µg/mL. The latter concentration corresponded to the ULOD. The injection of a blank sample after a sample with an absorption > 1500 mAU was necessary. Therefore, dilution of samples in cases of intoxications and injection of a water sample following DOA samples with a high immunological result were carried out.

3.2.5.6 Linearity

For linearity studies concentrations of the PCS 2 ranging from 0.1-20.0 µg/mL were extracted and analysed (n = 6). Performing unweighted linear regression between the analyte peak area and the analyte concentration revealed that linearity for the analytes was obtained from 0.2-15.0 µg/mL for codeine, EDDP, morphine, from 0.25-15 µg/mL ($R^2 \geq 0.995$) for neostigmine bromide and scopolamine ($R^2 \geq 0.995$) and 0.2-5.0 µg/mL for MDA ($R^2 = 0.993$), respectively. The small range of linearity of MDA compared to the other drugs was due to its higher molar absorption.

3.2.5.7 Limit of Detection

The LLOD may be defined as the theoretically lowest concentration of a compound giving a spectrum that can be matched against the spectra library. The lowest peak area required, yielding a spectrum that could be reliably matched, was 140 000 counts (λ = 205 nm). The detection limits for different analytes varied according to differences in extraction efficiency and molar absorption. Thus, the lower limit of detection was defined as the lowest concentration of the PCS 2 analytes yielding a S/N > 3.0 (λ = 205 nm). The upper limit of detection (ULOD), determined by the highest concentration of a compound giving a spectrum that could be matched against the spectra library,

was 15.0 µg/mL or 5.0 µg/mL (MDA), respectively. For samples with high substance concentrations e.g. acute intoxications, the ULOD might not always be sufficient. In such cases the sample has to be diluted. Dilution into the validated range of calibration showed to be linear.

3.2.5.8 Batch-to-Batch Reproducibility

Batch-to-batch reproducibility of the extraction and analytical column was examined, investigating three different batches of each column type. Overall, the RSD of the peak areas (PA) was ≤ 3.8% (n = 3) and the RRTs did not deviate at all from extraction column to extraction column. The RSD of the RRTs between the analytical column batches was < 4% (n = 6), and ≤ 0.2% for the within-run precision (n = 6), respectively.

In Table 3-8 the batch-to-batch reproducibility from extraction column to extraction column is summarised, in Table 3-9 the RRT parameters for the investigated analytical column batches are shown.

Table 3-8 *Batch-to-batch reproducibility within extraction columns calculated on the peak area (n = 3 each)*

	MDA	**Scopolamine**	**Morphine**	**Codeine**	**EDDP**	**I.S.**
$\overline{X}_{(PA^*\text{-batch 1})}$	4948506	1154956	2938342	3214995	3119922	10741130
$\overline{X}_{(PA^*\text{-batch 2})}$	4961439	1173724	2998423	3241703	3172792	11130974
$\overline{X}_{(PA^*\text{-batch 3})}$	4948389	1241199	3070487	3253578	3157610	11577358
$\overline{X}_{(PA^*\text{-batch 1-3})}$	4952778	1189960	3002417	3236758	3150108	11149821
SD	7501	45356	66163	19761	27222	418433
RSD (%)	0.15	3.81	2.20	0.61	0.86	3.75

** PA: peak area*

Table 3-9 *Relative retention time (RRT) parameters for investigated analytical column batches (n =6)*

Batch	**$RRT_{(MDA)}$**	**$RRT_{(Scopolamine)}$**	**$RRT_{(Morphine)}$**	**$RRT(_{Codeine})$**	**$RRT_{(EDDP)}$**	**$RRT_{(I.S.)}$**
1	0.57	0.62	0.68	0.79	0.86	1.00
2	0.57	0.62	0.70	0.81	0.83	1.00
3	0.53	0.57	0.65	0.77	0.84	1.00
$\overline{X}_{(RRT\ batch\ 1-3)}$	0.56	0.60	0.68	0.79	0.84	0.56
SD	0.02	0.02	0.02	0.02	0.01	0.02
RSD (%)	3.39	3.91	3.04	2.07	1.48	3.39

3.2.5.9 Calibration for Semi-Quantitative Determination

As is described in 1.1.4, quantitation in urine is difficult due to individual variation in excretion and the lack of availability of twenty-four-hour urine. However, the Remedi^{TM}-HS library (BioRad) provides response factors for semi-quantitative calibration. Therefore, it was assessed whether semi-quantitative calibration could be carried out with the developed system. Calibration of the following analytes was investigated: 6-AM, amphetamine, atropine, BEC, codeine, EDDP, MDA, MDMA, methamphetamine, methadone, morphine, nortilidine, psilocin, scopolamine. The results for the mean recovery, within-day precision, limits of detection (LLOD and ULOD), linearity by range and slope are summarised in Table 3-10. In 2.2.6, Table 2-3 the immunological assay result (in-house cut-off) is given, which had to be exceeded in order to allow analysis by the developed method. In cases with lower immunological assay results, more sensitive chromatographic methods had to be chosen. In Fig. 3-9 linearity diagrams of the investigated analytes are shown.

Table 3-10 *Calibration results (n = 6)*

Substance	**Mean recovery**	**Within-day precision**	**Linearity**		**Slope**	**Intercept**
	(%) ± SD	**RSD (%)**	**c (µg/mL)**	**R^2**		
Amphetamines						
Amphetamine	99.2 ± 0.2	0.4	0.1 - 10.0	0.9962	0.27	+ 0.05
MDA	96.9 ± 0.7	0.8	0.1 - 5.0	0.9926	0.56	+ 0.09
MDMA	84.2 ± 3.8	4.3	0.1 - 5.0	0.9970	0.55	+ 0.06
Metamphetamine	99.3 ± 0.4	0.5	0.1 - 10.0	0.9992	0.26	+ 0.02
Cocaine						
BEC*	96.4 ± 9.5	9.9	0.2 - 10.0	0.9979	0.21	- 0.01
Opiates						
Codeine	90.6 ± 1.2	0.2	0.1 - 15.0	0.9997	0.40	+ 0.02
Morphine	83.2 ± 2.9	3.5	0.1 - 15.0	0.9947	0.35	+ 0.03
6-AM	78.6 ± 0.5	2.8	0.1 - 15.0	0.9996	0.35	+ 0.03
Opioids						
EDDP	94.3 ± 1.8	1.9	0.1 - 15.0	0.9999	0.44	- 0.01
Methadone	97.0 ± 2.1	1.3	0.1 - 15.0	0.9995	0.33	+ 0.01
Alkaloids/Others						
Atropine	93.2 ± 2.0	2.4	0.2– 15.0	0.9996	0.14	+ 0.01
Psilocin	96.4 ± 1.6	0.8	0.1– 15.0	0.9950	0.40	+ 0.10
Scopolamine	78.7 ± 4.9	7.2	0.2– 15.0	0.9977	0.13	+ 0.03
Nortilidine	93.5 ± 1.8	4.0	0.1– 15.0	0.9984	0.12	+ 0.02

**BEC method*

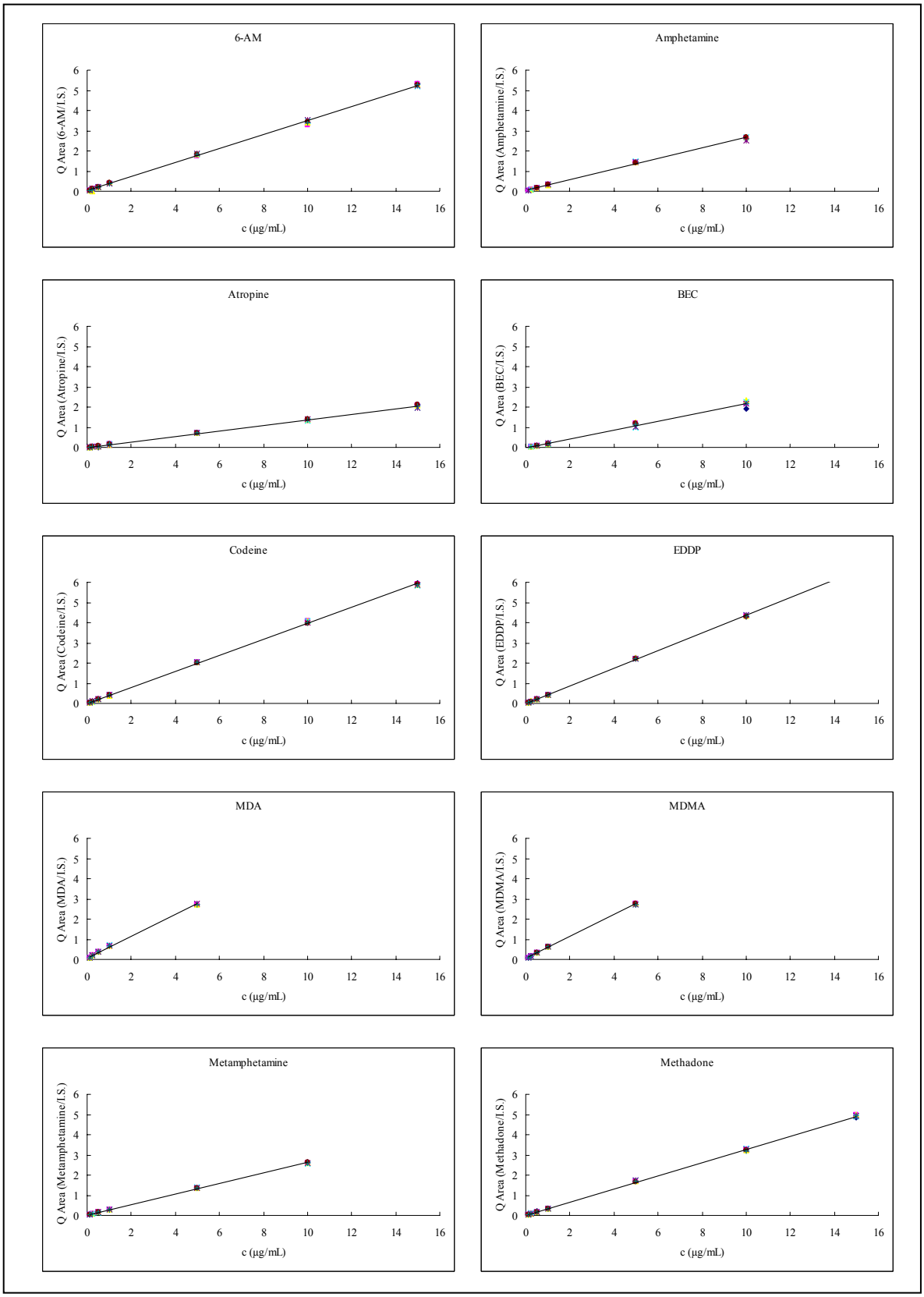


Fig. 3-9 *Linear regression for semi-quantitative analysis*

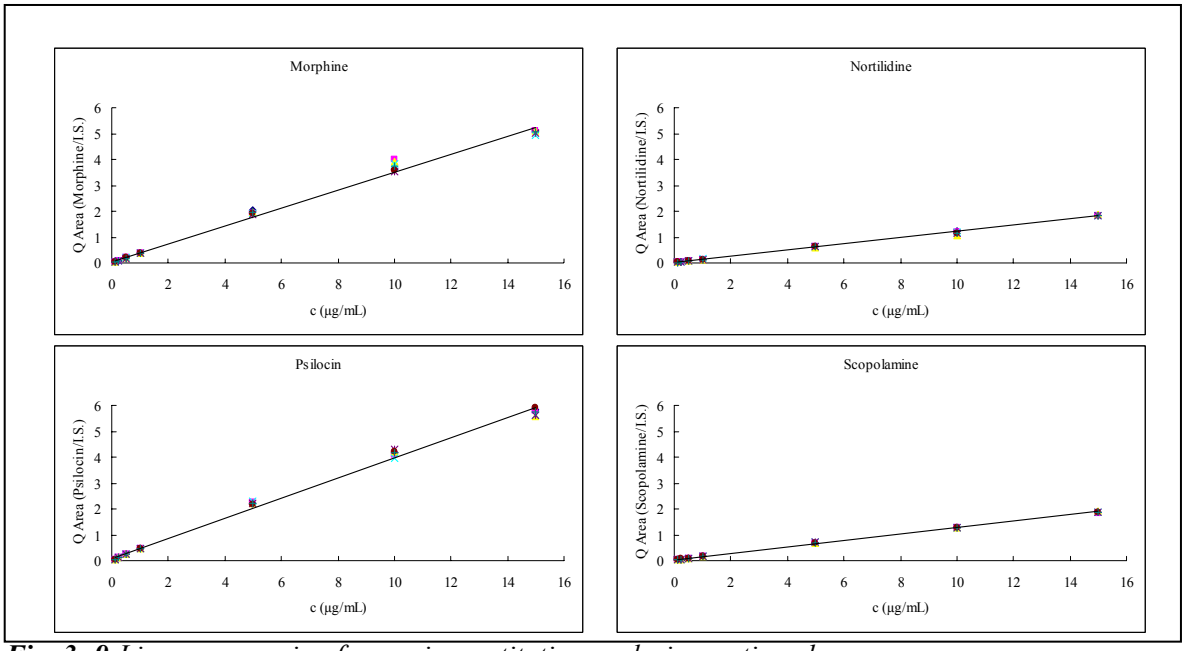


Fig. 3- 9 *Linear regression for semi-quantitative analysis, continued*

Within-day precision was ≤ 9.9% for all investigated analytes (Table 3-10). No outliers were registered (Grubbs-test ≤ ± 1.822, 5% significance niveau). Calculation of analyte concentration was carried out by linear regression according to Eq. 5 (2.2.4.9). Accuracy for the semi-quantitative calculation was -6 - +14% (Eq. 6, 2.2.4.9) and between-day precision was ≤ 11.3% for the PCS 1* sample analytes (Table 3-11).

Table 3-11 *Accuracy and between-day precision of the new PCS* (n = 6)*

Analyte	Nominal concentration (µg/mL)	Calculated concentration (µg/mL) ± SD	Relative error (%)	Between-day precision RSD (%)
6-AM	1.00	1.14 ± 0.09	+14	7.8
Codeine	1.00	0.96 ± 0.07	-4	7.3
EDDP	1.00	0.99 ± 0.17	-1	0.2
MDA	1.00	1.07 ± 0.09	+7	8.7
Morphine	1.00	0.94 ± 0.08	-6	9.3
Tilidine	1.00	0.96 ± 0.11	-4	11.3

In summary, all investigated analytes showed sufficient linearity and therefore allowed semi-quantitative calculations over the tested concentration range. Due to the higher absorption MDA, MDMA and amphetamine, methamphetamine showed linearity over a smaller concentration range of 0.2-5.0 µg/mL and 0.2-10.0 µg/mL, respectively. The lower linearity of MDA may be also explained by the higher absorption which led to a break of the calibration curve at c ≥ 5 µg/mL. Accuracy was -6% - +14% RE, within- and between-day precision were < 15% RSD and therefore met the requirements of the FDA guidelines for precision [96]. Consequently, semi-quantitative analysis with the developed method is possible. Accuracy was exemplary determined using the new

PCS* (only at one concentration). Therefore, if quantitation is required, calibration and determination of accuracy over the complete concentration range should be performed. However, due to the above mentioned limitations of quantitation in urine, semi-quantitative analysis should be carried out with caution, bearing in mind that reliable data can only be obtained from 24 h urine or in relation to a scaling parameter (e.g. creatinine).

3.2.6 Method Modifications for Critical Compounds

3.2.6.1 Benzoylecgonine Method

The cocaine metabolite BEC was only detected in high concentrations ≥ 1.0 µg/mL by the on-line extraction HPLC-DAD method for basic compounds in urine. Therefore the BEC method was developed in order to increase the BEC recovery. This was achieved by reduction of the wash step with acetonitrile/water (90/10, v/v) to 0.5 min and extension of the loading and wash step with 0.01 M phosphate buffer to 4.5 min in comparison to the earlier described extraction method for basic compounds (3.2.2), taking advantage of the early elution of BEC prior to interfering matrix compounds (RT 14.2 min). Although the extract showed more matrix interferences in the chromatogram, the sensitivity for BEC was improved and the LLOD was decreased by factor 5 ($LLOD_{(BEC)}$ BEC method: 0.2 µg/mL, $LLOD_{(BEC)}$ method for basic compounds 1.0 µg/mL). This means, that the LLOD of the BEC method is below the immunological cut-off value (300 ng/mL). Therefore, the method provides sufficient sensitivity for BEC confirmation analysis. In Fig. 3-10 the chromatograms of a BEC positive sample at the immunological cut-off concentration (300 ng/mL) analysed with the screening method for basic compounds in urine (1) and the BEC method (2) are shown.

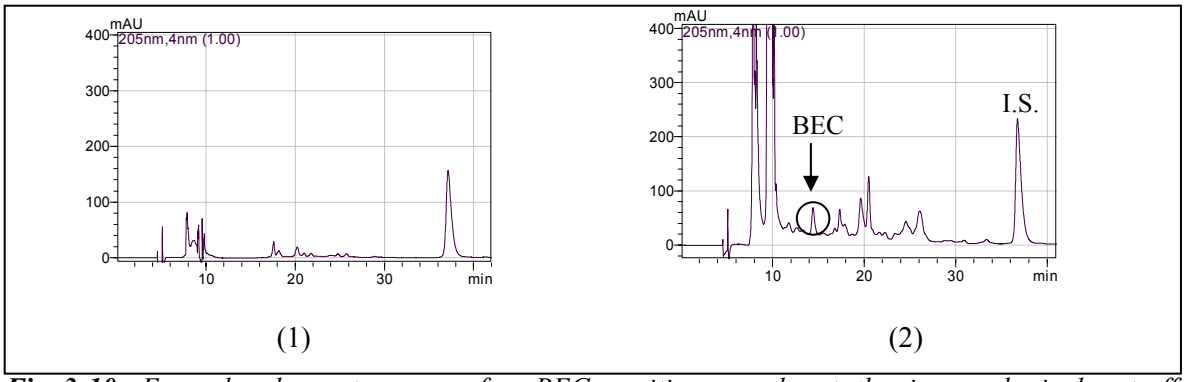


Fig. 3-10 *Example chromatograms of a BEC positive sample at the immunological cut-off concentration (c = 300 ng/mL) analysed by the screening method for basic compounds in urine (1) and the BEC method (2).*

Whereas in (1) the BEC peak was not detectable, in (2) the BEC peak was clearly selectively detectable, although overall the chromatogram showed more matrix interferences. During the

analytical run, the extraction column had to be washed with acetonitrile/water (90/10, v/v) to clean the extraction column and avoid carry-over into the following sample analysis.

3.2.6.2 Method for Late Eluting Compounds

Some compounds with more than two nitrogen atoms (aripiprazol, clozapine, olanzapine, opipramol, pipamperone, quetiapine, risperidone, ziprasidone) were not analysed within an appropriate analysis time by the method due to strong interactions between the dications and the SCX material, which led to RTs > 8 h (late eluting compounds). As a consequence, the dications were eluted from the extraction column into a reversed phase column, taking advantage of the column switching valves. With this approach the possibility to analyse late eluting compounds that could not be eluted on the SCX material was achieved and could be used when samples were screened negative with the method for basic compounds and other methods within the scope of systematic toxicological analysis. In Fig. 3-11 an example chromatogram of a clozapine positive sample is shown, where clozapine and its metabolite norclozapine were identified. In comparison to the long retention on the coupled SCX columns, the RT on the coupled Nucleosil^{TM} 100 C8 columns was short and close to the injection peak, directly behind the I.S. (neostigmine bromide). Both effects were due to the same reason, the relatively high polarity of the compounds.

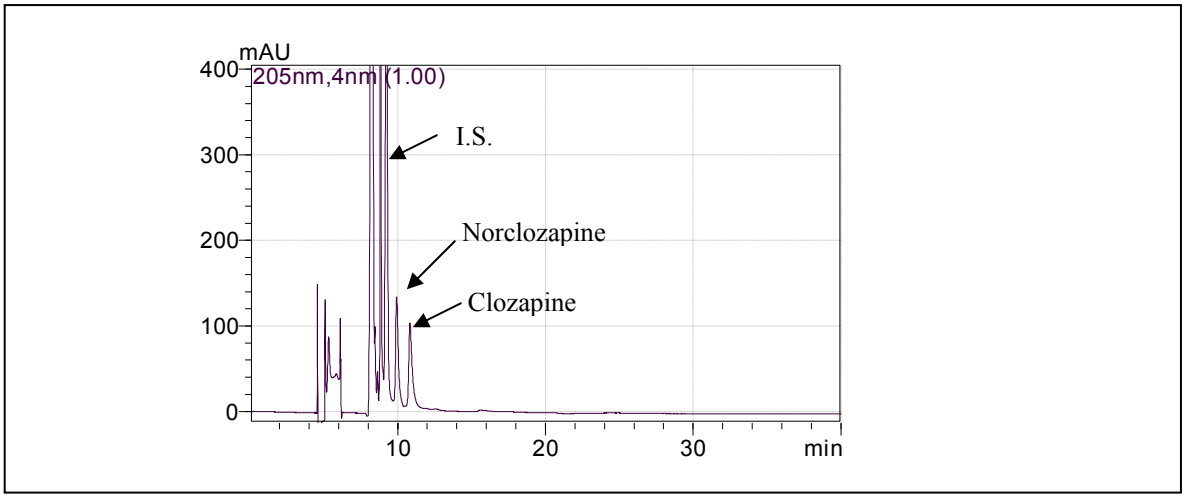


Fig. 3-11 *Example chromatogram with the LEC method*

3.2.7 Analysis of Authentic Urine Samples and Comparison with the Remedi^{TM}-HS

In order to investigate the applicability of the developed method for the toxicological routine use, 405 authentic urine samples were analysed and the analysis results were compared to those obtained from an existing urine screening system (Remedi^{TM}-HS) by parallel analysis. Besides the use of both systems in cases of acute intoxications within STA, DOA confirmation analysis was investigated.

3.2.7.1 STA

The results of investigated cases of general unknown screenings within the scope of STA when urine samples were available besides plasma samples are summarised in Table 3-12. Except for the detected alkaloids, all compounds were also detected in plasma within routine STA at the laboratory of the Institute of Toxicology-Clinical Toxicology and Poison Control Centre Berlin. Additional information from urine analysis was achieved in the cases of alkaloid intoxications. The developed method showed positive results in two more cases for the alkaloids psilocin and scopolamine than the Remedi^{TM}-HS, which in both cases was due to better peak separation by the developed method.

In comparison to the Remedi^{TM}-HS, clozapine, olanzapine, opipramol, quetiapine and risperidone were not identified by the developed method. This was because of their strong retention on the used HPLC material which led to RTs > 8 h as investigations on its retention behaviour showed (2.2.5.2 and 3.2.6.2). To avoid carry over of those substances into another sample analysis, a column wash was programmed after every tenth injection. For the analysis of these late eluting compounds the LEC method was run, using on-line extraction with subsequent separation on a C8 reversed phase column (2.2.5.2). As the latter late eluting compounds could be effectively and quantitatively analysed in plasma/serum, the qualitative LEC method was only used when no plasma/serum was available.

Except for the LEC, the parallel analysis of STA samples in both investigated systems proved, that the systems can be used alternatively for the compounds listed in Table 3-12. Both methods were suitable as additional methods for toxicological screening of basic drugs in urine as the wide range of identified compounds showed. As expected, due to the extraction mechanisms, none of the systems allowed extraction of neutral and acidic substances. As a consequence an on-line extraction method for the automated analysis of benzodiazepines was established in the same system (3.4).

The investigation of alkaloid positive samples showed that the additional screening of urine is necessary as long as no more sensitive methods for plasma analysis are available, as the alkaloids could not be detected in plasma due to their short half-lives in blood and/or the concentration below the required LLOD for plasma analysis.

Table 3-12 *Comparison of the developed method with the Remedi^{TM}-HS for STA*

Substance	Total of detected drugs	Detected drugs by developed method	Detected drugs Remedi^{TM}-HS
Alkaloids			
Atropine	5	**5**	3
Psilocin	2	**2**	1
Scopolamine	5	**5**	4
Antidepressants/Neuroleptics			
Amitriptyline	3	3	3
Citalopram	8	8	8
Clomethiazol	1	1	1
Chlorprotixene	1	1	1
Clomipramine	1	1	1
Clozapine	11	*	11
Diltiazem	1	1	1
Doxepine	14	14	14
Fluoxetine	1	1	1
Flurazepam	1	1	-
Olanzapine	10	*	10
Opipramol	3	*	3
Pipamperone	1	*	1
Promazine-S-oxide	5	3	**5**
Promethazine	2	2	2
Risperidone	4	*	4
Venlafaxine	4	4	4
Other compounds identified			
Ambroxol	1	1	1
Carvedilol	1	1	1
Metoclopramid	3	3	3
Metoprolol	3	3	3
Sildenafil	2	**2**	1
Tocainide	3	3	3
Urapidil	1	1	1
Verapamil	1	1	1
Xylometazoline	2	2	2
Zopiclone-Metabolite	4	4	4

**Identification by LEC method possible*

3.2.7.2 DOA Confirmation Analysis

Urine samples that were presumptively positive for DOA using immunological pre-screening and GC-MS confirmation analysis were evaluated.

Table 3-13 lists the compounds detected by the two HPLC systems in cases of DOA confirmation analysis. As is shown, the developed method and the Remedi^{TM}-HS showed comparable results when opiates and opioids (methadone and its metabolite EDDP) were analysed. The developed method presented better results concerning the detection of morphine (+ 8%-points), normorphine (+ 36%-points), 6-AM (+ 4%-points) and codeine (+ 5%-points). Norcodeine was better identified by the Remedi^{TM}-HS (+ 33%-points). Unsuccessful confirmation was mostly due to concentrations below the limit of detection (developed method: 0.1-0.3 µg/mL (3.2.5.5), Remedi^{TM}-HS system 0.2-0.5 µg/mL [55]). Another reason for unsuccessful identification was the appearance of co-medication peaks in the chromatogram, which might have overlapped the expected peak. Norcodeine, normorphine and oxycodone were not investigated with GC-MS because they had not been included in the routine program for DOA confirmation at the laboratory of the Institute of Toxicology-Clinical Toxicology and Poison Control Centre Berlin, but where identified by the HPLC methods. Tilidine and dihydrocodeine were analysed due to the suspicion of abuse of these substances and successfully confirmed by HPLC-DAD only. Tilidine showed many metabolites in urine besides its major metabolites bisnortilidine and nortilidine in both systems.

MDA was identified in one more case by the developed method, whereas the Remedi^{TM}-HS identified its parent compound MDMA in one more case.

In general, the confirmation of cocaine (positive identification of its main metabolite BEC) by the HPLC methods was too low. Therefore the modified on-line extraction method, BEC method, was used for positively screened urine samples in cases of cocaine abuse and showed benefits in the recovery of BEC (increase from 45% to 80%).

Validation results showed that the lower limit of detection of the BEC method was 0.2 µg/mL (3.2.5.9, Table 3-10) and therefore was five times lower compared to the Remedi^{TM}-HS system.

Overall, both compared HPLC systems showed to be valuable for DOA confirmation screening (amphetamines, cocaine, opiates, methadone) above the given limit of detection and replaced time- and work-intensive specialised analyses such as GC/MS methods in 81% (developed method) and 78% (Remedi^{TM}-HS) of the evaluated cases. In Fig. 3-12 and Fig. 3-14 DOA example chromatograms of the developed method are given. Fig. 3-13 and Fig. 3-15 show example chromatograms of the same samples used for Fig. 3-12 and Fig. 3-14 analysed in the Remedi^{TM}-HS system.

Table 3-13 *Comparison of chromatographic methods for DOA confirmation analysis*

Substance	Total amount	GC/MS	Developed method		Remedi^{TM}-HS	
		Number confirmed	Number confirmed	%	Number confirmed	%
Opiates						
6-AM	55	55	45	82	43	78
Codeine	118	118	94	80	88	75
Dihydrocodeine	1	n.i.	1	100	1	100
Morphine	127	127	109	86	99	78
Norcodeine	3	n.i.	2	67	3	100
Normorphine	11	n.i.	11	100	7	64
Noscapine	1	1	1	100	1	100
Oxycodone	1	n.i.	1	100	1	100
Opioids						
Methadone (EDDP)	51	51	42	82	43	84
Tilidine	5	n.i.	5	100	4	80
Amphetamine derivatives						
Amphetamine	32	32	32	100	32	100
Ephedrine	1	1	01	100	1	100
Fencamfamine	1	1	1	100	1	100
MDA	15	15	15	100	14	93
MDMA	17	17	16	94	17	100
Methoxyamphetamine	1	1	1	100	1	100
Cocaine						
BEC	46	46	21 (37)*	45 (80)*	25	54
Cocaine	18	18	13	72	11	61
Total	*504*	*483*	*410 (426)**	*81(85)**	*392*	*78*

GC/MS analysis was carried out following solid phase extraction (Bond Elut Certify^{TM}, 130 mg, 3 mL-cartridge, Varian, Darmstadt, Germany) on a GC 17A coupled to a QP-500 mass spectrometer (both from Shimadzu Europe GmbH, Duisburg, Germany) [103].
*n.i. = not investigated, *BEC method*

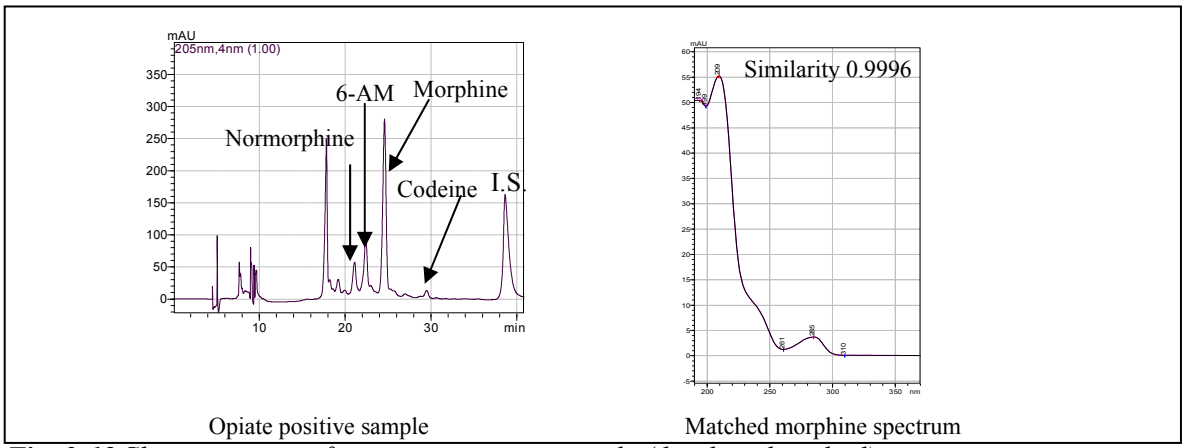


Fig. 3-12 *Chromatogram of an opiate positive sample (developed method)*

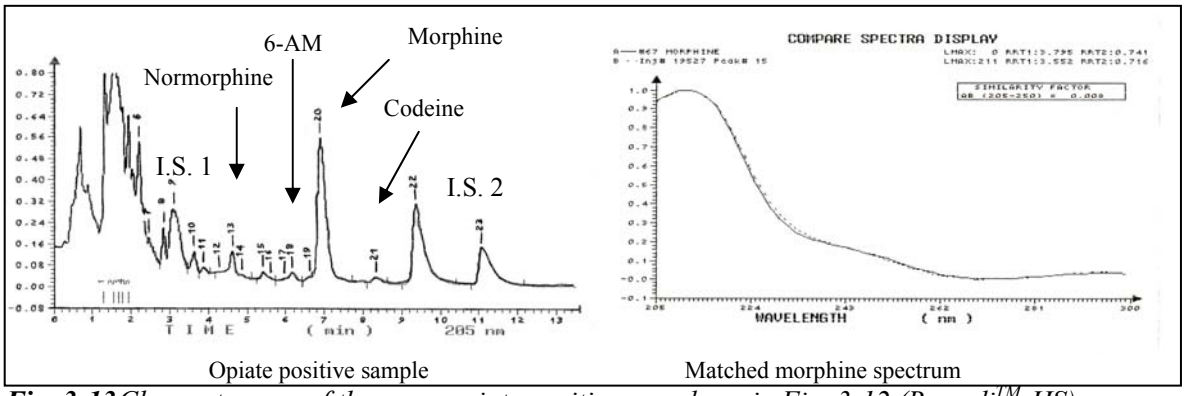


Fig. 3-13 *Chromatogram of the same opiate positive sample as in Fig. 3-12 (Remedi^{TM}-HS)*

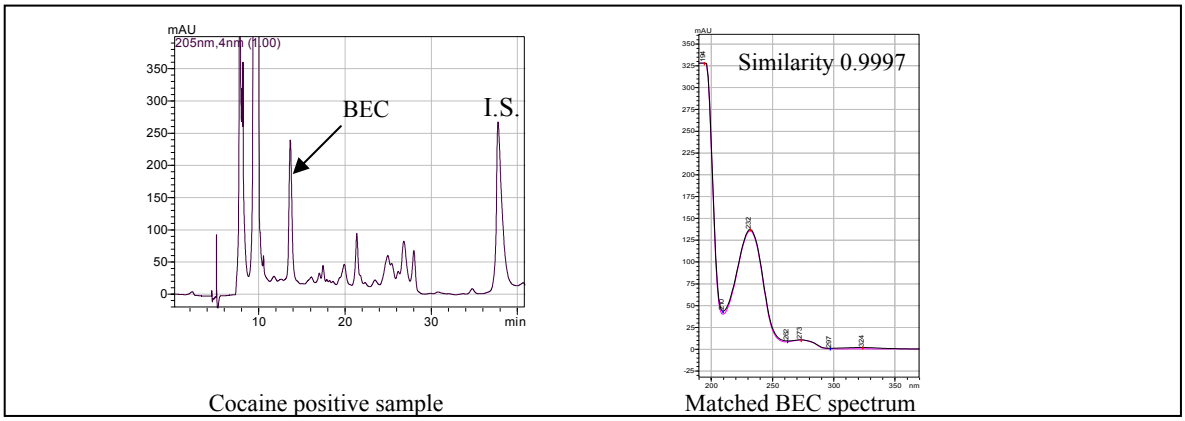


Fig. 3-14 *Chromatogram of a cocaine positive sample (developed method)*

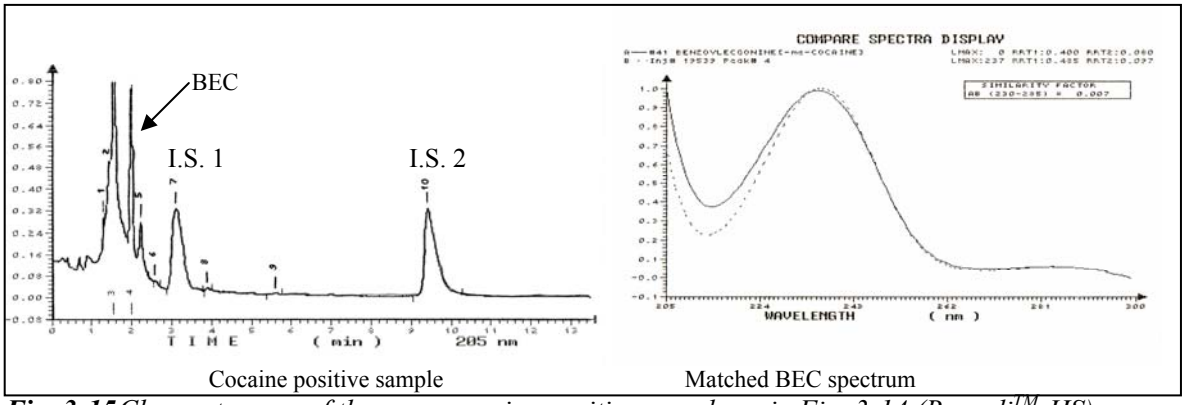


Fig. 3-15 *Chromatogram of the same cocaine positive sample as in Fig. 3-14 (Remedi^{TM}-HS)*

In summary, in cases of STA basic xenobiotics were identified by both systems. For the analysis of clozapine, opipramol, quetiapine and risperidone the LEC method had to be used with the developed system.

Due to a larger window of detection and/or higher concentrations in urine compared to plasma/serum, information about compounds which have a short half-life in blood could be gained.

Concerning the confirmation analysis of DOA, the developed method gave comparable results to the Remedi^{TM}-HS system and can be used in this field of application. The presented HPLC methods showed reliable results within the investigated limit of detection and allowed simple analysis of basic analytes over a wide range of polarities.

Both methods do not require sample derivatisation and can work unattended. An apparative comparison of both systems is summarised in Table 3-14.

Table 3-14 *Comparison of analytical characteristics between the developed method and the Remedi^{TM}-HS*

Parameter	Developed method	Remedi^{TM}-HS
Extraction Columns	1 + Pre-(guard)cartridge	2
Analytical Columns	2 + Pre-(guard)cartridge	2 + Saturator column and filter
Total columns	*3*	*5*
Lower limit of detection	0.10 – 0.25 µg/mL	0.20 – 0.50 µg/mL
Time for analysis	41 min	20 min
Equipment	Common laboratory material Modern computer equipment and software	Bio-Rad (company-dependent) Dated computer equipment and software (MS-DOS^{TM})
Flexibility	Additional methods can be set-up by the operator, e.g. BEC method, LEC method, plasma-/serum-analysis, method for neutral and acidic compounds	Method cannot be changed by the operator

In conclusion, the developed system demonstrated to be an adequate alternative to the Remedi^{TM}-HS drug profiling system. It can be used for the same fields of application, but offers the advantages of common HPLC equipment, laboratory material and modern computer software in order to keep maintenance and costs low. As can be seen from Table 3-14 the number of columns was reduced in comparison to the Remedi^{TM}-HS. In addition, the developed system is more flexible, as methods can be set-up or modified by the operator as the establishment of the BEC method showed.

3.2.8 Routine Use

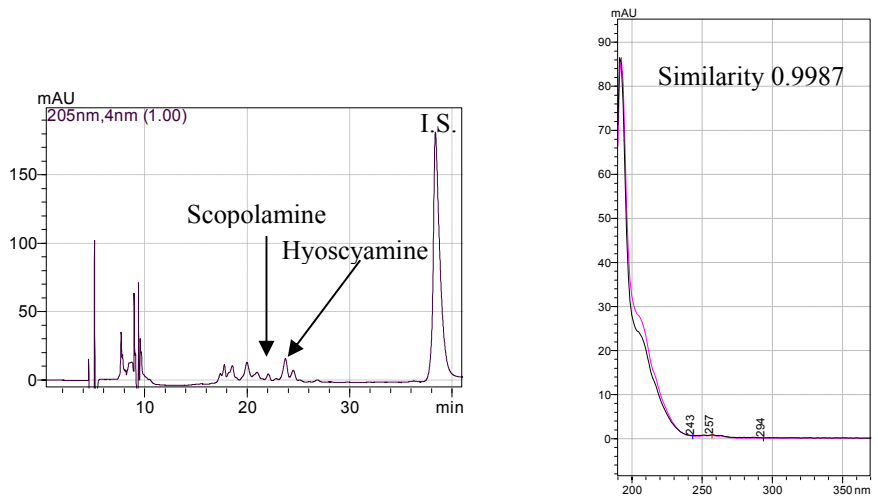
The developed method has been applied to clinical toxicological routine use including emergency analysis within STA and DOA confirmation screening. The following examples illustrate the applicability of the system reporting of two alkaloid intoxications and two drug screen confirmations.

3.2.8.1 Case 1

A 15-year old boy was brought to hospital after he had ingested Jimson weed seeds together with four other friends. He complained about headache and blurred sight. His skin was warm, the mucous membrane dry and his pupils wide (mydriasis). The patient was kept under observation and

a urine sample was sent to the laboratory of the Institute of Toxicology-Clinical Toxicology and Poison Control Centre Berlin.

Fig. 3-16 shows the results of the urine analysis by the developed method, which confirmed the Jimson weed intoxication. Scopolamine and hyoscyamine (or the racemate atropine) derive from certain plants, especially Deadly nightshade and Jimson weed. Both alkaloids are therapeutically used because of their anticholinergic qualities, but due to the effect on the central nervous system have a misuse potential [25]. Scopolamine and hyoscyamine were identified by spectra comparison and matching of the retention and RRT. As scopolamine and hyoscyamine differ only little in their chemical structures, they reveal similar spectra. Therefore, secure peak identification could only be carried out by comparison of the RRTs, which were 0.65 for hyoscyamine and 0.61 for scopolamine, respectively.



Chromatogram case 1 Matched hyoscyamine spectrum

Fig. 3-16 *Chromatogram case 1*

3.2.8.2 Case 2

A 5-year old girl was admitted to hospital by her mother because of hallucinogenic symptoms with suspicion of intoxication. The mother reported the girl had seen red trees, had been talking and singing to fishes, was restless and could not walk straight. After questioning of what had happened, the girl reported that she and her father had eaten mushroom soup made of “magic mushrooms”. During the examination and observation in hospital the symptoms decreased, but it was conspicuous that the girl had not slept for over 20 hours and showed no signs of tiredness. A urine and a plasma sample were investigated with the developed screening method for basic compounds in urine. The psychoactive compound psilocin were detected in the urine sample. The plasma sample was screened negative.

Psilocin, the dephosphorylated pharmacologically-active in vivo metabolite of psilocybin, the main psychoactive compound of Psilocybe mushrooms, is available for analysis in biological fluids [25]. As psilocin is partially excreted in the glucuronide conjugated form [26], glucuronide hydrolysis was performed prior to psilocin analysis to extend the time of detectability for psilocin in urine.

Fig. 3-17 shows the results of the drug screening performed on the urine sample following glucuronide hydrolysis. No other drugs were found. The girl left hospital after one day without any sequel. The case was handed over to the police.

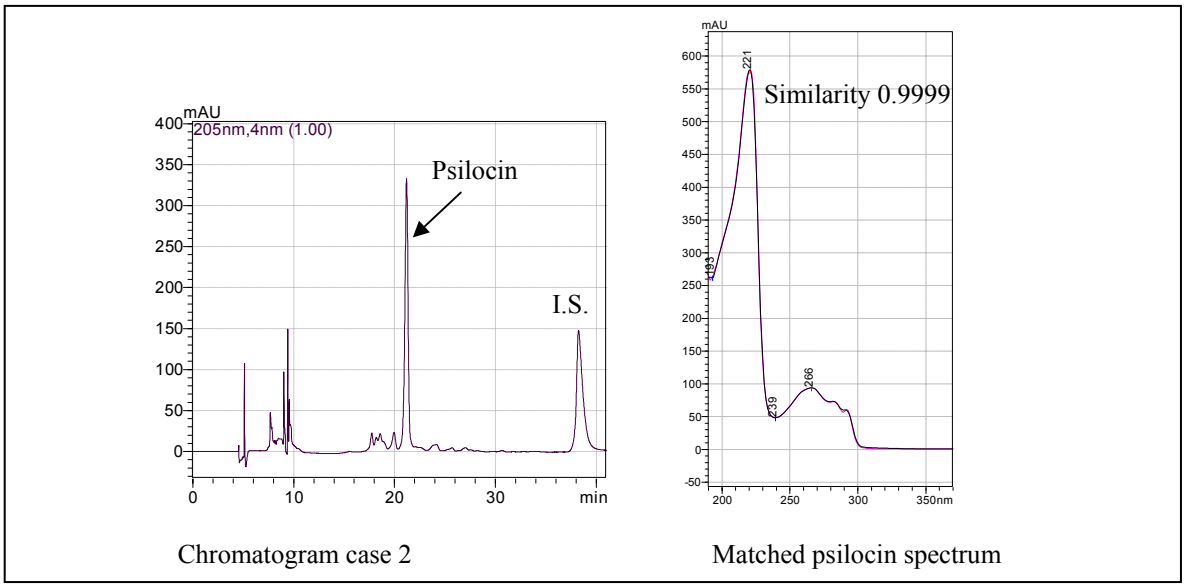
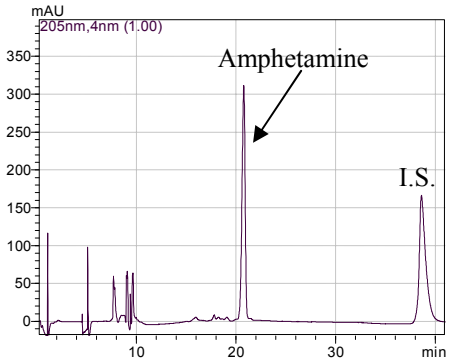
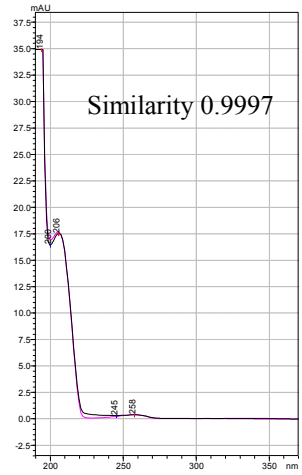


Fig. 3-17 *Chromatogram case 2*

3.2.8.3 DOA Cases

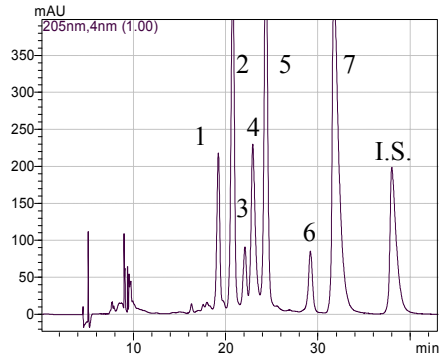
The following cases represent cases of DOA confirmation analysis following immunological pre-screening (CEDIA® DAU [102]). Fig. 3-18 shows the chromatograms obtained from the analysis of urine samples screened positive for amphetamines (case 3) and ecstasy, heroin and methadone (case 4). The subjects had to undergo urine control screening within the scope of a drug control program. As can be seen from Fig. 3-18, the sample analyses confirmed the positive results of the pre-screening. In case 3 the intake of amphetamine, in case 4 the abuse of ecstasy (MDMA, MDA), heroin (6-AM, codeine, morphine) and the intake of methadone (EDDP, methadone) was confirmed.

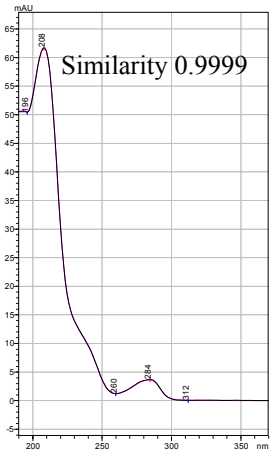




Chromatogram case 3

Matched amphetamine spectrum





Chromatogram case 4
1: MDA, 2: MDMA, 3: 6-AM, 4: Morphine
5: Methadone, 6: Codeine, 7: EDDP

Matched 6-AM spectrum

Fig. 3-18 *Chromatogram cases 3 and 4*

Cases 1 and 2 gave examples of STA in urine in which compounds that have short half-lives in blood were effectively detected in urine. Cases 3 and 4 exemplary demonstrate the successful applicability of the system to DOA confirmation screening, where presumptive positive samples from initial immunoassay screening, had to be confirmed by chromatographic methods. In 7.7 in the appendix more example chromatograms of STA and DOA cases are shown.

The results of the first year's experience of DOA confirmation screening with the developed method are summarised in Table 3-15. Altogether 3115 samples were presumptively positive by immunological pre-screening; 289 of the positive samples required confirmation analysis with a chromatographic method for legal validity. With the developed method confirmation results were achieved in 167 cases of in total 205 samples, which had compound concentrations above the LLOD of the developed method.

Table 3-15 *Results of drugs of abuse confirmation screening*

Screened substance/ substance group	**Number of immunological positive pre-screened samples**	**Number of pre-screened samples which required confirmation analysis**	**Number of samples with concentrations above the LLOD of the developed method**	**Number confirmed by the developed method**	***Percentage confirmed of all confirmed samples by the developed method, %***
6-AM	279	78	66	51	*65*
Amphetamine	15	5	5	5	*100*
Cocaine	490	33	33	30	*91*
Methadone	1225	18	15	14	*78*
Opiates	1106	155	86	67	*43*
Total	*3115*	*289*	*205*	*167*	*58*

In conclusion, in 58% of all cases, or 81% of the cases with concentrations > LLOD, respectively, work and more time intensive methods (GC-MS) were replaced by the developed method. The results corresponded to those achieved by the parallel analysis with the Remedi^{TM}-HS and GC-MS analysis described in 3.2.7, where samples with concentrations above the LLOD were investigated. Of all investigated DOA cases in 2006, confirmation results for opiates were comparably low (43%) by the developed method. This low percentage might be due to partial excretion as glucuronides, which were not extracted by the developed method. For example, as opiate-glucuronides cross-react with the opiate immunoassay, the concentration of free and therefore extractable opiates can be low despite a relatively high immunological result. First investigations on opiate hydrolysis prior to sample analysis doubled the detection rate for opiates. Consequently, the following strategy for DOA confirmation analysis was drawn from the presented results:

- All opiate positive samples (immunological cut-off ≥ 300 ng/mL) should be analysed by the developed method but have to be hydrolysed prior to analysis in order to increase the confirmation rate to ≥ 80%.
- 6-AM positive samples (immunological cut-off ≥ 10 ng/mL) should be analysed by GC-MS in order to avoid repetitive analysis (HPLC-DAD and GC-MS) as this parameter shows a low confirmation rate by the developed method (65%).
- All BEC samples (immunological cut-off ≥ 300 ng/mL) can be analysed with the BEC method without hydrolysis (confirmation rate 90%).
- All amphetamine (immunological cut-off ≥ 1000 ng/mL) and all methadone-positive samples (immunological cut-off ≥ 100 ng/mL) can be analysed with the developed method without hydrolysis (confirmation rate 100% and 80%, respectively).

3.3 Screening Method for Plasma

The screening method for plasma was established in the developed system to allow screening of plasma besides urine (2.3). Parallel plasma analysis was carried out in order to evaluate the results obtained in the developed system in comparison to a reference system in routine use (7.3).

3.3.1 Validation

The adaptation of the toxicological screening method in plasma into the established system was checked by a suggested performance test for accuracy control (2.3 and [22]). For the determination of the dead time (t_0) the histamine peak (c = 0.1 mg/mL) was investigated showing the following result: t_0 ± standard deviation (SD) = 2.84 min ± 0.002 min (n = 6). The accuracy of the auto sampler was determined by the peak area of caffeine (c = 0.1 mg/mL), which fulfilled the demand being between 260 and 280 nm. The calculation of the RT and RRT was set to MPPH (I.S., c = 0.1 mg/mL). The measured RT for MPPH ± SD was 15.31 min ± 0.02 min (n = 6). Benzene (c = 1.0 mg/mL) vibration bands were between 240 and 270 nm, which was in accordance with the demands for good resolution, precision and reproducibility of the UV spectra measurement. Thus, overall, the results of the investigations met the given criteria [22]. In Fig. 3-19 an example chromatogram of the evaluation is shown.

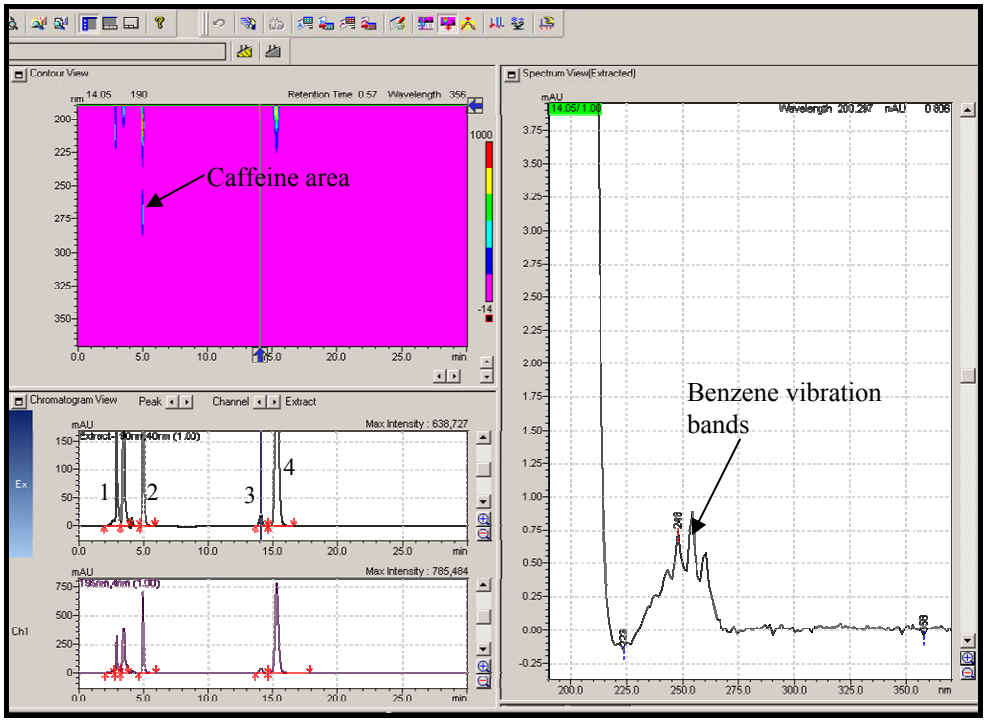


Fig. 3-19 *Validation of the toxicological screening method (left top corner: caffeine area between 260 nm and 280 nm, left bottom corner: chromatogram of performance test for accuracy control (peak identification: 1: Histamine, 2: Caffeine, 3: Benzene, 4: MPPH (I.S.)), right corner: benzene spectrum with benzene vibration bands)*

First within-day precision of the system and of the set-up method was determined showing the following results for the PCS-PA analytes: Within-day precision of the system was ≤ 4.6% RSD for the peak areas and ≤ 0.05% RSD for the RT (Table 3-16). Between-day-precision of the method was ≤ 5.8% RSD for the peak areas and ≤ 2.5% RSD for the RT of the PCS-PA analytes (Table 3-17).

Table 3-16 *Within-day precision of the system (n = 6)*

	Dipyridamol (peak area)	**MPPH (peak area)**	**Diazepam (peak area)**	**Dipyridamol (RT)**	**MPPH (RT)**	**Diazepam (RT)**
$\overline{X}$	102624	435212	339726	11.8	15.9	24.6
SD	4726	9001	7838	0.01	0.01	0.01
RSD, %	4.6	2.1	2.3	0.05	0.03	0.02

Table 3-17 *Between-day precision of the method (n = 6)*

	Dipyridamol (peak area)	**MPPH (peak area)**	**Diazepam (peak area)**	**Dipyridamol (RT)**	**MPPH (RT)**	**Diazepam (RT)**
$\overline{X}$	117730	464608	384784	11.9	15.8	24.6
SD	6828	19513	14237	0.30	0.14	0.32
RSD, %	5.8	4.2	3.7	2.49	0.88	1.32

The analysis of PCS-PA with the toxicological screening method for plasma (n = 6) by the developed system and by a reference HPLC system in routine use in order to determine the system-to-system-precision resulted in a RSD ≤ 6.6% ($RSD_{(developed\ method)} \leq 4.6\%$, $RSD_{(reference\ system)} \leq 3.7\%$) for the analysed compounds. The accuracy (RE, %) from system to system was -8.9% - -6.2% for the investigated analytes (Table 3-18) and in general showed a lower peak area as trend for the analysis by the developed method. The validation data with RSD values < 15% confirmed the successful set-up of the method. Therefore, the adaptation of results obtained from previous investigations on the reference system, e.g. RRTs set to MPPH, was justified.

Table 3-18 *System-to-system precision and accuracy (n = 6)*

	Dipyridamol ($X_{(peak\ area)}$ ± SD)	**MPPH ($X_{(peak\ area)}$ ± SD)**	**Diazepam ($X_{(peak\ area)}$ ± SD)**
Developed system	102624 ± 4726	435212 ± 9001	339726 ± 7838
System in routine use	109457 ± 4091	472874 ± 13579	372773 ± 11307
System-to-system precision, %	4.6	5.9	6.6
Accuracy from system-to-system (RE), %	-6.2	-8.0	-8.9

3.3.2 Compound Identification

Compound identification was carried out using the commercial spectra library of Pragst et al. [22] and an in-house spectra library with compound and metabolite spectra that were recorded during

the years of routine use of the method. Criteria for positive identification were the same as mentioned before in 2.2.2.1. The chromatograms were recorded at 210 nm.

3.3.3 Analysis of Clinical Plasma Samples

Analysis of clinical samples that were previously analysed by the reference system in routine use (7.3) using the method for toxicological screening for plasma showed comparable qualitative results. Quantitative results (system-to-system accuracy, RE, %) of the investigated samples ranged from -56.5% - + 40% with no recognisable trend whereas in most cases (70%) accuracy was ≤ ±20%. The low accuracy might be due to the fact that in most of the cases, the analysed samples were stored in the fridge 5-8 °C for more than 4 weeks after the analysis in the reference system and before the analysis in the developed system. This hypothesis could be supported by the measurement of samples at the same time prepared by the same person, where accuracy was ≤ ±10% from system-to-system.

In Table 3-19 the qualitative results of the parallel analysis of a selection of samples is summarised. The comparison of the results showed that in 12 out of 15 cases identic results were achieved. Unsuccessful identification might be due to stability problems and/or matrix interferences from the monovettes. Generally, hydroxy-risperidone (sample 4), morphine and codeine (sample 9) elute close to the injection peak and are difficult to detect in cases of increased matrix interferences. In Fig. 3-20 example chromatograms of both systems are shown, where differences in peak height and width are recognisable.

Table 3-19 *Qualitative comparison from system-to-system*

Sample	Developed system	Reference system in routine use
Sample 1	Diphenhydramine Diazepam Methadone	Diphenhydramine Diazepam Methadone
Sample 2	Risperidone	Risperidone
Sample 3	Midazolam	Midazolam
Sample 4	Diphenhydramine Risperidone ---	Diphenhydramine Risperidone OH-Risperidone
Sample 5	---	Zonisamid
Sample 6	Trimipramine Diazepam Nordiazepam	Trimipramine Diazepam Nordiazepam
Sample 7	Dipenhydramine	Diphenhydramine
Sample 8	Diazepam Nordiazepam	Diazepam Nordiazepam

Sample	Developed system	Reference system in routine use
	Temazepam	Temazepam
Sample 9	Nordiazepam	Nordiazepam
	---	Morphine, Codeine
Sample 10	Midazolam	Midazolam
	Ofloxacine	Ofloxacine
	Methylaminoantipyrine	Methylaminoantipyrine
Sample 11	Quetiapine	Quetiapine
	Methylaminoantipyrine	Methylaminoantipyrine
Sample 12	Paracetamol	Paracetamol
	Diazepam	Diazepam
	Nordiazepam	Nordiazepam
	Temazepam	Temazepam
	Oxazepam	Oxazepam
Sample 13	Midazolam	Midazolam
	Diazepam	Diazepam
	Nordiazepam	Nordiazepam
Sample 14	Doxepine	Doxepine
	Nordoxepine	Nordoxepine
Sample 15	Carbamazepine	Carbamazepine
	Carbamazepineoxide	Carbamazepineoxide

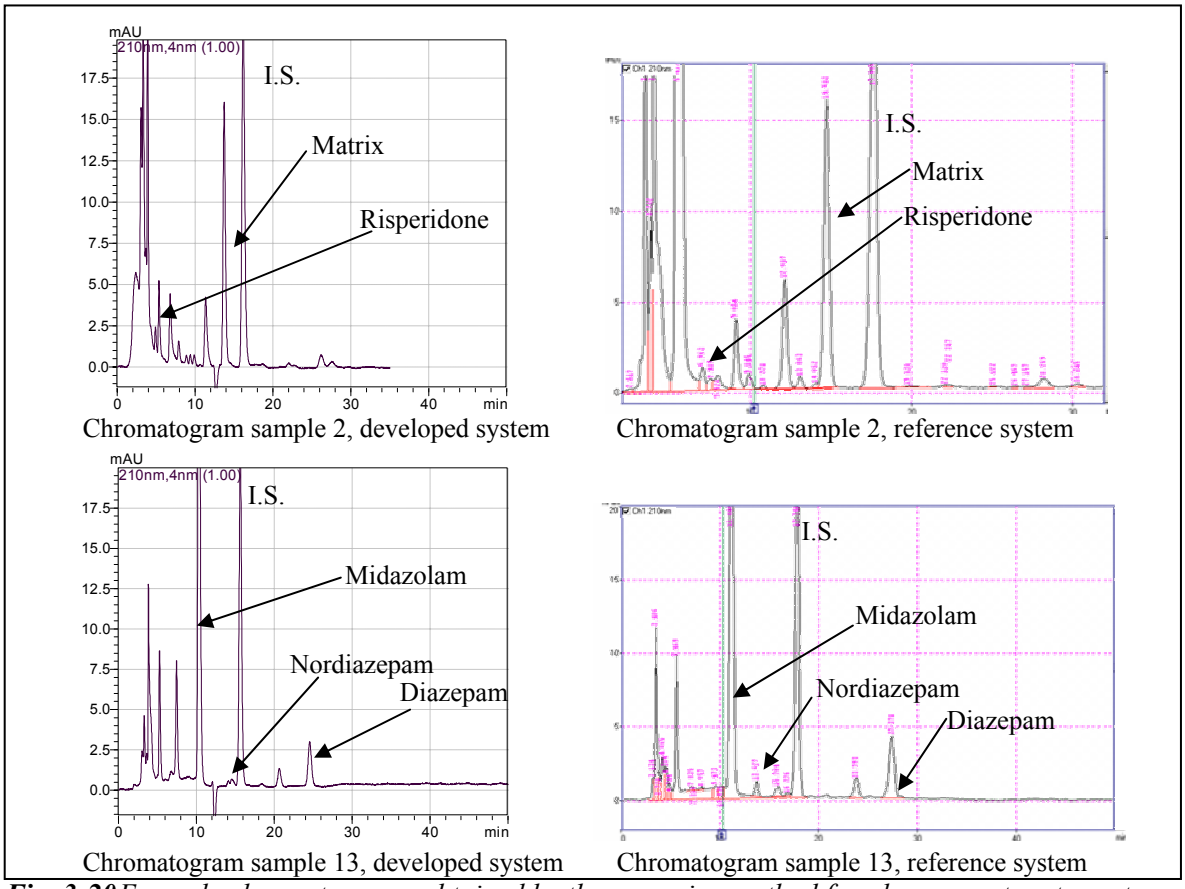


Fig. 3-20 *Example chromatograms obtained by the screening method for plasma, system-to-system comparison*

As can be seen from the chromatograms in Fig. 3-20, RTs of the set-up method were approximately 1 to 1.5 min shorter than in the reference system in routine use. This might be due to differences in the equipment, such as the use of a mixing chamber in the mobile phase flow line for the reference system in routine use. Different dead times for each system were measured: 2.84 min for the developed system, 3.64 min for the system in routine use, respectively. However, deviation of the RRT from system-to-system was < 5% and no changes in the peak order were noticed.
In conclusion, the use of column switching valves allowed the set-up of a further method for screening in plasma in the same analytical system. The system-to-system validation results (3.3.1) obtained from the analysis of PCS-PA indicated that previous data for quantitation of the system in routine use could be adapted. This was proven by parallel analysis of clinical plasma samples prepared at the same time for system-to-system comparison (system-to-system precision ≤ ±10%). Reanalysis of plasma samples in the developed system after more than four weeks after the analysis in the reference system led to poor precision results.

3.4 Screening Method for Neutral, Weakly Acidic and Weakly Basic Compounds in Urine

The screening method for neutral, weakly acidic and weakly basic compounds in urine was especially designed for the analysis of the toxicologically relevant group of benzodiazepines and was further assessed for the analysis of barbiturates because these compounds were not extracted via the weak cation-exchange material used for the extraction of basic compounds.
Integration of this method into the analytical system meant that the solvent choice was limited to the already used solvents for the on-line extraction of basic compounds and the toxicological screening method in plasma. Therefore, except for the employed columns and the consistence of the sample loading and the first wash solvent, the method did not vary from the earlier described methods concerning flow rates, order of wash steps and use of pumps.

3.4.1 Analytical Separation

In order to keep costs for material low, only the already established analytical columns used for the toxicological screening method in plasma (Nucleosil^{TM} 100 C8) and the SCX columns used for the on-line extraction method of basic compounds, were investigated. For the analytical separation of PCS-BDP and PCS-BARB under isocratic conditions, better separation results were achieved on the coupled Nucleosil^{TM} 100 C8 columns than on the SCX columns. This might be due to the fact that the investigated benzodiazepines differ only little in their basic properties, which are responsible for the cross reactions with the SCX material. Only, bromazepam provides a third basic nitrogen atom (pyridine N, pK_a = 5.2), which possibly led to a slightly better separation.

Furthermore, the C8 columns were already tested for the analysis of a wide range of toxicologically relevant compounds including benzodiazepines and barbiturates, because of its use for toxicological screening in plasma. In Fig. 3-21 an example chromatogram of PCS-BDP on the described columns is shown.

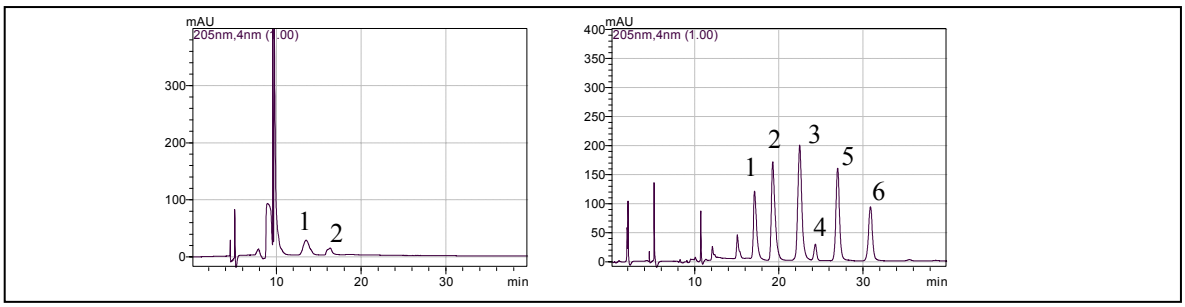


Fig. 3-21 *Example chromatograms of PCS-BDP on SCX material (left, 1: overlapped PCS-BDP analytes, 2: Bromazepam) and C8 material (right, 1: Bromazepam, 2: Demoxepam, 3: Oxazepam, 4: Nordiazepam, 5: MPPH, 6: Temazepam)*

The composition of mobile phase 1, optimised for the on-line extraction method of basic compounds, could not be changed due to apparative limitations as only one solvent can be transported by pump C. Therefore, mobile phase 1 instead of mobile phase 2, which was used for the analytical separation on the C8 material, had to be chosen for the analytical separation. Although mobile phase 1 contained 4.5% more of 0.05 M phosphate buffer pH 2.3 than mobile phase 2, the flow rate had to remain at 0.7 mL/min to allow maximum separation, e.g. of oxazepam and nordiazepam. In general, all investigated benzodiazepines showed good separation over the chromatogram (see RT in the specific library (7.4 in the appendix)). The I.S. MPPH was adapted to this method, as it showed good results under the given conditions. The detection wavelength was set to 205 nm and time for analysis including on-line extraction was 50 min.

3.4.2 On-line Extraction

At pH 6 neutral, weakly acidic and weakly basic compounds are not charged, or more precisely depending on the pK_a not completely charged, thus 0.01 M phosphate buffer pH 6.0 was used as loading buffer. It could be shown, that when applying the sample with 100% 0.01 M phosphate buffer pH 6.0 onto the extraction material, early retention of the target analytes on the C18 pre-(guard)cartridge material occurred. Therefore, optimisation of the consistence of the loading buffer by varying the phosphate buffer/(acetonitrile/water (90/10, v/v)) ratio and determination of the sample break-through was performed. In Fig. 3-22 the circuit diagram of the sample loading step is shown, illustrating, that all analytes have to pass the pre-(guard)cartridge before they reach the extraction column.

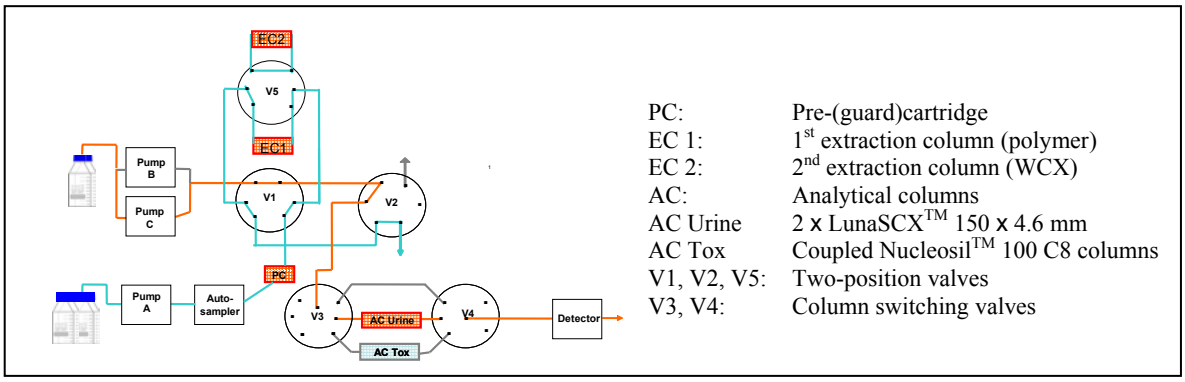


Fig. 3-22 *Circuit diagram of the sample loading step (light blue), the analytes pass the pre-(guard)cartridge column before reaching the extraction column*

The phosphate buffer content was decreased to 90% 0.01 M phosphate buffer pH 6.0 and the acetonitrile/water (90/10, v/v) content increased to 10%, respectively. With this approach passing of the pre-(guard)cartridge and retention of the target analytes on the extraction column was achieved. The results of the loading buffer optimisation are summarised in Table 3-20.

Table 3-20 *Results of the loading buffer optimisation*

Buffer content (%)	Retention pre-column	Break-through extraction column
100	yes	no
95	yes	no
90	**no**	**no**
85	no	yes
80	no	yes

In the following wash step the acetonitrile/water (90/10, v/v) fraction was increased to 20% as absence of matrix compounds was verified and only little compound break-through (< 5%) was obtained. Higher organic fractions led to an early compound break-through and changes in RTs.

Before eluting the analytes, the flow through the extraction column was reversed and the extraction column was washed with water. With the combination of a wash in the forward and backward mode, sufficient clean-up by removing interferences prior to elution was achieved. In comparison to the earlier described on-line extraction method for basic compounds, basic interferences which are protonated at pH 6 were not retained on the apolar material and therefore did not co-elute with the target compounds of this method. Finally, the analytes were eluted with the mobile phase 1 due to the organic fraction of 31.5% and protonation of weakly basic analytes.

Comparison of polymer (electroneutral) extraction materials from different manufacturers showed comparable results when using polymer material. Therefore the same brand used for the extraction method of basic compounds (StrataX, Phenomenex) was chosen, to simplify the analytical system concerning same column types and cartridge holder. In Fig. 3-23 example chromatograms obtained from the analysis of different column materials are shown.

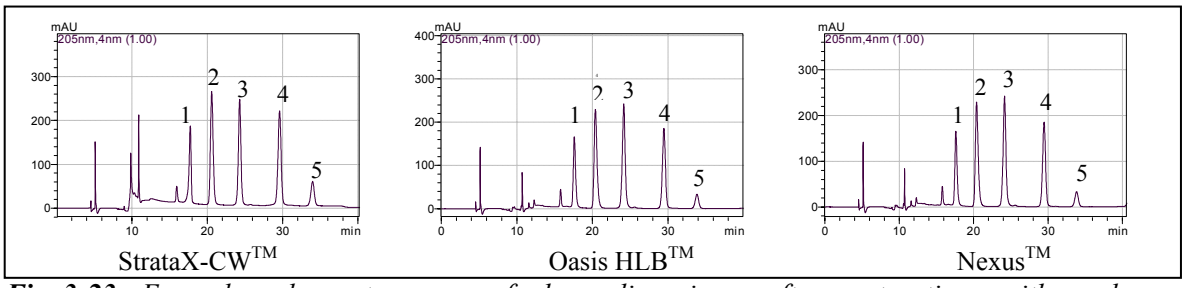


Fig. 3-23 *Example chromatograms of benzodiazepines after extraction with polymer (electroneutral) material from different manufacturers. Peak identification: 1: Bromazepam, 2: Demoxepam, 3: Oxazepam, 4: Nordiazepam, 5: Temazepam*

3.4.3 Glucuronide Hydrolysis

Most benzodiazepines undergo intensive phase 1 and 2 metabolism [107] and are mainly excreted as glucuronide conjugates in urine [97, 107, 108]. Therefore, glucuronide hydrolysis had to be performed prior to analysis as the very polar glucuronides could not be analysed with the developed method. In order to keep time for glucuronide hydrolysis low, the minimum hydrolysis time, where maximum deglucuronidation could be achieved was determined. It could be shown, that a hydrolysis time of 30 min at 45 °C was sufficient and that longer hydrolysis time did not result in considerably better deglucuronidation. Increase of the peak areas of oxazepam (1) and temazepam (4) with increased hydrolysis time was ≤ 7% from 30 to 180 min. Hydrolysis with HCl at 80 °C for 30 min following hydrolysis instructions described in the literature [109] led to less matrix interferences in the chromatogram, but to a lower deglucuronidation rate. Furthermore possible acidic ring cleavage to benzophenones may occur [105]. In Fig. 3-24 exemplary chromatograms of the same benzodiazepine positive sample analysed after different hydrolysis times are shown.

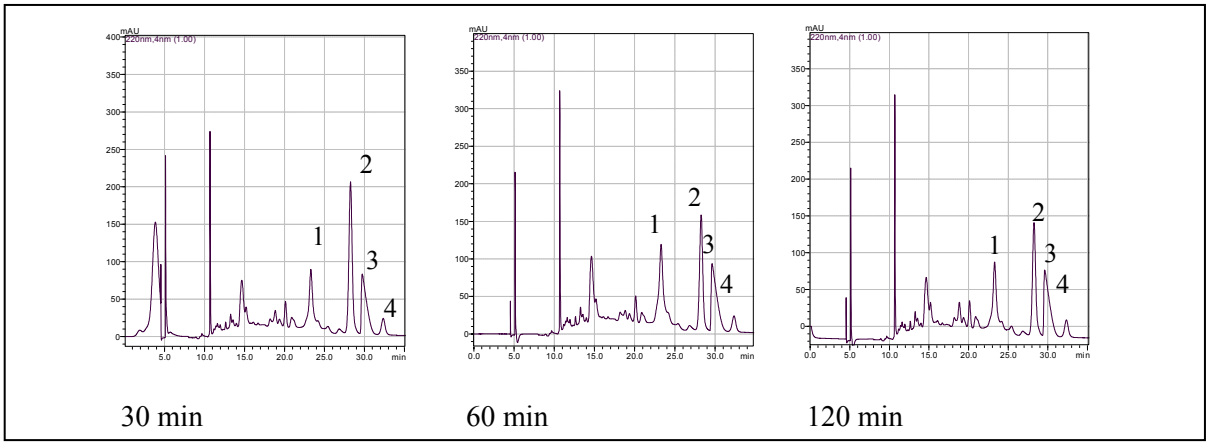


Fig. 3-24 *Benzodiazepine positive sample analysed after 30, 60 and 120 min of hydrolysis at 45 °C, (Peak identification: 1: Oxazepam, 2: MPPH, 3:EDDP , 4: Temazepam)*

3.4.4 Final Procedure

As described previously, the developed method differs only concerning the employed columns and the consistence of the sample loading and the first wash solvent from the on-line extraction HPLC-DAD method for basic compounds. Therefore, the description of flow rates, column switching and pumps in 3.2.3 can be adapted and is not repeated. A short summary is given in Table 3-21.

Table 3-21 *Event table of the final method for neutral, weakly acidic and weakly basic compounds in urine*

Working step	Time (min)	Pump A		Pump B		Pump C	
		Flow rate (mL/min)	Solvent*	Flow rate (mL/min)	Solvent*	Flow rate (mL/min)	Solvent
Loading	0 - 2.5	2.0	1	0	-	0.7	4
1st Wash	2.5 - 4.5	2.0	2	0	-	0.7	4
Preparation for the 2nd wash	4.5 - 5.0	2.0	4	3.0	3	0.7	4
2nd Wash	5.0 - 6.0	1.2	4	3.0	3	0	-
Elution and analytical separation	6.0 - 45.0	0.5	4	0	-	0.7	4
Analytical separation, wash of the extraction column	45.0 - 47.5	2.0	2	0	-	0.7	4
Analytical separation, conditioning of the extraction column	47.5 - 50.0	2.0	1	0	-	0.7	4

**1 = loading buffer: 90% 0.01 M phosphate buffer pH 6.0/10% (acetonitrile/water (90/10, v/v))*
2 = first wash solvent: 80% 0.01 M phosphate buffer pH 6.0/20% (acetonitrile/water (90/10, v/v))
3 = second wash solvent: water
4 = mobile phase1: 68.5% 0.05 M phosphate buffer pH 2.3/31.5% (acetonitrile/water (90/10, v/v))

3.4.5 Compound Identification

The retention and spectral data of barbiturates and benzodiazepines and their metabolites in urine have been successfully stored in a method-specific library (appendix, 7.4), chromatograms were recorded at 205 nm. The choice of 265 nm as detection wavelength, where benzodiazepines show good absorption, was investigated. At this wavelength interfering matrix components showed low absorption results, but unfortunately, the same effect applied for the I.S. (MPPH). Therefore, under the chosen conditions with MPPH as I.S., the benefits at 265 nm of good benzodiazepine absorption and low absorption of interferences could not be taken into account.

3.4.6 Validation

The performance control samples PCS-BDP 1 and 2 and PCS-BARB 1 and 2 consisting of five different benzodiazepines plus the I.S. and five different barbiturates plus the I.S. in buffer (1) and

urine matrix (2), respectively (2.1.1), were used for evaluation following the FDA guideline for bioanalytical method validation [96]. To evaluate the developed method, PCS-BDP and PCS-BARB were used. In Fig. 3-25 an example chromatogram of the performance control samples is shown. All validation data are summarised in Table 7-13 and 7-14 (7.6, appendix).

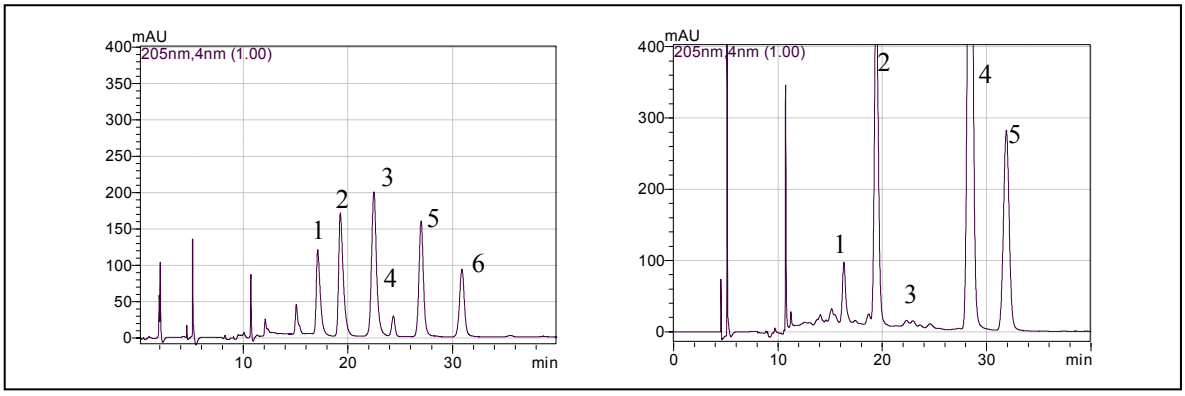


Fig. 3-25 *Chromatograms of PCS-BDP 1 (left, 1: Bromazepam, 2: Demoxepam, 3: Oxazepam, 4: Nordiazepam, 5: MPPH, 6: Temazepam) and PCS-BARB 1 (right, 1: Cyclobarbital, 2: Pentobarbital, 3: Crotylbarbital, 4: MPPH, 5: Methohexital)*

3.4.6.1 Selectivity/Specificity

No interferences with the signals of the analytes (S/N > 3) were detected analysing urine samples obtained from six healthy volunteers. In Fig. 3-26 example chromatograms of a blank urine sample and a blank urine sample spiked with I.S. are shown. Except from switching peaks occurring from the on-line extraction procedure, no interfering peaks with a S/N > 3 are visible.

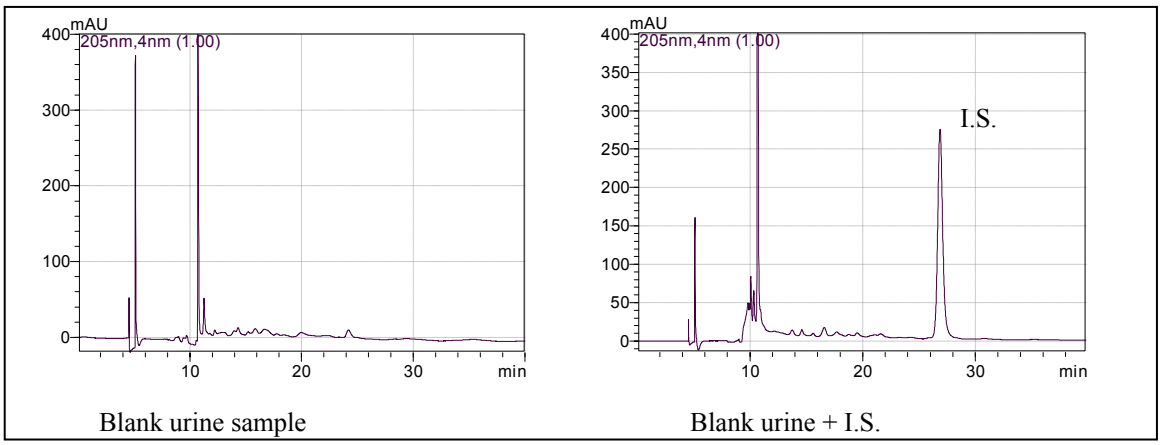


Fig. 3-26 *Example chromatograms of a blank urine sample and a blank urine sample spiked with I.S.*

3.4.6.2 Stability

Stock solutions and PCS-BDP 1 stored in the freezer, showed stability over the investigated time range of 28 days. Mean concentrations ranged from 84-119% compared to the freshly prepared

performance control sample (n = 3). Due to the low recovery and precision results for PCS-BARB 1 and 2 (3.4.6.3, 3.4.6.4), stability investigations were only carried out for PCS-BDP 1. Detailed stability data are summarised in Table 7-11 in the appendix (7.5).

3.4.6.3 Recovery

Recovery calculated from the average peak areas (arithmetic mean) of the extraction of the PCS-BDP 1, (n = 6) and PCS-BDP 2 (n = 6) at three calibrator concentrations in relation to the average peak areas (arithmetic mean) of the direct injection (n = 6) was ≥ 63-95% for the analysed benzodiazepines, RSD ≤ 8.4%. Matrix influence on the recovery was 28% for the early eluting bromazepam and ≤ 12% for all other analytes.

The investigated barbiturates of PCS-BARB 1 and PCS-BARB 2 (each n = 6) showed very low recovery (cyclobarbital 11%, 6.7% RSD, crotylbarbital 13%, 11.3% RSD, phenobarbital 4.6%, 6.0% RSD) except for methohexital (72%, 1.5% RSD) and pentohexital (62%, 3.2% RSD). Matrix influence on the recovery was ≤ 12% for all PCS-BARB 2 analytes.

Recovery ± SD of MPPH (I.S.) was 43.7% ± 1.3 and no pH dependency of the extraction recovery occurred as investigations at pH 4.5, 6.0, 7.0 and 9.0 showed. According to the FDA guidelines [96] recovery does not need to be 100% as long as the extent of recovery of the analytes and I.S. is consistent, precise and reproducible. The investigations therefore met the latter criteria.

3.4.6.4 Precision

Precision of the system was < 4.0% RSD for the peak area and ≤ 0.3% RSD for the RT of all PCS-BDP 1 and PCS-BARB 1 analytes (arithmetic mean level 1-3). The results for the within-day precision of the method (n = 6) ranged from 1.8-8.4% RSD for PCS-BDP 2, 1.5-11.3% RSD for PCS-BARB 2, respectively (level 1-3), and thus met the acceptance criteria for bioanalytical method validation of the FDA guideline [96]. Results for between-day precision were ≤ 14.7% RSD for the peak area and ≤ 3.0% RSD for the RRT.

Unfortunately, no benzodiazepine glucuronides were available as reference standard substances to measure precision following glucuronide hydrolysis as will be performed in routine use. Therefore analysis of three consecutive hydrolysed samples from the same origin was determined in order to calculate precision following hydrolysis. The following precision results were obtained: bromazepam: 1.7% RSD, demoxazepam: 0.5% RSD, oxazepam: 0.5% RSD, nordiazepam: 8.3% RSD, MPPH: 2.4% RSD, temazepam: 6.4% RSD. These results corresponded to the within-day precision results without hydrolysis.

3.4.6.5 Carry-Over Experiments

Within carry-over experiments, injecting blank urine samples after each sample of the following concentrations 5.0, 10.0, 15.0 and 20.0 µg/mL, no carry-over effect was observed. However, in order to keep good separation quality and to increase column life time, a column wash and injection of a blank sample should be performed after every tenth injection and after intoxication samples with high compound concentrations.

3.4.6.6 Linearity

Calibration curves of the PCS-BDP 2 and PCS-BARB 2 analytes with concentrations ranging from 0.05-20.0 µg/mL were analysed (n = 6) for the description of the relationship between peak area (detection response) and concentration. Linearity was obtained from 0.05-10.0 µg/mL for bromazepam, demoxepam, oxazepam, temazepam ($R^2 \geq 0.997$) and 0.1-20.0 µg/mL for nordiazepam and MPPH ($R^2 \geq 0.999$), respectively. In the case of barbiturates, only methohexital and pentohexital showed linearity over the following concentration range: 0.1-20.0 µg/mL ($R^2 \geq 0.995$).

3.4.6.7 Limit of Detection

In accordance to the method for basic compounds, the lower limit of detection (LLOD) was defined as the lowest concentration of the PCS-BDP 2 and PCS-BARB 2 analytes yielding an S/N > 3.0. The LLOD was 0.03 µg/mL for demoxepam, oxazepam and temazepam, 0.04 µg/mL for bromazepam and 0.1 µg/mL for nordiazepam, methohexital and pentobarbital. All other barbiturates showed LLOD values > 1 µg/mL.

The upper limit of detection (ULOD) was 10.0 µg/mL for bromazepam, demoxepam, oxazepam and temazepam or 20.0 µg/mL for nordiazepam, methohexital and pentobarbital, respectively. For the other barbiturates the ULOD is most probably higher than the evaluated highest concentration due to the low extraction efficiency.

3.4.6.8 Batch-to-Batch Reproducibility

Batch-to-batch reproducibility of the extraction column was examined, investigating two different batches. The RSD of the peak areas was ≤ 11.4% (n = 3) and the RRTs did not deviate from extraction column to extraction column. Batch-to-batch reproducibility of the C8 columns was not specifically tested as several batches over many years of routine use showed good results.

In summary, the developed method showed to be reliable for the analysis of benzodiazepines as sufficient recovery, good linearity and precision results were obtained.

In the case of barbiturates, only methohexital and pentobarbital showed good results concerning the latter mentioned parameters. All other PCS-BARB analytes showed poor recovery and linearity and therefore can only be analysed qualitatively in acute intoxication cases where high barbiturate concentrations are expected.

3.4.7 Analysis of Authentic Benzodiazepine Samples

To investigate the applicability of the developed method for the analysis of authentic urine samples following glucuronide hydrolysis, 70 urine samples that were presumptively positive for benzodiazepines by immunological pre-screening (immunological cut-off benzodiazepines: 200 ng/mL) within DOA confirmation screening were evaluated. Creatinine values ranged from 0.6-3.8 g/L. Unfortunately, no clinical barbiturate positive samples were available for analysis.

In 74% of the evaluated samples, the benzodiazepine positive result was confirmed. Most frequently detected benzodiazepines (in 95% of the cases) were oxazepam and temazepam. Additionally nordiazepam (7%), demoxepam (2%) and in other cases hydroxymidazolam (2%), flurazepam (1%), lorazepam (1%) and tetrazepam (1%) were detected. Unsuccessful confirmation was due to overlapping spectra in three cases. In all other cases the immunological result was < 1000 ng/mL when no benzodiazepines were chromatographically detected. The detailed results of the evaluation are shown in 7.8 in the appendix. The sensitivity of the method seemed to be not sufficient for the latter samples. Consequently, only samples with immunological pre-screening results ≥ 1000 ng/mL can be analysed by the developed method and more specific methods have to be chosen for those samples below immunological values of 1000 ng/mL, respectively.

3.4.8 Routine Use

The method has been applied to toxicological routine use in order to confirm immunological pre-screening results in cases of suspected benzodiazepine abuse and acute benzodiazepine or barbiturate intoxications.

In the following examples (Fig. 3-27), clinical cases of benzodiazepine positive samples (immunological result for benzodiazepines ≥ 1000 ng/mL) with different benzodiazepines analysed within DOA screening are shown. A comparison between plasma and urine samples analysed within STA will be given in the next chapter. During the years 2003-2006 only 3 barbiturate intoxications were investigated at the Institute of Toxicology-Cinical Toxicology and Poison Control Centre Berlin and only plasma/serum was available as sample material.

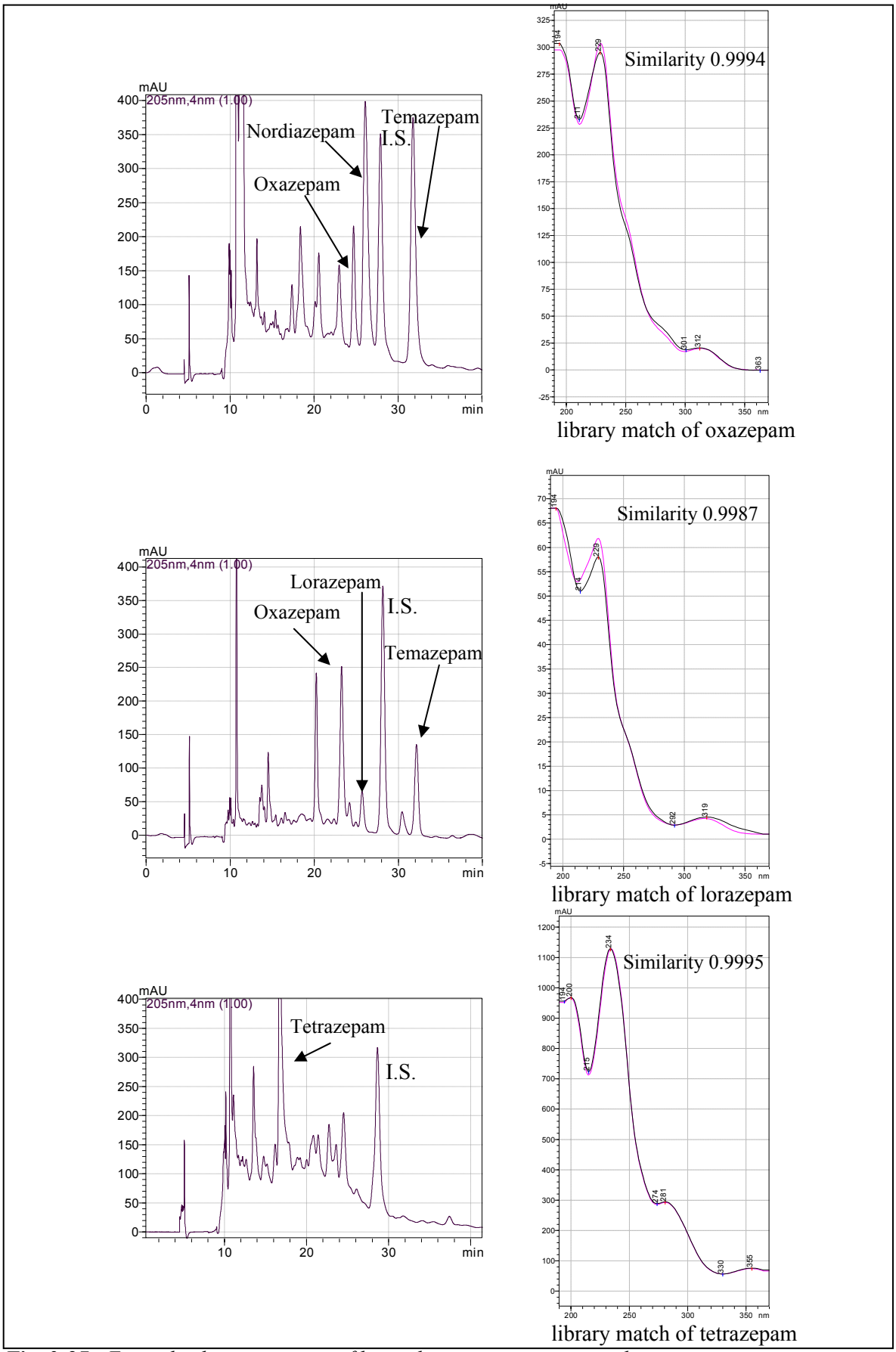


Fig. 3-27 *Example chromatograms of benzodiazepine positive samples*

As can be seen from Fig. 3-27, the method for neutral, weakly acidic and weakly basic compounds allowed simple analysis of clinical benzodiazepine samples above the given immunological pre-screening result of ≥ 1000 ng/mL. In contrast to the investigated urine samples of healthy volunteers and negatively screened patient samples, chromatograms of most clinical samples showed peaks of matrix compounds. However, these latter interfering matrix peaks did not influence the identification of the target compounds.

3.5 Systematic Toxicological Analysis with the Analytical System

3.5.1 Samples Spiked with Reference Standards

The analysis of spiked samples with reference standards of toxicological relevant and/or frequently prescribed compounds resulted in method-specific libraries for each of the set-up methods as has been described in the according compound identification chapters 3.2.4, 3.3.2 and 3.4.5 and in 7.4 of the appendix.

Compounds that were not identified by the on-line extraction method for basic compounds in urine nor by the method for neutral, weakly acidic and weakly basic compounds in urine, because they were neither extracted due to their pK_a-values nor analytically separated on the employed column material have been listed in a "negative list" (7.9, appendix).

Some of the compounds of the negative list were identified alternatively by the toxicological screening method for plasma. As a result, the importance of the combination of different extraction and separation methods for STA in order to identify a maximum range of xenobiotics can be stated.

3.5.2 Authentic Toxicological Samples

With the analysis of authentic toxicological samples, the libraries were expanded with spectra of metabolites which often were not commercially available.

In Table 3-22 the exemplary investigation of 16 cases with the developed system where plasma and urine were sent for analysis is shown. All 16 samples were previously analysed within the scope of STA using immunological screening and chromatographic methods, ethanol was determined by a GC-Headspace technique (1.1, Fig.1-1). These results are shown as final STA results in the 2nd column of Table 3-22. After evaluation of the final STA results, analysis of the samples in the developed system was carried out and the results summarised as follows: In the 3rd column the results of repeated screening in plasma with the developed system by the toxicological screening method for plasma when sufficient material was provided are summarised. In the 4th and 5th column the results of urine analysis are given obtained from the analysis with the developed on-line extraction HPLC-DAD methods.

Table 3-22 *Exemplary investigation results of 16 cases analysed with all three established methods in comparison to the final STA result*

Case Nr.	Final STA result (HPLC, GC, immunological methods*)	Screening method for Plasma	Screening method for basic compounds in urine	Screening method for neutral weakly basic and weakly acidic compounds in urine
1	Negative	No material	Negative	Negative
2	Medazepam Diazepam Nordiazepam Cocaine* Benzodiazepines*	Medazepam Diazepam Nordiazepam	Ketamine BEC**	Medazepam Nordiazepam Oxazepam
3	Midazolam Amphetamines* Benzodiazepines* Ethanol	Midazolam	MDA, MDMA Amphetamine Lidocaine-Metabolite 1, Lidocaine	Midazolam-Metabolite
4	Chlorprotixene Metoclopramide	No material	Chlorprotixene Metoclopramide Metoclopramide-Metabolite Chlorprotixene-Metabolite	Chlorprotixene-Metabolite
5	Metamizol-Metabolite Pantoprazol Benzodiazepines*	Metamizol-Metabolite Caffeine Pantoprazol	Metamizol-Metabolite	Pantoprazol
6	Diazepam Benzodiazepines* Ethanol	Diazepam	Lidocaine	Oxazepam
7	Amitriptyline Nortriptyline Tricyclic antidepressants*	No material	Nortriptyline Ambroxol Ciprofloxacine	Amitriptyline Nortriptyline
8	Negative	Negative	Negative	Negative
9	Lorazepam Benzodiazepines*	Lorazepam	Negative	Lorazepam
10	Opiates* 6-AM (only in urine)	No material	Morphine 6-AM Codeine	Papaverine
11	Diphenhydramine	No material	Diphenhydramine-Metabolite	Diphenhydramine Diphenhydramine-Metabolite

** immunological method, **BEC method*

Table 3-22 *Exemplary investigation results of 16 cases analysed with all three established methods in comparison to the final STA result, continued*

Case Nr.	Final STA result (HPLC, GC, immunological methods*)	Screening method for Plasma	Screening method for basic compounds in urine	Screening method for neutral weakly basic and weakly acidic compounds in urine
12	Diphenhydramine	No material	Diphenhydramine Diphenhydramine-Metabolite	Diphenhydramine Desmethyldiphen-hydramine
13	Flurazepam Desalkylflurazepam Hydroxycarbazepine Benzodiazepines*	No material	Tilidine Tilidine-Metabolite	Flurazepam Flurazepam-Metabolite
14	Salicylic acid*	No material	Negative	Negative
15	Negative	Negative	Citalopram Citalopram-Metabolite	Diphenhydramine Diphenhydramine-Metabolite
16	Negative	No material	Citalopram Citalopram-Metabolite	Negative

** immunological method, **BEC method*

With the on-line screening methods the results of the STA were confirmed according to the chemical properties of the analysed compounds: basic compounds were identified with the on-line extraction method for basic compounds (2.2, 3.2), benzodiazepines and other neutral, weakly basic and weakly acidic compounds were confirmed with the corresponding method (2.4, 3.4). In cases of positive immunological screening (*), the compounds were specified with the basic on-line extraction method. In case 3 MDA, MDMA and amphetamine were confirmed, in case 10 heroin abuse was identified. In case 2 the cocaine positive result was not confirmed by the on-line extraction HPLC-DAD method due to its relatively low concentration, but in a second run using the BEC method (**). In case 7 the identification of nortriptyline confirmed the immunological positive tricyclic antidepressant (TCA) finding. Salicylic acid (case 14) could not be identified by the on-line extraction methods for urine screening, as it was not extracted.

In five cases additional information was obtained from urine screening. In case 7 ciprofloxacine (antibiotic) and ambroxol (expectorant) were detected. More toxicologically relevant information was obtained from case 2 and 13 where ketamine (narcotic) and tilidine (analgesic), respectively, were additionally found in urine. In comparison to plasma analysis citalopram, citalopram- and diphenhydramine-metabolites were identified in case 15 and 16, respectively. All five cases demonstrate the longer detectability of drugs in urine compared to blood.

Case number 2, 3, 5, 6, 9 and 13 represent benzodiazepine positive cases. Except for case 5 were the positive immunological result was below 1000 ng/mL and therefore below the in-house cut-off

value for the developed on-line method, in all other cases the benzodiazepine intake was confirmed. The results of specified benzodiazepines corresponded to those of STA investigations, although the main metabolites were identified.

Unfortunately, only in six cases plasma/serum was available for repeated screening with the developed analytical system using the toxicological screening method for plasma. However, when plasma screening was performed, the results obtained from previous plasma screening with the reference system in routine use were confirmed.

In summary, the analysis of clinical samples with the developed system proved its use for toxicological routine applications. Results of previously performed STA were confirmed and further information about basic compounds was obtained in 5 cases from urine screening. Thus, it could be proven that investigation of different sample specimens, besides different analytical techniques, can lead to more information about the intoxication case.

Overall, the results well corresponded to achieved data of this thesis, such as the in-house cut-off value for benzodiazepines and BEC in combination with the better detection of BEC by the BEC method.

3.5.3 Quality Assessment Schemes

External quality assessment schemes for urine analysis were screened by the developed urine screening system in order to control the reliability of the methods. A summary of investigated external quality assessment schemes for urine analysis as an example for how to handle the different methods in the developed system is summarised in Table 3-23. It can be seen, that most of the included compounds were successfully analysed by the according urine methods. Failed identification was due to concentration below the LLOD (barbiturates (1.), phencyclidine (1.)), coelution (6-AM (2.)) or the lack of extraction (clomethiazole (3.)). Nordiazepam (1.) was only identified in the higher concentrated C3 sample although the concentration in the C2 sample was above the LLOD. This might have possibly been caused by competitive displacement by the other included compounds on the polymer extraction material. Propoxyphene was not identified due to any available spectra in the UV-library, but was matched insufficiently with the norpropoxyphene spectrum.

Table 3-23 *External quality assessment schemes*

Included compounds	Concentration (µg/mL)	Quality assessment results: Drugs detected by the screening method for basic compounds in urine	Drugs detected by the BEC method	Drugs detected by the LEC method	Drugs detected by the screening method for neutral, weakly acidic and weakly basic compounds in urine
1. Low opiates C2, C3 (Bio-Rad)					
Amphetamine	0.38, 0.63	x			
Barbiturates	0.15, 0.25				-
BEC	0.38, 0.63		x		
Codeine	0.23, 0.38	x			
EDDP	0.23, 0.38	x			
Hydroxyalprazolam	0.23, 0.38				x
Methadone	0.23, 0.38	x			
Methamphetamine	0.38, 0.63	x			
Morphine-glucuronide	0.23, 0.38	x*			
Nordiazepam	0.23, 0.38				x**
Norpropoxyphene	0.23, 0.38	x			
Oxazepam	0.23, 0.38				x
Phencyclidine	0.02, 0.03	-			
Propoxyphene	0.23, 0.38	-			
2. GTFCh UF 1/06					
Amphetamine	Not given	x			
6-AM	Not given	Overlapped by nortriptyline			
BEC	Not given		x		
Chlordiazepoxide	Not given				x
Ketamine	Not given	x			
Nortriptyline	Not given	x			
Morphine	Not given	x			
3. GTFCh UF 3/03					
Atropine	0.60	x			
Codeine	0.75	x			
Clomethiazole	0.50	-			
Norbuprenorphine	0.25	x			
Norclozapine	0.50			x	
Oxazepam	1.50				x
Pentobarbital	0.35				x

** following glucuronide hydrolysis*

***C3 sample only*

4 Discussion

In this thesis an analytical system including three chromatographic methods was set-up. Two automated screening methods for the determination of compounds in urine were developed and an existing screening method for plasma analysis was implemented in the system. The described results will be discussed in the following

- the screening method for basic compounds in urine (3.2),
- the toxicological screening method for plasma (3.3) and
- the screening method for neutral, weakly acidic and weakly basic compounds in urine (3.4).

Finally the routine use with the developed analytical system will be evaluated and future perspectives will be given.

4.1 Screening Method for Basic Compounds in Urine

With the developed screening method for basic compounds in urine a reliable and automated on-line extraction HPLC-DAD method has been provided for the simple analysis of alkaloids and further basic drugs that can be effectively detected in urine.

With the use of common laboratory material and standard HPLC equipment costs for analysis have been kept to a minimum (costs for solvents and reagents were approx. 0.90 €/analysis, and for columns + pre-(guard)cartridges 1.60 €/analysis, ∑ 2-3 €/analysis). Specialised techniques (e.g. GC-MS, approx. 6 €/analysis for consumables) as well as company-dependent methods and reagents (Remedi^{TM}-HS, Bio-Rad, approx. 13 €/analysis for consumables) can be replaced for the analysis of alkaloids and further basic drugs (STA) as well as amphetamine, cocaine/BEC, opiate and methadone/EDDP confirmation analysis of immunologically pre-screened samples (DOA). A method-specific spectra library with approx. 900 spectra including spectra of metabolites and possible occurring endogenous peaks has been provided for sample evaluation. Moreover, an existing spectra library with 2600 entries [22] is accessible for compound identification and therefore the analytical capability of the method is increased due to the enlarged database capacity [18, 76, 77].

4.1.1 Analytical Separation

The aim to separate basic compounds from a wide range of chemical structures and polarities was accomplished by the deployment of two coupled SCX columns for the analytical separation. The possibility to separate basic compounds on embedded polar phase material under basic mobile phase conditions as suggested in the literature [34, 110, 111] was not applicable due to incompatibility with the used spectra library and thus would have required complete set-up of a

new spectra library. Under the mobile phase conditions of the existing commercial library, separation of the target compounds on embedded polar phase material showed to be insufficient. Therefore SCX material was investigated, which in comparison to described methods on reversed phase material [11, 22, 23, 104, 112-115], allowed separation of polar substances like psilocin, atropine or scopolamine from the injection peak under acidic mobile phase conditions compatible with the spectra library [22]. The use of cation-exchange material at a pH level < 3 [79] and silica material with moderate cation-exchange properties at pH 6 [55, 116-118], respectively, has been described in the literature for successful separation of polar substances. In this thesis, it was shown, that the coupling of two 150 mm long SCX columns (300 mm cation-exchange material) was necessary for the separation of toxicologically relevant target compounds at pH 2.3, which in practice might occur together. For example, in comparison to coupled reversed phase and silica material [55], separation of methadone, morphine and 6-AM was achieved (3.2.8.3, Fig. 3-18). The coupling of 150 + 250 mm long SCX columns led to longer RTs but to only slightly better resolution of the target compounds, which showed already sufficient separation on the 2 x 150 mm SCX material. Neither on the coupled 2 x 150 mm SCX nor on the coupled 150 + 250 mm SCX columns 6-AM and scopolamine were separated under optimised conditions. As the gained 100 mm of separation phase did not lead to separation of the latter two compounds, only gradient elution or a different mobile phase composition might potentially give better resolution results. However, as the occurrence of 6-AM and scopolamine in biological samples was of minor toxicological importance compared to the separation of e.g. amphetamine/ methamphetamine, morphine/methadone/6-AM or scopolamine/hyoscyamine, more focus was put on the separation of these compounds which were sufficiently separated under the described conditions.

Basic compounds that dissociate to dications under the acidic mobile phase conditions are late eluting compounds (LEC) like clozapine, olanzapine, opipramol, quetiapine, which were not eluted on the SCX columns due to strong interactions with the HPLC material. All of the latter compounds have -besides another nitrogen atom- a piperazine partial structure. Piperazine is a base (pK_{a1} = 5.5, pK_{a2} = 9.8 [105]) which might be responsible for the long RT of the LEC. This hypothesis was supported by the RT of cinnarizine (1-benzhydryl-4-[(E)-3-phenylallyl]piperazine, a basic drug that does not show other basic atoms besides the piperazine nitrogens and elutes at 45 min. The same effect applied for cetirizine with two nitrogen atoms (RT 50 min). Together with a second basic component as in the case of the LEC, RTs exceeded the analysis time of the method (41 min). Other examples for LEC with a piperazine partial structure were aripiprazol, fluphenazine, perphenazine, perazine and ziprasidone. Whereas occurring metabolites of fluphenazine, perphenazine and perazine were detected within the 41 min run. In case of risperidone the piperidine nitrogen together with the second nitrogen atom (pyrimidine ring) might be responsible for the long RT. Therefore, these compounds had to be analysed with the LEC

method (3.2.6.2), which meant on the one hand that a second analytical run had to be carried out for the same sample. On the other hand, coelution of these compounds for urine analysis with the target analytes such as alkaloids and DOA, especially following hydrolysis, where high metabolite concentrations may occur as it has been described by Sticht et al. for the detection of psilocin by HPLC-DAD [100] was avoided. However, as the aim was to detect alkaloids and DOA, the detection of LEC in a second run, when required, presented no considerable limitation.
The systematic analysis of different partial structures with increased basicity may allow structuring the analytical separation properties of the employed columns. This was already tried with the elution order of investigated analytes stored in the library according to their chemical properties. But in comparison to the investigation of partial structures, where the influence of certain atoms or structures can be studied in particular, influence of the compound size, polarity and other hetero atoms seemed to play a role.

4.1.2 On-line Extraction

The use of on-line extraction permitted the direct injection of urine samples after dilution and centrifugation, which held sample preparation to a minimum and replaced tedious and time-consuming purification steps [31, 32]. As far as could be seen from literature investigations, the use of the WCX on-line extraction material for an automated screening method is new and allowed sufficient sample clean-up, extraction of the target analytes within reasonable and reproducible recoveries (≥ 79%, 3.2.5.3) and elution with the mobile phase 1 for subsequent diode-array detection with access to the existing pH-depending spectra library. It was concluded, that the fulfilment of the latter parameters could only be achieved with the WCX material, since the use of polymer (electroneutral) materials allowed the elution with the acidic mobile phase, but led to an early elution (break-through) of the analytes during the first wash step. Reduction of the wash time or the organic percentage in the first wash step would have led to less clean extracts and coelution of endogenous compounds at the RT of the target analytes. Extraction of the target analytes on SCX material allowed sufficient sample clean-up due to strong retention of the target analytes on the extraction material. Elution of the analytes from SCX material on the other side required a high percentage of organic solvents and a pH adjustment to pH > 10 to neutralise and then elute the analytes which was not compatible with the use of the spectra library.
In comparison to commonly used strong cation-exchange off-line extraction techniques [16, 20, 47] or semi-automated on-line extraction methods [119], the selective elution of a first extract containing neutral and acidic compounds and a secondly eluted extract containing basic compounds, was not possible with WCX material, as any neutral and acidic compounds would have been eluted in the first wash step from the extraction material. Moreover, gradient elution would have had to be established for fraction elution. Nevertheless, the use of WCX material

presented a simple, reliable and fully automated solution for the extraction of basic compounds and therefore can be used to replace more work-intensive sample preparation steps such as LLE or off-line SPE for the extraction of alkaloids [120-122] and opiates [18, 19] as has been most commonly used for this purpose besides the Remedi^{TM}-HS. In comparison to the latter mentioned drug profiling system, the developed on-line extraction method required only one extraction column (Remedi^{TM}-HS: two extraction columns) and increased extraction column shelf-life (> 300 injections) was achieved by the use of a pre-(guard)cartridge, which had to be replaced every 50 injections. This means that not only time for equilibration and replacement of the extraction column was saved by the use of the easier exchangeable pre-(guard)cartridge which does not require equilibration, but also costs were reduced to 25% due to the exceeded shelf-life of the more expensive extraction column: Costs for the extraction column were approx. 900 €/300 injections without the pre-(guard)cartridge and were reduced to approx. 230 €/300 injections with the use of the pre-(guard)cartridge.

4.1.3 Compound Identification

In previous publications [1, 52, 53, 78, 81] and research objectives [79] on the selectivity of substance identification by HPLC-DAD in toxicological analysis using the UV spectra library of Pragst et al. [22], it could be shown that HPLC-DAD presents an efficient analytical method with high selectivity and accuracy, thus fulfilling the requirements of STA. According to Herzler et al. [79], a threshold value for spectral correspondence (SI) ≥ 0.999 should be considered, which could be confirmed for secure positive identification of urine analysis within the scope of this thesis (3.2.4, 3.3.2, 3.4.5). Together with a narrow window for RRT comparison (RRT deviation ≤ ± 5%) using the method-specific spectra library, efficient selectivity between compounds with similar chromophoric systems, e.g. 6-AM and morphine, was achieved and thus allowed application for DOA confirmation analysis as well.

The fact that metabolites and their parent compounds as well as some classes of drugs may have similar UV spectra due to chemical structure relations can be very helpful for toxicological analysis and can present an advantage over mass selective methods, because information about the parent compound or yet not investigated substances related to certain classes of drugs can be obtained.

In the literature the advantages of retention indices (RI) over RRT such as better analytical precision and reproducibility have been discussed [11, 23, 123]. However, as the developed method showed good precision and batch-to-batch-reproducibility results (RSD ≤ 8%, 3.2.5) and used only one HPLC material for the analytical separation in comparison to two different analytical materials in the Remedi^{TM}-HS (which therefore requires I.S. 1 and I.S. 2), the use of RRT and of only one I.S. was justified.

With the daily analysis of the new PCS 1*, a simple strategy to monitor the developed method was performed. The evaluation of the new PCS 1* was used to control the analytical separation and extraction behaviour as well as a quality control sample for DOA confirmation analyses.

4.1.4 Validation

Validation was carried out following international guidelines [96] with the exemplary use of the PCS. Sufficient selectivity, stability, recovery, precision and good linearity were achieved. According to different organizations [90-94], selectivity and the LOD are the most important parameters for the validation of qualitative methods. Besides the analysis of blank urine samples within the validation studies, the developed method proved to provide sufficient selectivity as could be shown from the analysis of real clinical samples. The achieved LLOD (0.1-0.3 µg/mL) will be discussed in the following for STA and DOA cases: The LLOD showed to be sufficient in cases of alkaloid intoxications as the examined examples demonstrated (3.2.8.1 and 3.2.8.2). In the literature urinary peak concentrations of psilocin ranging from 0.02 µg/mL to 0.9 µg/mL are described, which were observed after ingestion of psychoactive doses of 212 ± 25 µg/kg by volunteers [97]. These values were doubled after glucuronide hydrolysis was performed [97, 124]. Therefore glucuronide hydrolysis has to be carried out following the aim to increase the detectable amount of psilocin in a sample above the LLOD of the method [100]. Grieshaber et al. reported of total urinary psilocin concentrations ranging from 0.03-0.2 µg/mL in cases of abuse [85]. If the abuse of psilocin cannot be confirmed by the developed method, methods with lower LLODs such as GC-MS [85] or LC-MS [120] should be considered. However, it could be shown that psilocin analysis carried out by the Remedi^{TM}-HS could be easily replaced by the developed method and that the achieved LLOD for psilocin was sufficient for diagnosis in acute intoxication cases (3.2.7.1, 3.2.8.2). False negative results due to psilocin instability must be avoided by storage of the samples in the freezer. Psilocin stability in frozen samples was given for 28 days as reanalysis within this time period showed.

Only few analytical methods for the determination of hyoscyamine and scopolamine have been published [125]. In most cases off-line SPE [126, 127] or LLE [121, 122] were used prior to chromatographic analysis with UV, MS or coulometric detection. An integrated on-line extraction HPLC-UV method for the determination of hyoscyamine in human plasma using restricted access material (RAM) has been described with a LLOD of 25 ng/mL [128]. As therapeutic concentrations in plasma range from 2.6 ng/mL to 3.1 ng/mL and from 0.4 ng/mL to 1.0 ng/mL, for hyoscyamine and scopolamine, respectively [129] the latter method can only be used in cases of atropine intoxications or when atropine is used as antidote in cases of organophosphorous insectizide poisoning [128]. According to literature investigations, so far, only few urinary concentrations of the latter alkaloids have been described. A urinary hyoscyamine concentration of

1.5 µg/mL was reported by Corbett et al. [130] in a fatal case. As five cases of suspicious hyoscyamine and scopolamine intoxications were confirmed by the developed method, sufficient sensitivity for this field of application was assumed. Moreover, atropine (D,L-hyoscyamine) was positively identified in an external quality assessment scheme (c = 0.6 µg/mL) of the Gesellschaft für Toxikologische und Forensische Chemie (GTFCh, 3.5.3).
In STA cases except alkaloid intoxications, where high concentrations are expected, sample dilution (1/5, v/v) with 0.01 M phosphate buffer pH = 6 or water showed to be advantageous in order to not exceed the ULOD (15 µg/mL) and protected the analytical system from contamination.

The LLOD covered approximately 60% of the investigated DOA cases sent to the Institute of Toxicology-Clinical Toxicology and Poison Control Centre Berlin in the year 2006 (3.2.8.3, Table 3-15) and therefore could replace work-intensive methods such as GC-MS [103] in a lot of cases. Carrying out opiate hydrolysis prior to analysis, the detection rate was increased from ~60% to ~80%. For the remaining 40% (20%) (samples with DOA concentrations below the LLOD of the method), a more sensitive method has to be provided for confirmation analysis.
Further emphasis throughout validation of the method was put on the robustness of the method; especially on the batch-to-batch reproducibility of the extraction and analytical columns, to allow the simple column exchange by the operator. Both, the analytical and extraction columns showed good reproducibility results. Overall, stable RRT and batch-to-batch precision were achieved on the used SCX columns (3.2.5), which had to be exchanged approximately every 500-1000 injections. Precision from extraction column to extraction column was ≤ 3.8% (n = 6) and the RRTs did not deviate at all. Therefore, it can be stated, that the developed method proved to be robust with reproducible RRT from batch-to-batch and shelf-life of the analytical columns was doubled in comparison to the Remedi^{TM}-HS (250 guaranteed injections/column).

4.1.5 Method Modifications for Critical Compounds

The set-up of the BEC and LEC method presents the given opportunity and versatility of the system to modify established methods according to special purposes and requirements.
The BEC method decreased the LLOD of BEC from 1.0 µg/mL to 0.2 µg/mL compared to the previously used screening method for basic compounds in urine and therefore led to an increase of possible cocaine confirmations from 45% to 80%. The confirmation rate of BEC in the Remedi^{TM}-HS was 54% for the investigated samples, LLOD 1.0 µg/mL (3.2.7.2, Table 3-13). Moreover, the sensitivity of the BEC method for cocaine/BEC confirmation analysis was in accordance to the immunological cut-off value (300 ng/mL). Besides the decreased LLOD of the BEC method, BEC showed an appropriate retention behaviour on the SCX columns, as it was well separated from the injection peak. This demonstrates another advantage over the Remedi^{TM}-HS, where BEC eluted

close to the injection peak and therefore was difficult to detect in many cases (3.2.7.2, Fig. 3-14 and Fig. 3-15).

With the LEC method a solution for the analysis of late eluting compounds that could not be detected on the SCX columns was pursued as has been previously discussed in 4.1.1. LEC that exceeded analysis time on the SCX columns due to high polarity and/or basicity, consequently showed short RT on the C8 columns and eluted close to the elution peak. Therefore analytical separation of these compounds on embedded polar phase material or weak cation-exchange columns as a compromise between SCX and C8 material might be a possibility for improved chromatography of these compounds.

Further methods can be installed in the analytical system taking advantage of the employed column switching valves.

4.1.6 Analysis of Authentic Samples and Comparison with the Remedi^{TM}-HS

The analysis of authentic samples proved the applicability of the developed method for toxicological screening within the given limits of detection and has been introduced to toxicological routine use at the laboratory of the Institute of Toxicology-Clinical Toxicology and Poison Control Centre Berlin since 2006 within the scope of STA when additional urine was available and for DOA confirmation analysis. Previously, a comparison with an existing automated urine screening system, the Remedi^{TM}-HS [55] was performed. In cases of STA the developed method showed better separation results for the analysis of alkaloids due to better quality of chromatography in combination with higher sensitivity and selectivity. Late eluting compounds had to be analysed in a second run with the LEC method (3.2.7.1, 4.1.1). Both methods produced comparable results concerning DOA confirmation analysis.

From the investigation evaluation it could be concluded that the developed method presents an adequate alternative method to the Remedi^{TM}-HS drug profiling system for the investigated fields of application and offers advantages of common HPLC equipment, laboratory material (columns, solvents) and modern computer software. The developed method should prove to be useful in laboratories that routinely perform confirmation analysis of immunoassay pre-screening and gives additional information concerning administered drugs. In critical specimens in which the immunoassay results were not verified (e.g. confirmation of heroin abuse by 6-AM analysis); a more sensitive method should be considered. Therefore GC-MS remains the “gold standard” for DOA confirmation screening in urine [4].

According to Sadeg et al. who described a 12 months` experience of toxicological screening with the Remedi^{TM}-HS in a general hospital in France [62], it can be also stated for the developed method, that it presents a valuable tool for additional urine screening within STA. The Remedi^{TM}-HS or alternatively the developed method should be considered as complementary methods to other

analytical techniques in order to allow maximum identification possibilities in general unknown cases. Therefore, ideally, neither of the HPLC-UV methods should be used as the only chromatographic method, because the LLOD might not be sufficient (DOA) and acidic and neutral compounds are not extracted (STA). This strategy is also suggested in the literature [1, 2, 4, 5, 28] and is in practice carried out in toxicological laboratories (1.1). Total running time per sample including sample extraction is 41 min (developed method) and approximately 20 min (Remedi^{TM}-HS), respectively. The shorter analysis time of the Remedi^{TM}-HS presents an advantage for fast diagnosis in intoxication cases. However, as mentioned above, both methods will be run as part of a complex analysis strategy within STA in acute intoxication cases, which will take approximately 2 hours. Thus, 41 min for analysis is still acceptable and means that measurement of approximately 32 samples per 24 h is possible.

Analytical differences between both methods are described in context with the corresponding chapters (4.1.1, 4.1.2, 4.1.5). A comparison of the used columns including their shelf-lives is summarised in 7.10 in the appendix.

4.2 Screening Method for Plasma

The versatility of the developed analytical system with the option to integrate further independent analytical methods in the system is presented by the establishment of the toxicological screening method for plasma. The successful set-up of the latter method demonstrates the advantage of the system in comparison to other screening methods where only a single method is run and cannot be influenced by the operator [55] or where only one sample matrix (plasma) can be analysed [119]. The combination of a known toxicological screening method for plasma with the novel screening methods for compounds in urine led to a broad spectrum screening system. Different to the Remedi^{TM}-HS where plasma analysis following ultrafiltration can be performed using the same method for urine screening [131], the toxicological screening method for plasma can be understood as a complementary method which allows analysis of neutral and weakly acidic compounds, such as benzodiazepines, as well. Therefore, a larger spectrum of substances is accessible when complementary methods are used, which was proven with different clinical cases. Benzodiazepines (3.3.3, sample 13) or carbamazepine (3.3.3, sample 15) for example, which showed too low basicity to be extracted via basic on-line extraction or the Remedi^{TM}-HS could be detected and quantified with the aid of the plasma method. A second run for plasma analysis following protein removal using the same screening method for basic compounds as carried out with the Remedi^{TM}-HS would not have identified these compounds due to the same extraction technique. With the toxicological screening method for plasma, a quantitative method where one can refer to therapeutic or toxic concentrations is provided. Thus quantitation of analgesics, antidepressants, barbiturates, benzodiazepines, psycho pharmaceutical concentrations in plasma is possible.

Therefore, besides diagnosis of acute intoxications, modified plasma analysis could be potentially used for the purpose of therapeutic drug monitoring (TDM), prerequisite calibration and validation for the relevant concentration range is carried out and correlation between pharmacokinetic and pharmacodynamic properties of the drug of interest are known.

A first attempt to replace LLE by on-line extraction showed that ultrafiltration prior to analysis had to be carried out to remove interfering proteins. As the gain ratio of ultrafiltration was very low (approx. 1/5), sample material in acute intoxication cases is often very limited and moreover, only the unbound fraction would be analysed, the LLE technique was preferred for the toxicological screening method for plasma. An alternative approach for future investigations would be sample extraction by on-line extraction with restricted access material (RAM) for protein removal as has been described by Singh and Santos et al. [132, 133].

4.2.1 Validation

All given criteria of the performance test for accuracy control [22], which was used to check the adaptation of the toxicological screening method for plasma in the system, were met (3.3.1). Moreover, investigations on within- and between-day precision, accuracy and system-to-system precision determined at the given concentration of PCS-PA were < 15%. Successful integration of the toxicological screening method for plasma in the analytical system was assumed and existing data for quantitation obtained from the reference system was adapted. As the above mentioned validation parameters were determined with the use of PCS-PA at a single concentration and not in plasma matrix, parallel analysis of clinical samples was carried out.

4.2.2 Analysis of Clinical Samples

Parallel analysis of clinical samples in addition to the measurement of the according PCS-PA verified the successful set up of the system: authentic samples which were analysed at the same time and prepared by the same person showed accuracy values ≤ 10% from system-to-system.

System-to-system precision of investigated clinical samples that were not analysed at the same time due to practical reasons, ranged from -56.5% - + 40% with no recognisable trend; in most cases (70%) accuracy was ≤ ±20%. This might have been caused by instability problems and/or matrix interferences from the monovettes. Therefore an appropriate time range for reanalysis < 4 weeks should be determined for future investigations. A qualitative comparison of the results of these samples showed that in 80% of the investigated cases identic results were achieved. In the literature successful set-up of the method in different HPLC systems in accordance with the use of the spectra library was carried out and controlled by the performance test for accuracy control [22]. The authors of the latter library proved that spectra measurement was independent from the DAD-

type as DAD-systems from three different manufacturers were investigated (Shimadzu, Bio-Tec, and Agilent) [22]. Accuracy problems from system-to-system as recognized by the parallel analysis of clinical samples within this thesis which were assumed to be due to instability were not described.

4.3 Screening Method for Neutral, Weakly Acidic and Weakly Basic Compounds in Urine

Benzodiazepines are widely prescribed drugs for a range of clinical disorders [134]. They are administered as anti-anxiety, muscle relaxants, tranquillisers and anticonvulsivants [135, 136]. Besides the extensive medical use, abuse of this class of compounds is a known problem. Three main groups of benzodiazepines based on their structure are available: 1,4-benzodiazepines (e.g. clonazepam, diazepam, flurazepam, and lorazepam), diazolobenzodiazepines (e.g. midazolam) and triazolobenzodiazepines (e.g. alprazolam, triazolam) [105, 134]. Overall, more than 30 benzodiazepines with active and inactive metabolites are known, of which diazepam (Valium®), flunitrazepam (Rohypnol®) and midazolam (Dormicum®) are most popular [137]. Therapeutic blood concentrations range from 0.5 µg/mL (flunitrazepam) to 3 mg/mL (chlordiazepoxide) [137].
Barbiturates lost their formerly high importance as tranquilizers and sleeping medication [107]. Nevertheless, methohexital and thiopental are still important narcotics, phenobarbital is widely used as antiepileptic and some obsolete barbiturates may occur in accidental or suicidal intoxication cases. In case of thiopental its metabolite pentobarbital besides other biotransformation products will be found in urine [97].
Numerous papers dealing with the determination of benzodiazepines have been published [35, 66, 134, 135, 138-141]. However, their purpose was to measure one or only a few compounds and accordingly following these methods, it is not possible to have weakly polar benzodiazepines, such as diazepam or medazepam, and very polar benzodiazepines, such as bromazepam or oxazepam on the same chromatogram. Besides from these methods, few broad screening methods for the determination of benzodiazepines by off-line SPE HPLC-DAD [137, 142] or immunoadsorption [143] have been used. A fully automated version is distributed by Bio-Rad. As described in 1.1.2.2, to use this method, the existing Remedi™-HS has to be equipped with special columns and reagent kits for benzodiazepine analysis, which would take at least 1 h including column equilibration. Or, if basic compounds and benzodiazepines have to be analysed at the same time, two Remedi™-HS systems have to be installed. Therefore, the developed system where automated switching between the methods is possible, presents an advantage.

4.3.1 Analytical Separation

With the employed Nucleosil^{TM} 100 C8 columns separation of 24 benzodiazepines including major metabolites in urine under isocratic conditions was achieved. Moreover, barbiturates and other coextracted neutral, weakly basic and weakly acidic compounds were separated. As the elution behaviour on the latter chromatographic material under acidic conditions is already experienced due to its use for the toxicological screening method for plasma [104], evaluation of chromatograms and combination of the installed methods during routine use is kept simple.
The analytes of PCS-BDP coeluted on the SCX columns due to relatively little differences in polarity under the investigated analytical conditions. Only bromazepam was separated because of protonation of the additional pyridine nitrogen under the mobile phase 2 conditions.

4.3.2 On-line Extraction

A study on sample preparation for the subsequent analysis of benzodiazepines, comparing LLE and different apolar off-line SPE materials was performed by He et al. [139]. It was shown, that SPE offered advantages over LLE, and polymer SPE material over C18 SPE material regarding the purity of extracts and the extraction efficiency. Moreover, He et al. demonstrated, that no differences between investigated polymeric cartridges were observed [139]. This was also found within the studies of this thesis for investigated polymer on-line SPE material (2.2.3.2, Table 2-2) regarding the extraction of benzodiazepines and barbiturates. Considerable differences on the other hand, were observed between the extraction efficiency of benzodiazepines (≥ 63%) and barbiturates (< 20% for the PCS-BARB analytes except for methohexital and pentobarbital (≥ 62%), 3.4.6.3). This was possibly due to their differences in chemical properties such as structure and pK_a-values. In comparison to barbituric acid (pK_a 5) the investigated dialkyl-substituted barbiturates present weak acids (pK_a~ 8.0) [105]. Benzodiazepines have weakly acidic (pK_a ~ 11) and weakly basic properties (pK_a ~ 2-5) and are less polar than barbiturates [105]. Under the developed extraction conditions the PCS-BDP were not charged and thus could be extracted by the polymer material. The basic framework (2,4,6-trihydroxypyrimidine) of the PCS-BARB analytes is rather polar which may explain the low extraction efficiency on the polymer material due to decreased apolar interactions. The same effect may be the reason for the low recovery of MPPH (40%) which may also be explained by its rather high polarity due to the hydantoin basic framework. Different to the PCS-BARB analytes the structure of MPPH includes two phenyl partial structures for interaction with the polymer extraction material. The better recovery and linearity of methohexital and pentobarbital could possibly be due to interactions of the side chains on C5 with the polymer material and the higher pK_a value in comparison to the other PCS-BARB analytes.

The attempt to load the PCS-BARB with acidic loading buffer (0.05 M phosphate buffer pH 2.3) onto the extraction column and thus avoid deprotonation in every case and include possible side products (e.g. malonic acid derivatives) was not successful. Probably, the investigated barbiturates showed still too low hydrophobicity to be retained on the apolar extraction material.
In conclusion, reliable results can only be obtained for benzodiazepines by the screening method for neutral, weakly acidic and weakly basic compounds in urine. In cases of barbiturate intoxications with sufficiently high concentrations, positive identification may be possible. However, plasma analysis by the toxicological screening method for plasma would be the method of choice for barbiturate detection.

4.3.3 Compound Identification

In accordance with the other installed methods, compound identification was carried out by spectra and RRT comparison with a spectra library. The detection wavelength was set to 205 nm. It could be shown that at a detection wavelength of 265 nm, also used by the Remedi^{TM}-HS system for benzodiazepine analysis to reduce the appearance of interfering compounds [144, 145], less matrix interferences were visible in the chromatogram due to lower absorption at this wavelength. As described in 3.4.5, the chosen I.S. MPPH did not absorb either at this wavelength. Consequently, to take advantage of this effect, another I.S. would have had to be chosen. For example the use of a benzodiazepine with a very low urinary excretion or a prodrug like prazepam would be thinkable. However, if the future routine use of the method will show that the method lacks selectivity and sensitivity due to occurring matrix interferences, a change in the extraction conditions would be preferable.

4.3.4 Validation

Like with the on-line extraction HPLC-DAD screening method for basic compounds in urine, exemplary validation of a PCS was carried out following the FDA guidelines [96].
It could be demonstrated, that reliable data for the analysis of benzodiazepines can be obtained by the developed method as sufficient recovery (≥ 63%), good linearity (≥ 0.997) and precision results (≤ 8.4%) were achieved (3.4.6, and 7.6, Table 7-13). Nordiazepam (8.3%) and temazepam (6.4%) showed the highest imprecision results possibly due to matrix influences at the RT of the latter compounds. However, the achieved data were still within the acceptance limits of the FDA [96]. Despite the relatively low LLOD (0.05-0.1 µg/mL) of the method determined within the validation investigations, the analysis of authentic clinical samples showed that an immunological pre-screening value of > 1000 ng/mL had to be exceeded to allow positive chromatographic confirmation (3.4.7). This may be explained by the fact, that immunoassays are specific for a

substance group including metabolites and not for a single compound. For example the positive immunological result for benzodiazepines in urine in case of diazepam intake, would include its metabolites oxazepam, nordiazepam, temazepam and their glucuronides whereas the chromatographic assay is specific for each compound. Therefore, for each compound the concentration of the LLOD has to be at least reached to allow detection. In the future it will be proved if the LLOD is sufficient for the field of toxicological routine use, such as diagnosis of acute intoxications and DOA confirmation screening. So far, the developed method replaced more specialised methods in 74% of the evaluated samples. Besides the most commonly detected metabolites demoxepam, nordiazepam, oxazepam and temazepam, other benzodiazepine metabolites e.g. hydroxymidazolam, hydroxyalprazolam were identified by the developed method.
In the case of barbiturates, only methohexital and pentobarbital showed good results concerning the investigated validation parameters. All other PCS-BARB analytes showed poor recovery and low linearity and therefore can only be analysed qualitatively in acute intoxication cases where high barbiturate concentrations are expected, which will lead to a sufficiently high peak in the chromatogram even at a low recovery. Unfortunately, due to a lack of clinical samples, the method could not be proven with authentic cases. On the other hand this shows the relatively low importance of barbiturates in cases of abuse today. Nevertheless, as mentioned previously, in cases with the suspicion of barbiturate intoxication, plasma analysis using the toxicological screening method for plasma should be carried out [22, 104].

4.4 Routine Use

With its high separation power and versatility (opportunity to screen either urine or plasma), the developed analytical system appeared to be a potentially valuable approach for toxicological screening.
With the set-up of the toxicological screening method for plasma most basic and neutral compounds are extracted and thus can be analysed and quantified within STA. The screening method for basic compounds in urine allows analysis of alkaloids which are difficult to detect in plasma due to their short half-lives and may provide additional information about basic drugs by detection of metabolites (STA). Furthermore, the method can be used for DOA confirmation analysis taking again advantage of the enlarged window of detection of urine analysis compared to blood. Due to the employed weak cation-exchange on-line extraction material, only basic compounds with a $pK_a \geq 6$ can be analysed. Therefore, an automated method for the on-line extraction of neutral, weakly acidic and weakly basic compounds from urine was established.
When in acute intoxication cases plasma and urine samples are available, first the plasma sample should be run because information about actual xenobiotic concentrations can be obtained. During this run the urine sample can be prepared and a sample aliquot can be hydrolysed for

benzodiazepine analysis. The plasma sample can then be evaluated while the urine samples are analysed. The total analysis time would be 160 min including equilibration.

The given examples of authentic cases in the respective method chapters (3.2.7, 3.2.8, 3.3.3, 3.4.7, 3.4.8) illustrated the varied analytical use of the system. This was also demonstrated with the exemplary analysis of clinical samples using all three established methods (3.5.2). Furthermore, the handling of the different urine screening methods was proven with performed external quality assessment schemes (3.5.3). Overall, it was shown, that the developed methods proved to be reliable for their intended field of application, but once again, it can be stated for STA analysis, that the developed methods should be run within a complex analysis strategy to allow identification of those compounds that cannot be identified with the developed methods for urine screening. Vice versa, additional urine analysis may give additional information about drugs not detected in plasma as was shown in 3.5.2. In Fig. 4-1 the analysis strategy presented in 1.1, Fig.1-1 for STA is shown, but modified with the integrated use of the developed system (presented in italic). The Remedi^{TM}-HS system is replaced by the screening method for basic compounds, the screening method for neutral, weakly acidic and weakly basic compounds is added and toxicological screening in plasma can also be performed in the developed system.

In DOA cases immunological pre-screening is recommendable in order to choose a confirmation method with sufficient sensitivity and to avoid false negative results due to coelution or analyte concentrations below the LLOD. Therefore, the following strategy for DOA confirmation analysis was developed as is described in 3.2.8.3, according to gained experience by confirmation analysis of authentic samples (Fig. 4-2). Except for the heroin marker 6-AM, which presents a critical parameter (decision whether heroin was abused or not) and moreover showed the lowest confirmation rate by HPLC-DAD, all listed DOA can be confirmed by HPLC-DAD in the first place. In order to increase the confirmation rate opiates require glucuronide (glc) hydrolysis prior to analysis, BEC samples should be run with the specific BEC method. Benzodiazepines with immunological value between 200 to 1000 ng/mL measured by CEDIA (above the cut-off and below the LLOD of the screening method for neutral, weakly acidic and weakly basic compounds) should be analysed by GC-MS to avoid double sample preparation and repeated analysis.

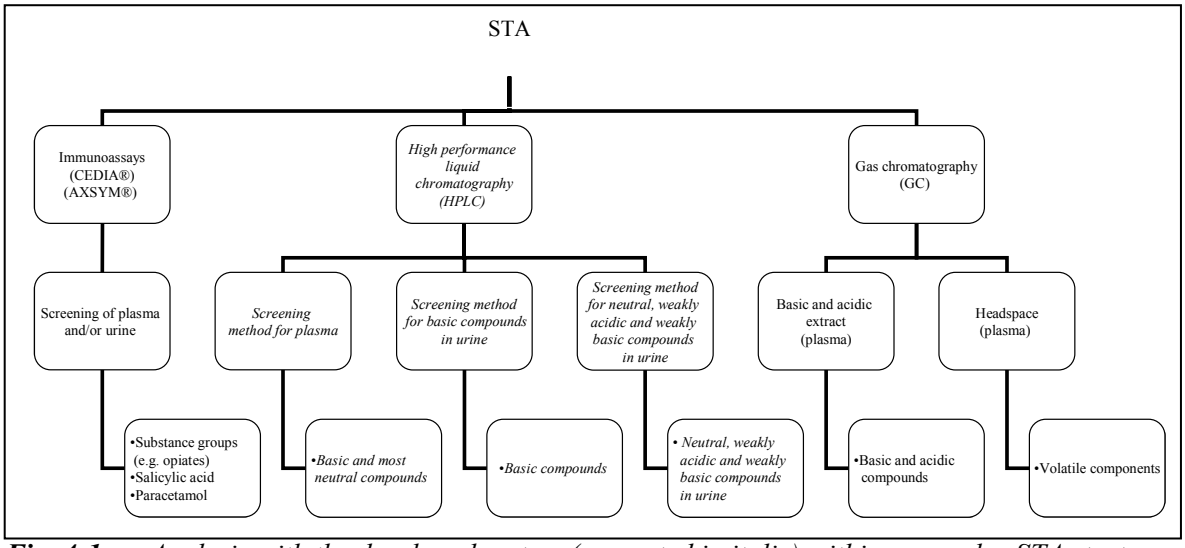


Fig. 4-1 *Analysis with the developed system (presented in italic) within a complex STA strategy*

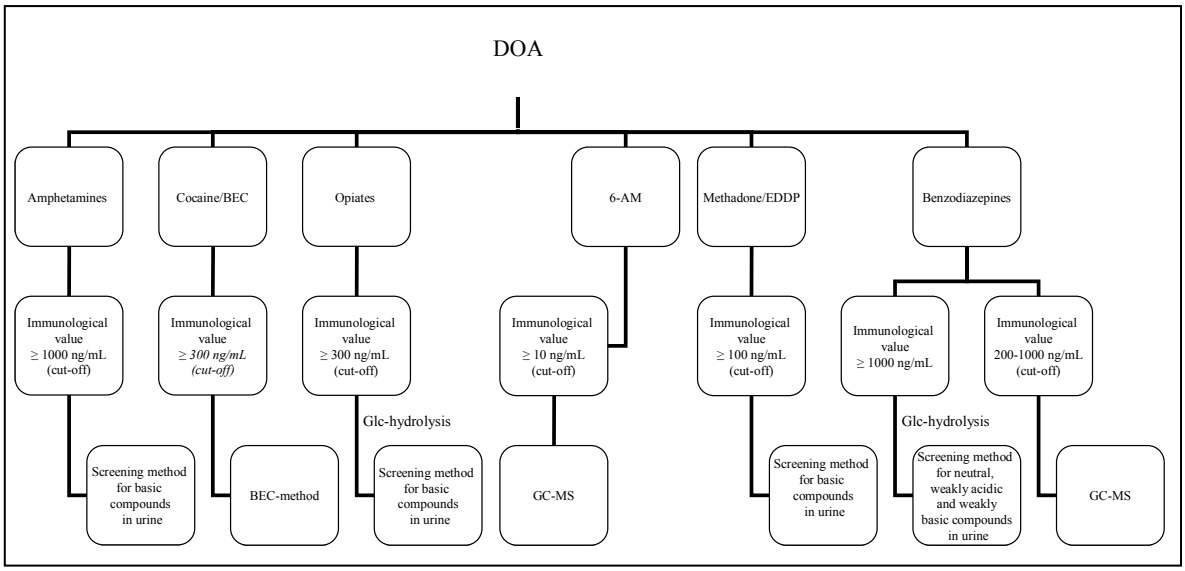


Fig. 4-2 *Strategy for DOA confirmation screening*

In conclusion, the use of switching valves allowed the combination of analytically different methods in one system and therefore provided efficient solutions for STA applications because basic, neutral, weakly acidic and weakly basic compounds can be screened in urine and also a complementary screening method for plasma analysis is provided. Depending on the number of samples and urgency of analysis, all three methods can be run for STA using just one analytical system. All integrated methods are simple, reproducible and based on common HPLC equipment as well as the same laboratory material (e.g. solvents, buffer) and software.

5 Conclusions and Future Perspectives

The scope of this thesis was the development of a chromatographic screening method for the determination of compounds in urine with main focus on those substances that are difficult to detect with common STA methods in plasma, such as alkaloids and other polar basic compounds in order to replace time-consuming and/or specialised methods formerly used for this purpose at the Institute of Toxicology-Clinical Toxicology and Poison Control Centre Berlin. This method was supplemented with two further methods: a toxicological screening method for plasma analysis and a method for the determination of neutral, weakly acidic and weakly basic compounds in urine. The following conclusions for the development can be drawn:

Firstly, a fully automated method for the qualitative determination of basic drugs from urine was established and validated. The use of on-line extraction replaced tedious and time-consuming purification steps and led to a system that could work unattended. The elution under isocratic conditions as well as the use of common HPLC solvents and equipment simplified the method and the method set-up. The analysis of authentic toxicological samples proved the utility for toxicological applications, such as the analysis of alkaloids and other basic compounds in general and demonstrated its applicability for STA and DOA confirmation analysis for amphetamine, cocaine/BEC, opiate and methadone/EDDP above the given LOD. A comparison with an existing urine screening method, the Remedi^{TM}-HS showed that the developed system presents an adequate alternative to the latter system, but offers higher versatility, modern equipment and sample analysis at low costs. The method has been successfully introduced to toxicological routine use and will eventually be commercially marketed as an alternative to the Remedi^{TM}-HS, which will be taken out of service in 2008. All obtained validation data met the criteria for the investigated parameters set in international guidelines for bioanalytical methods and confirmed the reliability of the method.

In conclusion, the developed on-line extraction HPLC-DAD method for basic compounds allowed simple and reliable determination of basic drugs in urine at low costs and is suitable for the routine use (STA and DOA confirmation screening) as results of authentic sample analyses showed.

Secondly, in addition to the on-line extraction HPLC-DAD method for basic compounds, a toxicological screening method for plasma was established in the analytical system. The plasma method covers most basic and neutral compounds that can be separated on the coupled Nucleosil^{TM} 100 C8 columns following LLE and allows quantitation of therapeutic and toxic concentrations. The set-up of the method was successfully controlled by a performance test and proven with the parallel analysis with a reference system.

Thirdly, an on-line extraction method for the determination of neutral, weakly acidic and weakly basic compounds in urine was developed and integrated in the analytical system. This method was

especially useful for the determination of the important group of benzodiazepines as could be concluded from the achieved validation results and as was illustrated with the analysis of clinical cases. Barbiturates could be identified qualitatively in high concentrations (> 1.0 µg/mL cyclo-, crotyl- and phenobarbital, > 0.1 µg/mL methohexital and pentobarbital).

Sample extraction as well as the switching between the different methods is fully automated and therefore easy to perform.

For all established methods, the use of the DAD system gave access to the spectra library of approximately 2600 spectra and additional spectra of the method-specific libraries and allowed the detection of metabolites by comparing the spectrum of the proposed metabolite and the parent compound. Thus, the spectra library will be continuously expanded with toxicological relevant compounds and metabolites which are not available as reference standards.

The developed system showed to be very useful and efficient for toxicological screening, as it covers a broad range of relevant compounds and particularly in its ability to screen either plasma or urine with complementary methods. In comparison to other chromatographic methods, no sample derivatisation (GC-MS) is required and direct injection of urine without carry-over problems (LC-MS) is possible.

The developed screening method for basic compounds in urine has been patented and will be distributed by Shimadzu under the name *Prominence* TOX.I.S.. Prominence describes the HPLC equipment series, TOX.I.S. stands for TOXicological Identification System. Therefore, it will be used in different fields of research resulting in inter-laboratory exchange concerning its advantages and limitations. Moreover, besides the continuous enlargement of the method-specific library through analysis of clinical routine samples at the Institute of Toxicology-Clinical Toxicology and Poison Control Centre Berlin, contributions from other laboratories are expected to lead to an increase of accessible spectra and RRT data.

Detailed characterisation of the substance spectrum for each method will be gained by continuous analysis of samples by all established methods, detecting gaps in either extraction or analytical separation for certain compounds in accordance to the used method.

Optimisation of the LEC method might be of interest for laboratories that previously performed analysis of LEC in urine by the Remedi^{TM}-HS. For this aim investigation of appropriate analytical columns will be necessary.

A further research objective will include the structure elucidation of appearing metabolites by MS in order to specify the library data. The expansion of the library with MS data will also give the opportunity of MS coupling. Thus, with the combination of UV and MS detection, higher sensitivity will be gained. However, a suitable volatile mobile phase component will have to be found for this purpose.

Replacement of LLE by automated on-line extraction for the toxicological screening method for plasma would be another interesting task, which would lead to a fully automated analytical system. To accomplish this aim, the use of RAM for protein removal may be an option.

Finally, more data about the screening method for neutral, weakly acidic and weakly basic compounds for toxicological routine use will be gained. Optimisation of the latter method concerning cleaner sample extracts will be subject to further investigations dealing with the testing of new on-line extraction materials.

6 Bibliography

[1] W. E. Lambert, J. F. Van Bocxlaer, A. P. De Leenheer, Potential of high-performance liquid chromatography with photodiode array detection in forensic toxicology, *J. Chromatogr. B Biomed. Sci. Appl.* **689:** 45-53, 1997.

[2] P. Marquet, Is LC-MS suitable for a comprehensive screening of drugs and poisons in clinical toxicology?, *Ther. Drug Monit.* **24:** 125-133, 2002.

[3] Leistungsbericht 2004/2005 Berliner Betrieb für Zentrale Gesundheitliche Aufgaben.

[4] H. H. Maurer, Position of chromatic techniques in screening for detection of drugs or poisons in clinical and forensic toxicology and/or doping control, *Clin. Chem. Lab. Med.* **42:** 11, 1310-1324, 2004.

[5] O. H. Drummer, Chromatographic screening techniques in systematic toxicological analysis, *J. Chromatogr. B Biomed. Sci. Appl.* **733:** 27-45, 1999.

[6] H. H. Maurer, Screening procedures for simultaneous detection of several drug classes used for high throughput toxicological analyses and doping control, *Comb. Chem. High Throughput Screen.* **3:** 467-480, 2000.

[7] J. P. Franke, R. A. de Zeeuw, Solid-phase extraction procedures in systematic toxicological analysis, *J. Chromatogr. B Biomed. Sci. Appl.* **713:** 51-59, 1998.

[8] A. Polettini, Systematic toxicological analysis of drugs and poisons in biosamples by hyphenated chromatographic and spectroscopic techniques, *J. Chromatogr. B Biomed. Sci. Appl.* **733:** 47-63, 1999.

[9] K. Pfleger, H. H. Maurer, A. Weber, Mass spectral and GC data of drugs, poisons, pesticides, pollutants and their metabolites, 2nd Edition, part 4, Wiley-VCH Verlag GmbH, Weinheim, Germany, 2000.

[10] A. Valli, A. Polettini, P. Papa, M. Montagna, Comprehensive drug screening by integrated use of gas chromatography/mass spectrometry and Remedi™-HS, *Ther. Drug. Monit.* **23:** 287-294, 2001.

[11] M. Bogusz, M. Erkens, Reversed-phase high-performance liquid chromatographic database of retention indices and UV spectra of toxicologically relevant substances and its interlaboratory use, *J. Chromatogr. A*, **674:** 97-126, 1994.

[12] J.-S. Chen, K.-J. Chang, R.-C. Charng, S. J. Lai, S. R. Binder, H. Essian, The development of a broad-spectrum toxicology screening program in Taiwan, *Clin. Toxicol.* **33:** 581-589, 1995.

[13] R. L. Fitzgerald, J. D. Rivera, D. A. Herold, Broad spectrum drug identification directly from urine, using liquid chromatography-tandem mass spectrometry, *Clin. Chem.* **45:** 1224-1234, 1999.

[14] J.-L. Veuthy, S. Souverain, S. Rudaz, Column-switching for the analysis of drugs in biologic samples, *Ther. Drug Monit.* **26:** 161-165, 2004.

[15] C. H. P. Bruins, C. M. Jeronimus-Stratingh, K. Ensing, W. D van Dongen, G. J. de Jong, On-line coupling of solid-phase extraction with mass spectrometry for the analysis of biological samples, 1. Determination of clenbuterol in urine, *J. Chromatogr. A*, **863:** 115-122, 1999.

[16] C. K. Lai, T. Lee, K. M. Au, A. Y. W. Chan, Uniform solid-phase extraction procedure for toxicological drug screening in serum and urine by HPLC with photodiode-array detection, *Clin. Chem.* **43:** 312-325, 1997.

[17] H. H. Maurer, Liquid chromatography-mass spectrometry in forensic and clinical toxicology, *J. Chromatogr. B Biomed. Sci. Appl.* **713:** 3-25, 1998.

[18] Y. Gaillard, G. Pepin, Use of high-performance liquid chromatography with photodiode-array UV detection for the creation of a 600-compound library. Application to forensic toxicology, *J. Chromatogr. A* **763:** 149-163, 1997.

[19] R. Dams, T. Benijts, W. E. Lambert, A. P. De Leenheer, Simultaneous determination of in total 17 opium alkaloids and opioids in blood and urine by fast liquid chromatography-diode-array detection-fluorescence detection, after solid-phase extraction, *J. Chromatogr. B Biomed Sci Appl.* **773:** 53-61, 2002.

[20] J. Yawney, S. Treacy, K. W. Hindmarsh, F. J. Burczynski, A general screening method for acidic, neutral and basic drugs in whole blood using Oasis MCX column, *J. Anal. Toxicol.* **26:** 325-332, 2002.

[21] H. K. Nordgren, O. Beck, Multicomponent screening for drugs of abuse, *Ther. Drug Monit.* **26:** 90-97, 2004.

[22] F. Pragst, M. Herzler, S. Herre, B.-T. Erxleben, M. Rothe, UV spectra of toxic compounds. Dr. Dieter Helm, Heppenheim, 2001.

[23] S. P. Elliott, K. A. Hale, Development of a high-performance liquid chromatography retention index scale for toxicological drug screening, *J. Anal. Toxicol.* **22:** 279-289, 1998.

[24] R. A. de Zeeuw, Recent developments in analytical toxicology: for better or for worse, *Toxicology Letters* **102-103:** 103-108, 1998.

[25] K. Möller, U. Nilsson, C. Crescenzi, Investigation of matrix effects of urine on a molecularly imprinted solid-phase extraction, *J. Chromatogr. B Biomed. Sci. Appl.* **811:** 171, 2004.

[26] V. Walker, G. A. Mills, Solid-phase extraction in clinical biochemistry, *Ann. Clin. Biochem*. **39:** 464-477, 2002.

[27] J. S. Fritz, Analytical solid-phase extraction.Wiley-VCH, New York, 1999.

[28] F. Pragst, H. H. Maurer, J. Hallbach, U. Staerk, W. R. Külpmann, F. Degel, H. J. Gibitz, in W. R. Külpmann (Editor), Klinisch-toxikologische Analytik. Wiley-VCH, Weinheim, 2002, p. 49.

[29] J. S. Fritz, M. Macka, Solid-phase trapping of solutes for further chromatographic or electrophoretic analysis, *J. Chromatogr. A* **902:** 137, 2000.

[30] T. R. Krishnan, I. Ibraham, Solid-phase extraction technique for the analysis of biological samples, *J. Pharm. & Biomed. Anal.*, **12:** 287-294.

[31] M. Moors, D. L. Massart, R. D. McDowall, Analyte isolation by solid phase extraction (SPE) on silica-bonded phases, classification and recommended practices, *Pure & Appl. Chem.* **66:** 2, 277-304, 1994.

[32] I. Liska, J. Krupcik, P. A. Leclercq, The use of solid sorbents for direct accumulation of organic compounds from water matrices–a review of solid-phase extraction techniques, *J. High Res. Chromatogr.* **12:** 577, 1989.

[33] V. Walker, G. A. Mills, Solid-phase extraction in clinical biochemistry, *Ann. Clin. Biochem.* **39:** 464-477, 2002.

[34] C. R. Mallet, Z. Lu, J. Mazzeo, U. Neue, Analysis of a basic drug by on-line solid-phase extraction liquid chromatography/tandem mass spectrometry using a mixed mode sorbent, *Rapid Commun. Mass Spectrom.* **16:** 805, 2002.

[35] A. Miki, Simultaneous determination of eleven benzodiazepine hypnotics and eleven relevant metabolites in urine by column-switching liquid chromatography-mass spectrometry, *J. Anal. Toxicol.* **26:** 87, 2002.

[36] E. Krämer, K.-A. Kovar, On-line coupling of automated solid-phase extraction with high-performance liquid chromatography and electrochemical detection, Quantitation of oxidizable drugs of abuse and their metabolites in plasma and urine, *J. Chromatogr. B Biomed. Sci. Appl.***731:** 167, 1999.

[37] P. M. Jeanville, E. S. Estape, I. Torres-Negron, A. Marti, Rapid confirmation/quantitation of ecgonine methyl esther, BEC and cocaine in urine using on-line extraction coupled with fast HPLC and tandem masss spectrometry, *J. Anal. Toxicol.* **25:** 69, 2001.

[38] T. R. Krishan, I. Ibraham, Solid-phase extraction technique for the analysis of biological samples, *J. Pharm. & Biomed. Analysis* **12:** 3, 287-294, 1994.

[39] V. Walker, G. A. Mills, Solid-phase extraction in clinical biochemistry, *Ann. Clin. Biochem.* **39:** 464-477, 2002.

[40] F. Degel, Comparison of new solid-phase extraction methods for chromatographic identification of drugs in clinical toxicological analysis, *Clin. Biochem.* **29:** 529-539, 1996.

[41] J. F. Wilson, B. L. Smith, P. A. Toseland, I. D. Watson, J. Williams, A. H. Thomson, N. E. Capps, G. Sweeney, L. N. Sandle, A survey of extraction techniques for drug of abuse in urine, *Forensic Sci. Int.* **119:** 23-27, 2001.

[42] M.-C. Heniion, Solid-phase extraction: method development, sorbents, and coupling with liquid chromatography, *J. Chromatogr. A* **856:** 3-54, 1999.

[43] D. T. Rossi, N. Zhang, Automating solid-phase extraction: current aspects and future prospects, *J. Chromatogr. A* **885:** 97-113, 2000.

[44] J. Hallbach, W. R. Külpmann, H. H. Maurer, F. Pragst, Strategie in der klinisch-toxikologischen Analyse, in W. R. Külpmann (Editor), Klinisch Toxikologische Analyse, Handbuch für Labor und Klinik, Verfahren, Befunde Interpretationen, Wiley-VCH Verlag GmbH, Weinheim, 2002.

[45] D. Hannak, W. R. Külpmann, F. Degel, H. Käferstein, H. H. Maurer, H.-J. Gibitz, F. Pragst, H. König, Messmethoden der klinisch-toxikologischen Analytik, in W. R. Külpmann (Editor), Klinisch Toxikologische Analyse, Handbuch für Labor und Klinik, Verfahren, Befunde Interpretationen, Wiley-VCH Verlag GmbH, Weinheim, 2002.

[46] G. Löffler, P. E. Petrides, P. C. Heinrich von Springer, Biochemie und Pathobiochemie, 7th edition, Springer Verlag, Berlin, 2002.

[47] X.-H. Chen, J.-P. Franke, J. Wijsbeek, R. A. de Zeeuw, Isolation of acidic, neutral and basic drugs from whole blood using a single mixed-mode solid-phase extraction column, *J. Anal. Toxicol.* **16:** 351-355, 1992.

[48] H. H. Maurer, Systematic toxicological analysis of drugs and their metabolites by gas chromatography-mass spectrometry, *J. Chromatogr. B Biomed. Sci. Appl.* **580:** 3-41, 1992.

[49] A. Polettini, A. Groppi, C. Vignali, M. Montagna, Fully-automated systematic toxicological analysis of drugs, poisons, and metabolites in whole blood, urine, and plasma by gas chromatography-full scan mass spectrometry, *J. Chromatogr. B Biomed. Sci. Appl.* **713:** 265-279, 1998.

[50] Z.-P. Huang, X.-H. Chen, J. Wijsbeek, J.-P. Franke, R. A. De Zeeuw, An enzymic digestion and solid-phase extraction procedure for the screening for acid, neutral and basic drugs in liver using gas chromatography for analysis, *J. Anal. Toxicol.* **20:** 248-254, 1996.

[51] S. P. Elliott, K. A. Hale, Development of a high-performance liquid chromatography retention index scale for toxicological drug screening, *J. Chromatogr. B Biomed. Sci. Appl.* **694:** 99-114, 1997.

[52] A. Tracqui, P. Kintz, P. Mangin, Systematic toxicological analysis using HPLC/DAD, *J. Forensic Sci.* **40:** 254-262, 1994.

[53] F. Pragst, M. Herzler, B.-T. Erxleben, Systematic toxicological analysis by high-performance liquid chromatography with diode array detection (HPLC-DAD), *Clin. Chem. Lab. Med.* **42:** 11, 1325-1340, 2004.

[54] F. Pragst, H. H. Maurer, J. Hallbach, U. Staerk, W. R. Külpmann F. Degel, H.-J. Gibitz, Messmethoden der klinisch-toxikologischen Analytik, in W.R. Külpmann (Editor), Klinisch Toxikologische Analyse, Handbuch für Labor und Klinik, Verfahren, Befunde Interpretationen, Wiley-VCH Verlag GmbH, Weinheim, 2002.

[55] S. R. Binder, M. Regalia, M. Biaggi-McEachern, M. Mazhar, Automated liquid chromatographic analysis of drugs in urine by on-line sample cleanup and isocratic multicolumn separation, *J. Chromatogr. B Biomed. Sci. Appl.* **472:** 325-341, 1989.

[56] P. Demedts, A. Wauters, F. Franck, H. Neels, Evaluation of the Remedi drug profiling system, *Eur. J. Clin. Chem. Clin. Biochem.* **32:** 409-417, 1994.

[57] M. Ohtsuji, J. S. Lai, S. R. Binder, T. Kondo, T. Takayasu, T. Ohshima, Use of Remedi HS in emergency toxicology for a rapid estimate of drug concentrations in Urine, Serum, and Gastric Samples, *J. Forensic Sci.* **41:** 5, 881-886, 1996.

[58] E. Pitarch, F. Hernandez , J. ten Hove, H. Meiring, W. Niesing, E. Dijkmann, L. Stolker, Hogendoorn, Potential of capillary-column-switching liquid chromatography-tandem mass spectrometry for the quantitative trace analysis of small molecules Application to the on-line screening of drugs in water, *J. Chromatogr. A* **1031:** 1-9, 2004.

[59] J.-S. Chen, K.-J. Chang, R.-C. Charng, S. Jason Lai, S. R. Binder, H. Essien, The development of a broad-spectrum toxicology screening program in Taiwan, *Clin. Toxicol.* **33:** 6, 581-589, 1995.

[60] S. R. Binder, M. Regalia, G. Sivorinovsky, Toxicological screening by liquid chromatography, *Trends Anal. Chem.* **8:** 10, 1989.

[61] S. D. Ferrara, L. Tedeschi, G. Frison, G. Brusini, F. Castagna, B. Bernardelli, D. Soregaroli, Drugs-of-Abuse testing in urine: statistical approach and experimental comparison of immunochemical and chromatographic techniques, *J. Anal. Toxicol.* **18:** 278-291, 1994.

[62] N. Sadeg, G. Francois, B. Petit, H. Dutertre-Catella, M. Dumontet, Automated liquid-chromatographic analyzer used for toxicology screening in a general hospital: 12 months` experience, *Clin. Chem.* **43:** 3, 498-504, 1997.

[63] A. Valli, A. Polettini, P Papa, M Montagna, Comprehensive Drug Screening by Integrated Use of Gas Chromatography/Mass Spectrometry and Remedi HS, *Ther. Drug Monit.* **23:** 287-294, 2001.

[64] J. Guitton, D. Durand, N. Bouillod, M. Manchon, Development of a solid/liquid extraction method for analysis of toxic drugs in serum using an automated liqud chromatograph: The Remedi, *Ann. Biol. Clin.* **52:** 111-115, 1994.

[65] R. L. Fitzgerald, J. D. Rivera, D. A. Herold, Broad spectrum drug identification directly from urine, using liquid chromatography-tandem mass spectrometry, *Clin. Chem.* **45:** 8, 1224-1234, 1999.

[66] J. L. Valentine, R. Middleton, C. Sparks, Identification of urinary benzodiazepines and their metabolites: comparison of automated HPLC and GC-MS after immunoassay screening of clinical specimens, *J. Anal. Toxicol.* **20:** 416-424, 1996.

[67] F. Musshoff, B. Madea, First experience with the Remedi HS urine benzodiazepine assay, *Clin. Chem. Lab. Med.* **36:** 10, 803-808, 1998.

[68] L. B. Baskin, D. L. Morgan, Drugs detected in patients suspected of acute intoxications, *Texas Medicine*, 50-58, 1997.

[69] W. Weinmann, N. Lehmann, M. Renz, Screening of drugs in serum and urine by LC/ESI/CID-MS and MS/MS with library searching, *Problems Forensic Sci.* **22:** 202-208, 2002.

[70] P. Marquet, G. Lachitre, Liquid chromatography-mass spectrometry: potential in forensic and clinical toxicology, *J. Chromatogr. B Biomed. Sci. Appl.*, **733:** 93-118, 1999.

[71] H. H. Maurer, Multi-analyte procedures for screening for and quantification of drugs in blood, plasma, or serum by liquid chromatography-single stage or tandem mass spectrometry (LC-MS or LC-MS/MS) relevant to clinical and forensic toxicology, *Clin. Biochem.* **38:** 310-318, 2005.

[72] M. Rittner, F. Pragst, W.-R. Bork, J. Neumann, Screening method for seventy psychoactive drugs or drug metabolites in serum based on high-performance liquid chromatography-electrospray ionization mass spectrometry, *J. Anal. Tox.* **25:** 115-124, 2001.

[73] P. Marquet, F. Saint-Marcoux, T. N. Gamble, J. C. Y. Leblanc, Comparison of a preliminary procedure for the general unknown screening of drugs and toxic compounds using a quadrupole-linear ion-trap mass spectrometer with a liquid chromatography-mass spectrometry reference technique, *J. Chromatogr. B Biomed. Sci. Appl.* **789:** 9-18, 2003.

[74] N. Venisse, P. Marquet, E. Duchoslav, J. L. Dupuy, G. Lachatre, A general unknown screening procedure for drugs and toxic compounds in serum using liquid chromatography-electrospray-single quadrupole mass spectrometry, *J. Anal. Toxicol.* **27:** 7-14, 2003.

[75] M. Tatsuno, M. Nishikawa, M. Katagi, H. Tsuchihashi, Simultaneaous determination of illicit drugs in human urine by liquid chromatography-mass spectrometry, *J. Anal. Toxicol.* **20:** 281-286, 1996.

[76] M. Bogusz, M. Erkens, Influence of biological matrix on chromatographic behaviour and detection of selected acidic, neutral and basic drugs examined by means of a standardized HPLC-DAD System, *J. Anal. Toxicol.* **19:** 49-55, 1995.

[77] G. Rücker, M. Neugebauer, G. G. Willems, Instrumentelle pharmazeutische Analytik, Lehrbuch zu spektroskopischen, chromatographischen und elektrochemischen Analysenmethoden, 2nd Edition, Wissenschaftliche Verlagsgesellschaft mbH, Stuttgart, 1992.

[78] M. Herzler, S. Herre, F. Pragst, Selectivity of substance identification by HPLC-DAD in toxicological analysis using a UV Spectra library of 2682 compounds, *J. Anal. Toxicol.* **27:** 233-242, 2003.

[79] M. Herzler, Über die Aussagesicherheit der Substanzidentifizierung mittels HPLC-DAD in der Systematischen Toxikologischen Analyse unter Verwendung einer selbsterstellten UV-Spektrenbibliothek mit 2682 Einträgen, Dissertation submitted to the Mathematic-Natural Science Faculty1 of the Humboldt University of Berlin, 2003.

[80] A. C. Moffat, M.D. Osselton, B. Widdop, Clarke`s analysis of drugs and poisons, 3rd Edition, The pharmaceutical press, London, 2004.

[81] E. M. Koves, Use of high-performance liquid chromatography–diode array detection in forensic toxicology, *J. Chromatogr. A* **692:** 103-119, 1995.

[82] E. Mutschler, Arneimittelwirkungen, Lehrbuch der Pharmakologie und Toxikologie, 7th Edition, Wissenschaftliche Verlagsgesellschaft mbH, Stuttgart, 1996.

[83] Psychrembel Klinisches Wörterbuch, 256th Edition, Walter de Gruyther, Berlin, 1990.

[84] H. Marquardt, S. G. Schäfer, Lehrbuch der Toxikologie, Wissenschaftsverlag, Mannheim, 1994.

[85] A. Grieshaber, K. A. Moore, B. Levine, The Detection of Psilocin in Human Urine*, J. Forensic Sci.*. **46:** 627-630, 2001.

[86] G. Aced, H. J. Möckel, Liquidchromatographie, 1st Edition, VCH, Weinheim, New York, Basel, Cambridge, 2001.

[87] V. Meyer, Praxis der Hochleistungs-Flüssigchromatographie, 8th Edition, Salle, Frankfurt am Main, 1999.

[88] S. Lindsay, Einführung in die HPLC, Analytische Chemie, Springer Verlag, Berlin, 1996.

[89] C. Jimenez, R. Ventura, J. Segura, Validation of qualitative chromatographic methods: strategy in antidoping control laboratories, *J. Chromatogr. B Biomed. Sci. Appl.* **767:** 341-351, 2002.

[90] Centre for Drug Evaluation and Research (CDER), Reviewer Guidance, Validation of chromatographic methods 1-29, 1994.

[91] Richtlinie der GFTCh, GFTCh-Weiterbildungsveranstaltung zur Validierung von Analysenverfahren, Kirkel, 13.-15.4.2000.

[92] International Conference on Harmonisation (ICH) Q2A, Validation of Analytical Methods (Definitions and Terminology) (October 1994); Analytical Validation-Methodology, (November 1996).

[93] S. Kromidas, R. Klinkner, R. Mertens, Methodenvalidierung im analytischen Labor, *Nachr. Chem. Techn. Lab.* **43:** 669-676, 1995.

[94] B. Fera, Zum Umfang der Validierung in der Analytik, *Nachr. Chem. Tech. Lab.* **46:** 28-34, 1998.

[95] A. J. Santos, J. C. Rodrigues, C. Fernandes, G. M. Titato, C. Alves, F. M. Lancas, Automated microcolumn-switching system for drug analysis by direct injection of human plasma, *J. Chromatogr. A* **1105:** 71-76, 2006.

[96] U.S. Food and Drug Administration (FDA), Guidance for industry, Biomedical method validation. http://www.fda.gov/cder/guidance/4252fnl.pdf

[97] Baselt, 6th Edition, Biomedical Publications, Foster City, California, 2002.

[98] F. Hasler, D. Bourquin, R. Brenneisen, F. X. Vollenweider, Renal excretion profiles of psilocin following oral administration of psilocybin: a controlled study in man, *J. Pharmaceut. Biomed. Anal.* **30:** 331-339, 2002.

[99] T. Kamata, M. Nishikawa, M. Katagi, H. Tsuchihashi, Optimized glucuronide hydrolysis for the detection of psilocin in human urine samples, *J. Chromatogr. B Biomed. Sci. Appl.* **796:** 421-427, 2003.

[100] G. Sticht, H. Käferstein, Detection of psilocin in body fluids, *Forens. Sci. Intern.* **113:** 403-407, 2000.

[101] R. Kysilca, M. Wurst, V. Pacakova, K. Stulik, L. Haskovec, High-performance liquid chromatographic determination of hallucinogenic indoleamines with simultaneous UV photometric and voltametric detection, *J. Chromatogr. B Biomed. Sci. Appl.* **320:** 44-420, 1985.

[102] CEDIA DAU® Microgenics Instruction Manual, Microgenics, Passau, 2004.

[103] Laboratory internal method for the determination of drugs of abuse in urine using off-line SPE-GC/MS, Institute of Toxicology-Clinical Toxicology and Poison Control Centre Berlin, 2006.

[104] F. Degel, W. Steimer, H. J. Birkhahn, D. Lampe, U. Demme, Neuroleptika und Antidepressiva, in W. R. Külpmann (Editor), Handbuch für Labor und Klinik, Klinisch-toxikologische Analytik, Verfahren, Befunde, Interpretationen, Wiley-VCH Verlag GmbH, Weinheim, 2002, 319-363.

[105] K. Eger, R. Troschütz, H. J. Roth, Arzneistoffanalyse, Reaktivität, Stabilität, Analytik, 4th Edition, Deutscher Apotheker Verlag, Stuttgart, 1999.

[106] M. Albinus, V. Hempel, Analgetika und Schmerztherapie, Medizinisch pharmakologisches Kompendium, 6th Edition, Wissenschaftliche Verlagsgesellschaft mbH Stuttgart, 1988.

[107] W. Forth, D. Henschler, W. Rummel, Allgemeine und spezielle Pharmakologie und Toxikologie für Studenten der Medizin, Veterinärmedizin, Pharmazie, Chemie, Biologie, sowie für Ärzte, Tierärzte, und Apotheker, 4th Edition, Bibliographisches Institut, Mannheim, Wien, Zürich, 1983.

[108] O. H. Drummer, The forensic pharmacology of drugs of abuse, Oxford University Press, New York, 2001.

[109] Bond Elut Certify™ Instruction Manual, Varian Darmstadt, 2001.

[110] S. I. Kawano, M. Takahashi, T. Hine, E. Yamamoto, N. Asakawa, On-line pretreatment using methylcellulose-immobilized cation-exchange restricted access media for direct liquid chromatography/mass spectrometric determination of basic drugs in plasma, *Rapid Commun. Mass Spectrom.* **19:** 2827-2832, 2005.

[111] A. Espada. A. Rivera-Sagredo, Ammonium hydrogen carbonate, an excellent buffer for the analysis of basic drugs by liquid chromatography-mass spectrometry at high pH, *J. Chromatogr. A,* **987:** 211-220, 2003.

[112] E. Yamamoto, K. Murata, Y. Ishihama, N. Asakawa, Methylcellulose-immobilized reversed-phase precolumn for direct analysis of drugs in plasma by HPLC, *Anal. Sci.,* **17:** 1155-1159, 2001.

[113] J.-T. Wu, H. Zeng, M. Qian, B. L. Brogdon, S. E. Unger, Direct plasma sample injection in multiple-component LC-MS-MS assays for high-throughput pharmacokinetic screening, *Anal. Chem.*, **72:** 61-67, 2000.

[114] A. Tracqui, P. Kintz, P. Mangin, High performance liquid chromatographic assay with diode-array detection for toxicological screening of zopiclone, zolpidem, suriclone and alpidem in human plasma, *J. Chromatogr. B Biomed. Sci. Appl.,* **616:** 95-103, 1993.

[115] S. D. Ferrara, L. Tedeschi, G. Frison, F. Castagna, Solid-phase extraction and HPLC-UV confirmation of drugs of abuse in urine, *J. Anal. Toxicol.,* **16:** 217-222, 1992.

[116] I. Jane, A. Mc Kinnon, R. J. Flannagan, High-performance liquid chromatography analysis of basic drugs on silica columns using non-aqueous ionic eluents. 2. Application of UV, fluorescence and electrochemical oxidation detection. *J. Chromatogr. B Biomed. Sci. Appl.,* **323:** 191, 1985

[117] I. Jane, The separation of a wide range of drugs of abuse by high-pressure liquid chromatography. A review. *J. Chromatogr. B Biomed. Sci. Appl.,* **111:** 227, 1975

[118] S.-O. Jannson, M.-L. Johannson, *J. Chromatogr. B Biomed. Sci. Appl.,* **395 :** 495, 1987.

[119] S. Sturm, A general unknown screening for drugs and toxic compounds in human serum, Dissertation submitted to the Faculty of Natural Sciences of the University Basel, Switzerland, 2005.

[120] T. Kamata, M. Nishikawa, M. Katagi, H. Tsuchihashi, Liquid chromatography-mass spectrometric and liquid chromatography-tandem mass spectrometric determination of hallucinogenic indoles psilocin and psilocybin in "magic mushroom" samples, *J. Forensic Sci.*, **50:** 336-340. 2005.

[121] M. Balikova, Collective poisoning with hallucinogenous herbal tea, *Forensic Sci. Int.*, **128:** 50-52, 2002.

[122] L. Kursinszki, H. Hank, I. Lszlo, E. Szöke, Simultaneous analysis of hyoscyamine, scopolamine, 6-ß-hydroxyhyoscyamine and apoatropine in solanaceous hairy roots by reversed-phase high-performance liquid chromatography, *J. Chromatogr. A*, **1091:** 32-39, 1091.

[123] M. Bogusz, Influence of elution conditions on HPLC retention index values of selected acidic and basic drugs measured in the 1-nitroalkane scale, *J. Anal. Toxicol.*, **15:** 174-178, 1991.

[124] F. Hasler, D. Bourquin, R. Brenneisen, Determination of of psilocin and 4-hydroxyindole-3-acetic acid in plasma by HPLC-ECD and pharmacokinetic profiles of oral and intravenous psilocybin in man. Pharm. Acta. Helv. **72:** 175-184, 1997.

[125] H. H. Maurer, Scopolamine-its role in clinical and forensic toxicology, 44th International meeting of the International Association of Forensic Toxicologists (TIAFT), Ljubljana, Slovenia, 26.08-01.09.2006, Abstract book, 42, 2006.

[126] R. Whelpton, P. R. Hurst, R. F. Metcalfe, S. A. Saunders, Liquid chromatographic determination of hyoscine (scopolamine) in urine using solid phase extraction, *Biomed. Chromatogr.*, **6:** 198-204, 1992.

[127] P. A. Steenkamp, N. M. Harding, F.R. van Heerden, B.E. van Wyk, Fatal datura poisoning: identification of atropine and scopolamine by high performance liquid chromatography/photodiode array/mass spectrometry, *Forensic Sci. Int.*, **145:** 31-39, 2004.

[128] O. Rbeida, B. Christiaens, Ph. Hubert, D. Lubda, K.-S. Boos, J. Crommen, P. Chiap, Intergrated on-line sample clean-up using cation exchange restricted access for the LC determination of atropine in human plasma coupled to UV detection, *J. Pharmaceut. Biomed. Anal.*, **36:** 947-954, 2005.

[129] Internal data base, Institute of Toxicology-Clinical Toxicology and Poison Control Centre Berlin, 2007.

[130] B. W. Corbet, A. J. Mc Bay, L. Kopjac, Atropine poisoning, *Bull. Int. Asso. For. Tox.*, **14:** 37-38, 1978.

[131] K. S. Kalasinsky, T. Schaefer, S. R. Binder, Forensic application of an automated drug-profiling system, *J. Anal. Toxicol.*, **19:** 412-418, 1995.

[132] S. S. Singh, M. Jain, H. Shah, S. Gupta, P. Thakker, R. Shah, B. B. Lohray, Direct injection, column switching-liquid chromatographic technique for the estimation of rabeprazole in bioequivalence study, *J. Chromatogr. B Biomed. Sci. Appl.,* **813:** 247-254, 813.

[133] A. J. Santos Neto, J. C. Rodrigues, C. Fernandes, G. M. Titato, C. Alves, F. M. Lancas, Automated microcolumn-switching system for drug analysis by direct injection of human plasma, *J. Chromatogr. A*, **1105:** 71-76, 2006.

[134] F. Guan, H. Seno, A. Ishii, K. Watanabe, T. Kumaszawa, H. Hattori, O. Suzuki, Solid-phase microextraction and GC-ECD of benzophenones for detection of benzodiazepines in urine, *J. Anal. Toxicol.*, **23:** 54-61, 1999.

[135] M. W. Mullett, J. Pawliszyn, Direct LC analysis of five benzodiazepines in human urine and plasma using an ADS restricted access extraction column, *J. Pharm. Biomed. Anal.* **26:** 899-908, 2001.

[136] O. H. Drummer, Methods for the measurement of benzodiazepines in biological samples, *J. Chromatogr. B Biomed. Sci. Appl.* **713:** 201-225, 1998.

[137] P. Mura, A. Piriou, P. Fraillon, Y. Papet, D. Reiss, Screening procedures for benzodiazepines in biological fluids by high-performance liquid chromatography using a rapid-scanning multichannel detector, *J. Chromatogr. B Biomed. Sci. Appl.,* **416:** 308-310, 1987.

[138] R. Lauber, M. Mosimann, M. Bührer, A. M. Zbinden, Automated determination of midazolam in human plasma by high-performance liquid chromatography using column switching, *J. Chromatogr. B Biomed. Sci. Appl.,* **654:** 69-75, 1994.

[139] H. He, C. Sun, X.-R. Wang, C. Pham-Huy, N. Chikhi-Chorfi, H. Galons, M. Thevenin, J.-R. Claude, J.-M. Warnet, Solid-phase extraction of methadone enantiomers and

benzodiazepines in biological fluids by two polymeric cartridges for liquid chromatographic analysis, *Chromatogr. B Biomed. Sci. Appl.,* **814:** 385-391, 2005.

[140] I. Deinl., G. Mahr, L. v. Meyer, Determination of flunitrazepam and its main metabolites in serum and urine by HPLC after mixed-mode solid-phase extraction, *J. Anal. Toxicol.*, **22:** 197-202.

[141] R. Kronstadt, I. Nystrom, M. Josefsson, S. Hodgins, Segmental ion spray LC-MS-MS analysis of benzodiazepines in hair of psychiatric patients, *J. Anal. Toxicol.*, **26:** 379-484, 2002.

[142] B. Ahrens, H.-J- Schwandt, H. Schütz, Screening, identification and quantitation of benzodiazepines in serum by solid phase extraction on a cyanopropyl phase using high performance liquid chromatography and photodiode array detection, Arzneim.-Forsch./Drug Res., **50:** 1057-1062, 2000.

[143] C. Franzelius, I. Ackermann, I. Deinl, L. Angermaier, G. Machbert, Simultaneous extraction of selected benzodiazepines and benzodiazepine-glucuronides from urine by immunoadsorption, *J. Anal. Toxicol.,* **22:** 359-362.

[144] A. Bucher, Erste Erfahrungen mit dem UBz- Quantifizierung von Oxazepam und verwandten Metaboliten sowie Kreatinin im Urin, Fachhochschule Isny an der Naturwissenschaftlich-technischen Akademie, 1995.

[145] S. R. Binder, D. L. King, United States Patent, Patent number 5,416,023, 1995.

[146] BioRad webpage, http://erlab.mc.ntu.edu.tw/remedi1.htm, last access 5.10.2007.

7 Appendix

7.1 Performance Control Samples

Table 7-1 *Compounds of the PCS with structure and pK_a values*

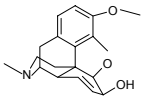
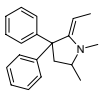
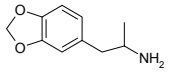
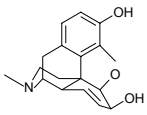
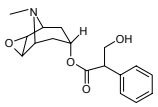
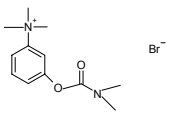
Name	Concentration (µg/mL)	Chemical structures	pK_a	Literature
Codeine	1.0		8.2	[97]
2-Ethyliden-1,5-dimethyl-3,3-diphenyl-pyrrolidine (EDDP)	1.0		9.6	[97]
3,4-Methylene-dioxy-amphetamine (MDA)	1.0		9.7	[80]
Morphine	1.0		8.0, 10.0	[97, 105]
Scopolamine	1.0		7.6	[105]
Neostigmine bromide (I.S.)	5.0		-	-

Table 7-2 *Compounds of PCS-BDP with structure and pK_a values*

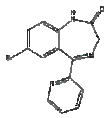
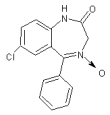
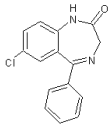
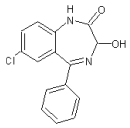
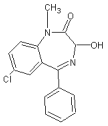
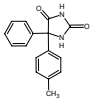
Name	Concentration (µg/mL)	Chemical structure	pK_a	Literature
Bromazepam	1.0		2.5, 5.2, 11.8	[105]
Demoxepam	1.0		4.5, 10.6	[80]
Nordiazepam	1.0		3.5, 12.0	[80]
Oxazepam	1.0		1.7, 11.6	[80]
Temazepam	1.0		1.6	[80]
MPPH (I.S.)	5.0		~ 8.0	[105]

Table 7-3 *Compounds of PCS-BARB with structure and pK_a values*

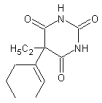
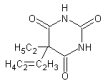
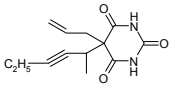
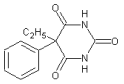
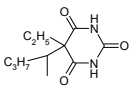
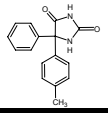
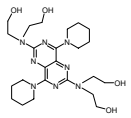
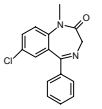
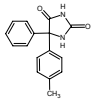
Name	Concentration (µg/mL)	Chemical structure	pK_a	Literature
Cyclobarbital	10.0		7.6	[80]
Crotylbarbital	10.0		7.7	[105]
Methohexital	10.0		8.3	[80]
Phenobarbital	10.0		7.4	[80]
Pentobarbital	10.0		8.0	[80]
MPPH (I.S.)	10.0		~ 8	[105]

Table 7-4 *Compounds of PCS-PA with structure and pK_a values*

Name	Concentration (mg/mL)	Chemical structure	pK_a	Literature
Dipyridamol	0.1		6.4	[80]
Diazepam	0.1		3.3	[80]
MPPH (I.S.)	0.1		~ 8.0	[105]

7.2 Remedi^{TM}-HS

Fig. 7-1 *Remedi^{TM}-HS system [146]*

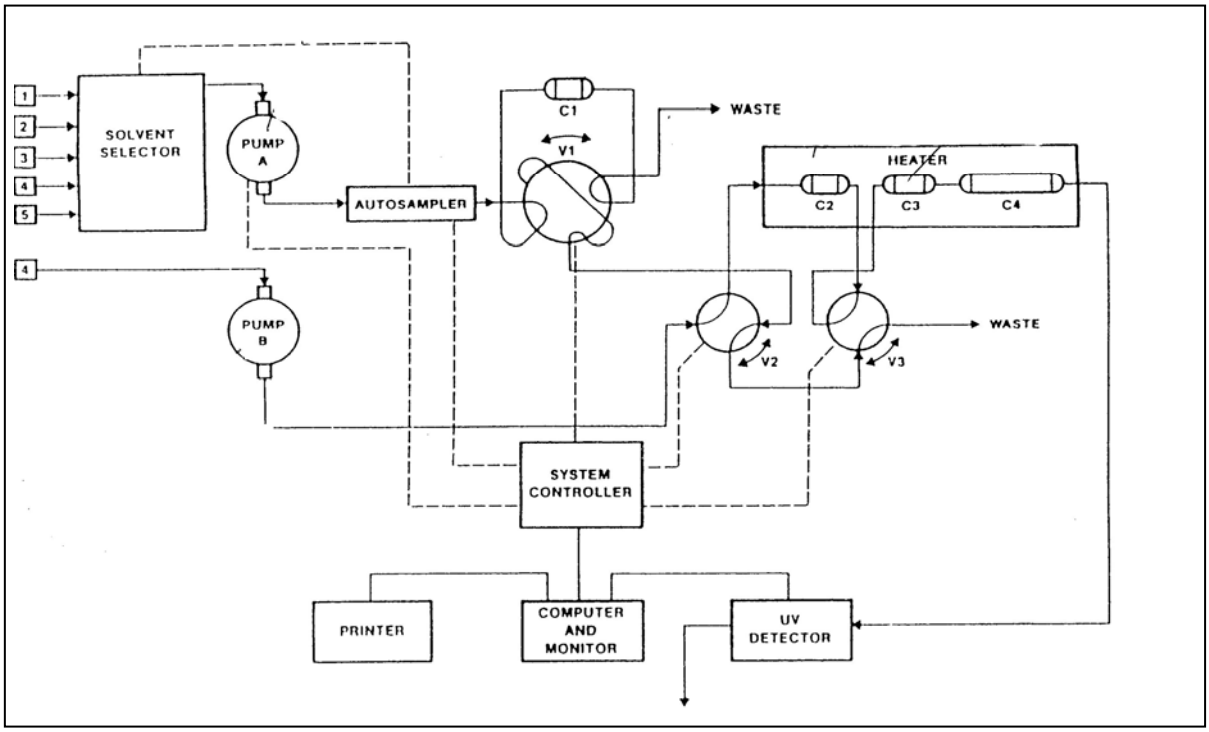


Fig. 7-2 *Circuit diagram of the Remedi^{TM}-HS system, V: valve, C1: purification column, C2: extraction column, C3 and C4: analytical columns [55]*

7.3 HPLC Equipment Reference System

Auto sampler (SIL-6B)	Shimadzu, Duisburg, Germany
DAD system (SPD-10 AVP)	Shimadzu, Duisburg, Germany
UV detector (SPD 10 AVP)	Shimadzu, Duisburg, Germany
Oven (CTO-6A)	Shimadzu, Duisburg, Germany
Shimadzu Class VP™ software	Shimadzu, Duisburg, Germany
System controller (SCL-6B)	Shimadzu, Duisburg, Germany
Communication module (CBM 10A)	Shimadzu, Duisburg, Germany
Two pumps (LC-9A)	Shimadzu, Duisburg, Germany
Monitor (L7031 TD)	Shimadzu, Duisburg, Germany
UV spectra of toxic compounds [22], Class VP™ files	Shimadzu, Duisburg, Germany
Degaser (DG 1310)	VDS Optilab, Berlin, Germany
Computer	Peacock Tiara

7.4 Libraries

Library of the screening method for basic compounds in urine, status 01/2007

Substance	Retention time (min)	Relative retention time	λ max (nm)
6-AM	23.226	0.60	208.07/284.37
Aconitine	19.570	0.51	195.75/231.79/273.13
Alfentanil	25.744	0.68	323.94
Alimemazine	27.157	0.70	193.14/252.12/301.17
Alprenolol	19.164	0.49	195.97/270.23
Ambroxol	27.101	0.72	209.84/245.86/309.88
Amfetaminil	20.779	0.54	193.14/257.16
7-Aminoflunitrazepam	18.827	0.54	242.65
Aminosuxethimide	17.581	0.46	193.14/236.66
Amisulpride	32.765	0.87	225.67/279.78
Amisulpride-Met.1	26.202	0.70	225.64/278.66
Amitriptyline	24.980	0.67	203.14/238.57
Amitriptylinoxide	21.461	0.58	203.63/238.39
Amlodipine	18.018	0.47	194.30/238.01/362.37
Amphetamine	21.980	0.56	194.30/204.91/256.95
Atenolol	20.655	0.54	194.30/224.79/273.31
Atomoxetine	19.007	0.52	195.32/269.67
Atracurium	26.854	0.72	200.51/230.66/280.28
Atracurium-Met.	22.500	0.60	200.12/231.87/280.36
Atropine	23.827	0.62	257.18
Benzatropine	23.886	0.62	194.30/258.29
BEC	14.144	0.36	194.30/231.65/273.29
Betaxolol	19.460	0.51	194.30/222.56/273.82
Biperidene	24.667	0.63	193.14/256.76
Bisnortilidine	19.110	0.49	193.14
Bisoprolol	20.682	0.55	195.78/224.62/271.99
Bufotenine	21.390	0.58	203.47/275.41
Bupivacaine	26.047	0.67	193.86/262.25
Buprenorphine	24.205	0.62	211.85/286.25
Buspirone	41.814	1.13	237.33/212.53/300.49
Carvedilol	22.447	0.58	214.41/250.70
Cathinone	23.671	0.61	197.32/248.31
Celiprolol	22.383	0.58	232.81/194.99/327.80
Cetirizine	50.718	1.36	193.14/230.64
Chloramphenicol	17.746	0.46	197.18/270.04
Chlorphenetazine	28.643	0.74	252.50/193.14/303.56
Chlorpheniramine	23.983	0.64	194.09/251.42
Chlorphenoxamine	23.927	0.64	194.18/258.37
Chlorphentermine	19.992	0.53	195.66/219.74/265.31
Chlorpromazine	28.546	0.77	255.08/307.01
Chlorprotixene	29.896	0.81	229.90/207.14/268.32/325.64
Cicletanine	19.339	0.52	194.63/216.05/284.44
Cimetidine	22.226	0.59	200.07
Citalopram	23.606	0.61	193.49/238.68/283.78
Citalopram-Met. 1	19.319	0.50	193.23/238.22/283.69
Citalopram-Met.1	19.667	0.54	193.14/238.44/272.70/283.07
Clemastine	22.052	0.57	195.00/259.38
Clenbuterol	36.450	0.54	212.03
Clobutinol	24.285	0.62	195.74/221.15/266.09
Clomipramine	25.546	0.68	252.28
Clomipramine-Met. 1	22.835	0.63	195.09/250.32
Clomipramine-Met.2	22.206	0.61	193.14/249.17

Substance	Retention time (min)	Relative retention time	λ max (nm)
Clonidine	25.940	0.70	198.62/270.94
Cocaine	28.190	0.71	195.60/232.01/274.09
Codeine	31.076	0.79	211.25/194.30/284.22
Cotinine	25.146	0.64	194.30/260.58
Cycloguanil	25.467	0.66	193.14/241.20
Cyproheptadine	25.843	0.67	224.12/194.30/289.63
Cytisine	34.281	0.94	195.92/304.58/232.23
Dimethoxy-bromo-methamphetamine	19.193	0.49	199.39/234.21/285.06
Desacetyldiltiazem	25.484	0.66	194.83/236.25
Desalkyl-flurazepam	37.501	1.03	228.77/307.49
Desipramine	22.473	0.60	193.14/251.44
Desmethyltrimipramine	23.189	0.62	193.89/250.95
Dihydrocodeine	31.399	0.83	208.69/283.26
Dioxopromazine	29.969	0.78	225.61/193.14/264.18/290.27/326.49
Diphenhydramine	22.972	0.61	193.14/258.13
Diphenhydramine-Met. 1	17.189	0.56	193.14/258.20
Diphenylpyraline	21.265	0.58	194.95/258.16/298.74
Dopamine	18.216	0.49	200.10/285.09
Dothiepine	25.609	0.65	193.14/229.40/302.45
Doxapram	25.914	0.68	195.00/258.31
Doxepin	26.063	0.69	205.00/293.34
Doxycycline	20.163	0.50	194.30/267.77/347.50
Doxycycline-Met.1	18.703	0.47	194.30/268.04/214.28/347.78
Droperidol	25.959	0.67	203.16/245.26/229.13/277.39
Duloxetine	20.650	0.56	216.19/290.19
EDDP	36.360	0.92	194.30/235.44
Unidentified endogeneous peak	17.935	0.45	193.14/221.56/273.35
Unidentified endogeneous peak	19.806	0.52	219.51/196.88/277.35
Unidentified endogeneous peak following hydrolysis	32.024	0.84	204.43/240.55
Unidentified endogeneous peak following hydrolysis	12.617	0.33	193.14/257.92
Unidentified endogeneous peak following hydrolysis	20.728	0.54	193.14/205.03/259.74
Unidentified endogeneous peak following hydrolysis	18.408	0.48	198.84/222.46/278.81
Unidentified endogeneous peak following hydrolysis	18.975	0.50	195.53/282.84/313.23
Ephedrine	20.755	0.54	204.61/256.71
Ergotamine	22.452	0.60	193.14/317.41
Fencamfamine	22.477	0.57	194.30/207.13/258.58
Fenfluramine	19.388	0.53	193.14/206.05/262.43
Fentanyl	28.550	0.74	256.85/324.27
Fexofenadine	17.084	0.46	193.14/259.09
Flecainide	18.300	0.50	198.68/298.27
Fluoxetine	19.191	0.51	193.84/226.71/263.44
Flurazepam	39.620	1.08	193.27/228.87/306.92
Fluvoxamine	17.789	0.48	250.35
Galantamine	32.636	0.87	211.65/288.93
Gallopamil	20.667	0.55	201.45/276.68
Haloperidol	25.395	0.68	194.52/246.08
Heroin	23.381	0.60	204.23/279.01
Hydroxy-methoxyamphetamine	21.174	0.54	199.47/226.19/279.24
Hydrocodone	24.258	0.73	205.74/280.66
Hydromorphone	25.937	0.66	204.41/280.65

Substance	Retention time (min)	Relative retention time	λ max (nm)
Hydroxyzine	33.792	0.86	193.14/230.70
Hyoscyamine	23.850	0.62	194.30/256.62
Imipramine	27.219	0.71	193.83/251.12
Ipratropiume	36.638	0.98	256.88/331.86
Ketamine	25.390	0.71	194.47/268.25
Ketamine-Met. 1	24.009	0.65	194.30/267.00
Ketamine-Met. 2	22.014	0.60	194.30/267.44
Ketamine-Met. 3	19.155	0.52	194.30/214.47/267.93
Ketotifen	30.358	0.79	203.45/298.12
Labetolol	20.797	0.54	205.71/193.14/302.58
Lamotrigine	20.807	0.54	208.13/264.67
Levofloxacine	33.573	0.88	294.28/226.03/193.14/325.65
Levofloxacine Met. 1	23.956	0.66	294.84/223.42/325.09
Lidocaine	25.536	0.67	193.94/262.48
Lidocaine-Met. 1	21.071	0.55	194.30/262.79
Lidocaine-Met. 2	19.166	0.50	194.30/262.83
Lisuride	34.120	0.87	193.14/225.36/239.88/312.10
LSD	35.259	0.94	232.01/312.60
LSD-Met. 1	33.015	0.88	225.66/312.14
LSD-Met. 2	29.506	0.78	232.65/315.70
LSD-Met. 3	28.635	0.76	225.32/312.38
Maprotiline	23.688	0.63	193.52/271.75/264.89
Methyl-1-(benzodioxol-5-yl)-butamine	21.055	0.53	199.57/234.40/285.25
MDA	21.444	0.54	199.28/233.96/284.87
Methylenedioxyamphet.	22.560	0.58	197.96/234.34/285.06
MDMA	22.607	0.57	199.45/233.95/285.05
Medazepam	31.243	0.89	194.23/253.42
Mefloquine	19.831	0.53	221.74/282.49/316.44
Melperone	31.101	0.83	196.18/246.09
Melperone-Met.1	24.600	0.67	196.69/245.42
Mepivacaine	27.189	0.73	193.14/262.37
Mescaline	21.810	0.55	203.98/268.40
Mesoridazine	40.574	1.10	261.01/238.53/307.58
Mesoridazine-Met. 1	30.908	0.85	200.85/262.90/238.62/311.76
Metaclazepam	29.115	0.79	194.52/252.18
Metamphetamine	23.466	0.59	194.30/205.31/256.99
Methadone	25.019	0.65	194.30/259.30/289.05
4-Methoxyamphetamine	19.358	0.53	196.42/223.34/274.20
4-Methoxymetamphetamine	21.419	0.59	196.21/223.60/274.27
Methylphenidate	22.757	0.60	193.14/256.84
Metoclopramide	32.640	0.87	213.08/274.05/309.04
Metoclopramide-Met.	27.243	0.71	214.11/271.77/307.49
Metoprolol	21.470	0.57	195.48/222.39/273.79
Metoprolol-Met. 1	19.175	0.54	196.02/223.27/272.10
Mexiletine	20.238	0.52	198.10/261.52
Midazolam	29.092	0.83	218.32
Moclobemide	27.920	0.71	197.96/238.77
Morphine	25.740	0.65	209.38/194.30/284.99
Moxonidine	25.005	0.68	196.46/231.09/325.50
Naloxone	22.366	0.60	203.93
N-Desmethyldiphenhydramine	19.599	0.66	193.14
N-Desmethyldoxepin	23.291	0.62	204.86/293.15
Nefazodone	23.344	0.62	193.14/209.40/248.46
Neostigmine bromide (I.S.)	39.468	1.00	260.14
Nicotine-Met.	25.888	0.66	203.80/258.60

Substance	Retention time (min)	Relative retention time	λ max (nm)
Noramidopyrine	20.707	0.59	194.30/260.21/241.55
Noramidopyrine-Met.	20.001	0.57	194.30/260.19/241.64
Norazepam	18.977	0.53	193.36/235.43
Norbuprenorphine	23.183	0.60	211.64/286.52
Norcitalopram	21.060	0.55	193.63/238.62/283.77/318.16
Norclomipramine	22.689	0.61	193.14/252.66
Norcocaine	20.368	0.57	195.24/231.71/274.51
Norcodeine	25.349	0.65	210.66/194.80/284.65
Norephedrine	19.830	0.50	193.14/205.14/256.40
Norfentanyl	24.299	0.63	256.50/324.15
Normaprotiline	21.520	0.57	193.65/271.75/264.88
Normethadone	20.985	0.57	193.14/259.56/292.37
Normethadone	21.659	0.56	194.30/259.36/292.32
Normorphine	20.656	0.56	209.42/194.29/285.49
Norpseudoephedrine	19.107	0.49	193.14/256.40
Norsertraline	23.829	0.70	194.80/272.97/265.63
Northioridazin-2-sulfoxide	30.908	0.85	200.85/262.90/238.62/311.76
Nortilidine	21.120	0.55	
Nortrimipramine	19.497	0.60	194.46/249.14
Nortriptyline	22.060	0.59	193.14/238.17
Norvenlafaxine	21.557	0.57	195.08/224.60/275.40
Norverapamil	20.886	0.56	200.67/229.74/278.04
Noscapine	26.148	0.70	212.48/312.15
Noxiptiline	23.658	0.61	193.14/248.93
Ofloxacine	32.138	0.85	294.34/226.07/193.14/325.71
Orciprenaline	16.589	0.43	198.44/277.79
Oxybutynine	19.105	0.48	193.14/256.85
Oxycodone	28.896	0.75	205.52/281.19
Oxymotphone	22.780	0.58	204.27/281.52
Papaverine	35.927	0.96	251.13/199.83/309.10/286.63
Paroxetine	21.265	0.57	293.52/234.20
Penbutolol	19.123	0.49	196.63/215.65/270.57
Pentamidine	17.412	0.46	196.16/258.96
Pentazocine	22.445	0.60	197.27/278.88
Perazine-Met. 4	23.914	0.62	194.30/248.91/304.00
Perazine-Met. 1	38.340	1.00	194.30/233.22/210.23/267.41/291.79
Perazine-Met. 2	32.198	0.84	233.18/194.30/267.90/292.47/335.92
Perazine-Met. 3	26.422	0.69	225.53/194.30/264.48/290.70/326.99
Perazine-Met. 5	21.245	0.55	194.30/248.98/303.11
Perazine-Met. 6	19.740	0.52	194.30/248.99/301.92
Periciazine	29.317	0.74	268.74/193.14/231.85
Pethidine	24.486	0.63	193.11
Pethidine-Met. 1	21.853	0.55	194.30/254.92/308.46
Pethidine-Met. 2	20.000	0.51	194.30/256.57
Pethidine-Met.3	19.314	0.49	194.30
Phertarmine	21.561	0.57	193.14/258.26
Pholedrine	19.522	0.49	193.14/222.72/275.21
Physostigmine	26.126	0.70	203.13/246.02/302.51
Pirenzepine	54.790	1.49	199.71/281.60
Pizotifene	26.837	0.95	193.14
p-Methoxyamphetamine	21.619	0.55	194.30/223.50/273.90
p-Methoxymetamphetamine	23.930	0.61	194.30/223.73/274.03
Pridinol	22.941	0.63	200.88/257.71
Procyclidine	24.913	0.66	193.14/256.74
Proguanil	33.353	0.91	196.61/256.74/231.69
Prolinthan	27.753	0.74	193.14/257.52

Substance	Retention time (min)	Relative retention time	λ max (nm)
Prolocaine	20.166	0.53	194.64/226.16
Promazin-S-oxide	34.140	0.92	231.46/271.38/298.01/341.52
Promazin-S-oxide-Met. 1	28.763	0.73	194.30/231.64/272.07/298.36/342.52
Propafenone	20.023	0.51	193.14/208.60/248.22/303.77
Propranolol	21.264	0.56	212.77/290.07
Pseudoephedrien	19.745	0.50	194.30/205.38/256.67
Psilocin	21.427	0.56	220.46/266.05
Remifentanil	24.392	0.64	193.14/257.64
Rifabutine	19.471	0.51	208.36/277.09/236.89/316.19/355.20
Ritodrine	15.320	0.41	197.62/221.94/274.52
Ropivacaine	26.104	0.69	193.14/262.27
Scopolamine	22.170	0.57	257.09
Sertindol	28.026	0.73	193.14/224.40/255.54/299.46
Sertraline	26.270	0.63	194.99/265.96
Sildenafil	25.022	0.68	223.21/213.49/193.84/292.85
Sildenafil-Met.	23.821	0.98	223.13/213.67/194.38/292.24
Sufentanil	25.858	0.69	193.14/226.60
Sulpiride	31.176	0.83	212.47/291.89
Talinolol	19.648	0.51	195.38/241.81/284.58
Terfenadine	18.428	0.49	193.14
Tetrazepam	32.752	0.86	237.63/204.49/280.43
Thebaine	30.289	0.43	203.93/284.57
Thiabendazole	28.585	0.76	195.22/301.63/243.53
Thioridazine	30.162	0.82	262.54/229.23/310.04
Thioridazin-2,5-disulfoxide	47.387	1.30	193.14/233.97/217.88/301.99/346.61
Thoncylamine	43.799	1.16	194.58/240.71/280.29/307.42
Tiapride	31.440	0.79	212.38/287.71
Tilidine	28.182	0.71	194.30/257.21
Tilidine-Met. 1	18.566	0.49	194.30/256.41
Tilidine-Met. 2	18.086	0.47	194.30/258.40
Tilidine-Met. 3	17.645	0.46	194.30/258.64
Timolol	21.858	0.56	296.64/193.14
Tizanidine	29.381	0.79	197.75/227.11/318.67
Tocainide	21.309	0.58	193.61/263.12
Tramadol	22.569	0.58	196.64/215.05/271.37
Tramadol-Met. 1	20.622	0.57	197.40/271.42
Tramadol-Met. 2	19.855	0.55	196.80/272.57
Tramadol-Met. 3	19.083	0.52	197.23/272.45/328.63
Tranylcypromine	20.780	0.53	195.12/262.32
Trazodone	31.235	0.83	211.02/248.74/314.63
Trichlorphone	25.031	0.67	200.93/261.40
Trifluperidole	21.158	0.53	193.14/246.30
Triflupromazine-Met. (S-oxide)	27.817	0.76	233.49/193.14/216.00/272.94/300.77
Triflupromazine-Met. (S-oxide)	24.747	0.67	233.34/215.94/193.14/273.14/301.18
Triflurpromazine	23.021	0.62	256.76/193.14/306.37
Trihexyphenidyl	25.466	0.66	193.14/256.59
Trimethoprime	22.728	0.59	203.94/270.41
Trimetoprim-Met. 1	20.114	0.56	202.82/273.74
Trimetoprim-Met. 2	19.306	0.54	205.08/269.21
Trimipramine	26.357	0.68	193.85/250.49
Trimipramine-Met. 1	18.267	0.56	195.17/248.69
Triperidene	29.009	0.75	193.14/256.87
Urapidil	37.353	1.15	203.62/267.86/237.23
Urapidil-Met. 1	28.896	0.95	195.84/267.81
Verapamil-Met.2	18.698	0.50	200.18/228.55/278.52
Venlafaxine	25.485	0.66	196.52/225.63/274.17/329.26

Substance	Retention time (min)	Relative retention time	λ max (nm)
Venlafaxin-Met. 1	20.291	0.58	196.34/224.52/275.19
Venlafaxin-Met. 2	18.536	0.53	195.75/223.90/275.02
Venlafaxin-Met. 3	17.574	0.50	194.76/223.85/275.26
Verapamil	22.894	0.59	200.45/229.95/278.05
Verapamil-Met.1	19.843	0.53	199.12/230.00/277.63
Verapamil-Met.3	18.380	0.49	200.89/229.87/277.74
Verapamil-Met.4	18.137	0.48	200.68/229.09/278.65
Veratrine	19.076	0.51	219.52/261.92/292.09
Xylometazoline	23.628	0.57	197.20/262.31
Yohimbine	31.561	0.82	219.43/270.71/349.87
Ziprasidone-Met.	35.552	0.94	208.46/314.53
Zolpidem	42.872	1.10	206.86/237.90/295.49
Zolpidem-Met.	27.784	0.72	205.22/297.85
Zopiclon-Met. 1	25.889	0.71	193.14/304.03
Zopiclon-Met. 2	19.819	0.64	193.14/304.12/214.55

Library of the Screening Method for Neutral, Weakly Acidic and Weakly Basic Compounds in Urine, Status 01/2007

Substance	Retention time (min)	Relative retention time	λ max (nm)
Alprazolam	40.284	1.35	194.30/221.29
Amobarbital	19.590	0.70	194.30
Bamipine	22.307	0.79	194.30/250.93/297.37
Brallobarbital	16.720	0.60	194.30
Bromazepam	17.099	0.60	233.77/194.30
Brotizolam	48.075	1.63	194.30/240.92
Butalbital	16.641	0.59	194.30
Carbamazepine	22.193	0.79	194.30/210.95/284.40
Carbromal	19.066	0.68	195.65
Chinine	11.157	0.40	207.18/245.49/341.04/318.23
Chlordiazepoxide	13.990	0.50	243.95/194.30/202.85/307.07
Chlorphenamine	13.843	0.48	194.30/221.75/262.75
Clobazam	35.974	1.28	229.67/194.30/288.80
Clomethiazole	19.400	0.69	251.26/194.30
Clonazepam	29.694	1.04	194.30/309.49
Clozapine	13.487	0.48	203.29/240.27
Colchicine	20.744	0.74	243.69/197.99/351.75
Crotylbarbital	23.682	0.77	194.30
Cyclicine	19.859	0.71	194.30/224.57/257.46
Cyclobarbital	16.670	0.54	194.30
Dantrolene	24.009	0.86	194.30/309.27/224.14
Demoxepam	19.846	0.69	236.24/194.30/305.98
Desalkylflurazepam	34.581	1.12	229.14/194.30/310.63
Diazepam	40.394	1.37	194.30/232.37/279.41/360.44
Diltiazem	22.691	0.81	194.30/236.84
Dimenhydrinat	20.354	0.73	194.30/257.93/299.61
Flumazenil	18.353	0.66	201.37/243.94/354.62
Flurazepam	21.268	0.76	194.30/228.65/307.79
Glisoxepide	32.378	1.15	194.30/229.36
Glutethimide	22.540	0.81	194.30/255.47/288.77
Heptabarbital	24.344	0.87	194.30
Hexobarbital	19.643	0.70	194.30
1-Hydroxymidazolam	17.492	0.61	194.30/218.12/345.00
4-Hydroxymidazolam	18.544	0.58	194.30/218.29
Leflunomide	31.798	1.14	291.40/246.95/200.46
Loprazolam	18.515	0.66	194.30/328.45/235.77
Lorazepam	25.824	0.90	194.30/229.14/316.38
Lormetazepam	37.286	1.26	194.30/230.58/315.74
Mesoridazine	22.316	0.80	260.96/194.30/238.21/307.79
Mesuximide	21.632	0.77	194.30
Methohexital	35.059	1.12	194.30/220.01
Methylphenobarbital	21.219	0.76	194.30
Midazolam	21.031	0.68	194.30/218.19
Mirtazepin	12.083	0.43	194.30/313.66/251.02
N-Desmethylchlordiazepoxide	14.233	0.48	194.30/245.21/299.23
Nitrazepam	23.879	0.83	194.30/216.82/260.82
Norclobazam	27.730	0.90	227.42/194.30/289.81
Nordiazepam	25.080	0.87	194.30/232.00/280.61
Opipramol	14.308	0.51	194.30/253.94/215.50
Oxazepam	23.363	0.81	228.86/194.30/313.54
Oxcarbazepine	17.740	0.63	194.30/255.09/305.43

Substance	Retention time (min)	Relative retention time	λ max (nm)
Pentobarbital	20.256	0.66	194.30
Phenmediphame	23.942	0.86	202.58/235.48/272.56
Phenobarbital	15.451	0.50	194.30
Quinapril	25.523	0.91	194.30/260.20
Ramipril	19.416	0.69	194.30/257.56
Risperidone	14.992	0.53	195.65/236.67/274.30
Secobarbital	21.800	0.78	194.30
Secubutabarbital	15.368	0.55	194.30
Temazepam	33.841	1.14	197.22/230.46/310.18
Tetrazepam	16.686	0.54	234.46/199.31/280.81/352.47
Thonzylamine	15.494	0.55	194.30/240.44/280.32/307.27
Tiagabine	36.498	1.29	194.30/257.62
Trapidil	13.961	0.49	302.11/220.17/198.62
Triflurpromazine	17.970	0.64	233.47/194.30/215.46/272.76/300.27
Zopiclone	22.382	0.80	194.30/302.85

7.5 Stability Investigations

Table 7-5 *Stability investigations on PCS 2 stored at room temperature*

Analyte	Recovery$_{(day\ 0\text{-}28)}$, % (Mean, ± SD)	Recovery range, %	RSD, %
Codeine	97 ± 3.8	91-100	3.8
EDDP	85 ± 4.8	78- 91	5.5
MDA	97 ± 9.4	77-100	9.7
Morphine	96± 10.3	84-108	10.5
Scopolamine	40 ± 33.1	14- 94	85.8
I.S.	97 ± 8.4	88-103	7.4

Table 7-6 *Stability investigations on PCS 2 stored at 5-8 °C (fridge)*

Analyte	Recovery$_{(day\ 0\text{-}28)}$, % (Mean, ± SD)	Recovery range, %	RSD, %
Codeine	98 ± 8.6	97-106	8.3
EDDP	99 ± 4.8	78- 91	5.5
MDA	95 ± 4.1	89- 99	4.4
Morphine	98 ± 7.0	87-102	7.1
Scopolamine	64 ± 22.2	43- 95	34.9
I.S.	99 ± 8.2	98-104	8.3

Table 7-7 *Stability investigations on PCS 2 stored at -15 °C (freezer)*

Analyte	Recovery$_{(day\ 0\text{-}28)}$, % (Mean, ± SD)	Recovery range, %	RSD, %
Codeine	99 ± 5.5	94-106	5.4
EDDP	102 ± 5.5	98-104	5.3
MDA	98 ± 5.2	94-103	5.2
Morphine	95 ± 5.8	90-102	6.1
Scopolamine	93 ± 2.5	89- 96	2.7
I.S.	104 ± 7.6	92-111	6.9

Table 7-8 *Stability investigations on PCS 1 stored at 5-8 °C (fridge)*

Analyte	Recovery$_{(day\ 0\text{-}28)}$, % (Mean, ± SD)	Recovery range, %	RSD, %
Codeine	99 ± 1.1	97-101	1.1
EDDP	100 ± 1.4	98-102	1.4
MDA	98 ± 1.1	96-101	1.1
Morphine	98 ± 6.0	89-103	6.1
Scopolamine	95 ± 8.8	83-102	9.3
I.S.	98 ± 9.1	85-109	9.4

Table 7-9 *Stability investigations on PCS 1 stored at -18 ± 3 °C (freezer)*

Analyte	**Recovery$_{(day\ 0\text{-}28)}$, % (Mean,** ± SD)	**Recovery range, %**	**RSD, %**
Codeine	98 ± 2.3	94-100	2.3
EDDP	98 ± 1.2	98-102	1.2
MDA	99 ± 1.2	97-100	0.9
Morphine	99 ± 6.0	90-102	2.2
Scopolamine	98 ± 4.6	90-102	4.7
I.S.	99 ± 1.5	97-101	1.6

Table 7-10 *Stability investigations on the new PCS 1* stored at -18 ± 3 °C (freezer)*

Analyte	**Recovery$_{(day\ 0\text{-}67)}$, % (Mean,** ± SD)	**Recovery range, %**	**RSD, %**
6-AM	96 ± 2.2	91-103	2.3
Codeine	99 ± 8.6	81-102	8.6
EDDP	99 ± 4.1	96-103	4.1
MDA	98 ± 5.1	97-100	5.2
Morphine	98 ± 6.4	90-103	6.5
Tilidine	96 ± 4.6	91-104	4.8
I.S.	95 ± 9.7	87-104	10.2

Table 7-11 *Stability investigations on the PCS-BDP stored at -18 ± 3 °C (freezer)*

Analyte	**Recovery$_{(day\ 0\text{-}67)}$, % (Mean,** ± SD)	**Recovery range, %**	**RSD, %**
Bromazepam	104 ± 10.4	94-116	10.0
Demoxepam	102 ± 4.9	95-105	4.8
Nordiazepam	97 ± 11.2	96-104	11.4
Oxazepam	100 ± 3.1	85-106	3.0
Temazepam	95 ± 9.4	84-119	9.8
MPPH (I.S.)	105 ± 15.0	87-105	14.3

7.6 Validation Data

Table 7-12 *Recovery, RSD, LLOD and linearity data of the screening method for basic compounds in urine, PCS 2*

Analyte	Recovery$_{(mean\ level\ 1\text{-}3)}$ (%) ± SD	RSD$_{(recovery)}$ (%)	RSD$_{(RRT)}$ (%)	LLOD (µg/mL)	Linearity	
					Range (µg/mL)	R^2
Codeine	90.6 ± 1.2	1.3	0.2	0.1	0.20 – 15.00	0.9997
EDDP	94.3 ± 1.8	1.9	0.2	0.1	0.20 – 15.00	0.9999
MDA	96.9 ± 0.7	0.8	0.2	0.1	0.20 – 5.00	0.9926
Morphine	83.2 ± 2.9	3.5	0.2	0.1	0.20 – 15.00	0.9947
Scopolamine	78.7 ± 4.9	7.2	0.2	0.2	0.25 – 15.00	0.9977
I.S.	72.7 ± 2.4	3.3	0.2	0.2	0.25 – 15.00	0.9949

Table 7-13 *Recovery, RSD, LLOD and linearity data of the screening method for neutral, weakly acidic and weakly basic compounds in urine, PCS-BDP 2*

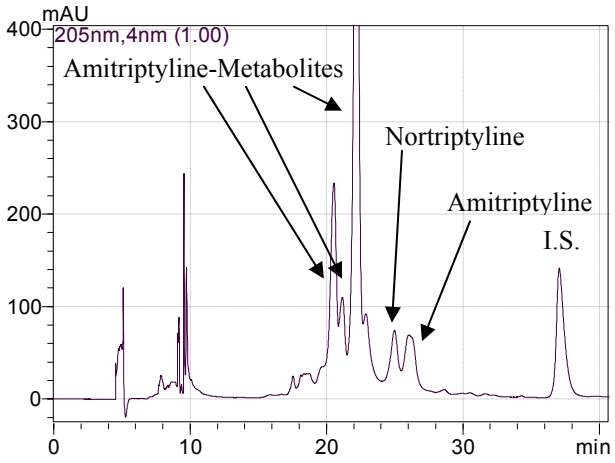
Analyte	Recovery$_{(X\ Level\ 1\text{-}3)}$ (%) ± x (SD)	RSD $_{(Recovery)}$ (%)	RSD $_{(RRT)}$ (%)	LLOD (µg/mL)	Linearity	
					Range (µg/mL)	R^2
Bromazepam	95.0 ± 8.0	6.5	0.1	0.04	0.05-10.00	0.9993
Demoxepam	63.0 ± 2.6	4.1	0.2	0.03	0.05-10.00	0.9992
Nordiazepam	95.7 ± 9.0	8.4	0.1	0.10	0.10-20.00	0.9990
Oxazepam	93.7 ± 1.7	1.8	0.3	0.03	0.05-10.0	0.9997
Temazepam	93.7 ± 6.5	6.1	0.5	0.03	0.05-10.00	0.9970
MPPH (I.S.)	43.7 ± 1.3	3.0	0.1	0.03	0.05-20.00	0.9993

Table 7-14 *Recovery, RSD, LLOD and linearity data of the screening method for neutral, weakly acidic and weakly basic compounds in urine, PCS-BARB 2*

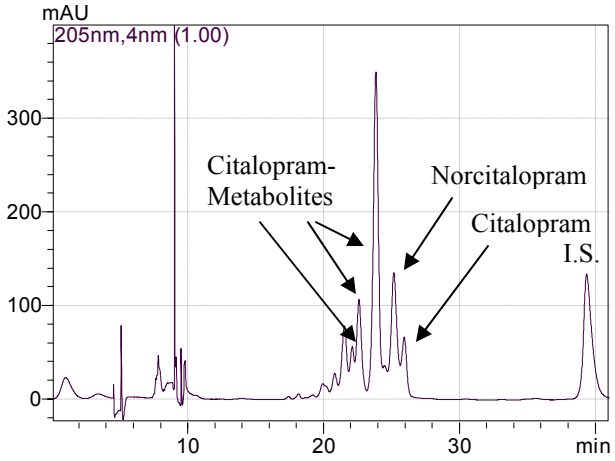
Analyte	**Recovery$_{(X\ Level\ 1\text{-}3)}$ (%) ± x(SD)**	**RSD $_{(Recovery)}$ (%)**	**RSD $_{(RRT)}$ (%)**	**LLOD (µg/mL)**	**Linearity**	
					Range (µg/mL)	**R^2**
Cyclobarbital	10.7 ± 0.7	6.7	0.01	1.0	5.00-40.00	0.9890
Crotylbarbital	13.0 ± 1.5	11.3	0.10	1.0	1.00-20.00	0.9870
Methohexital	71.9 ± 1.1	1.5	0.02	0.1	0.10-20.00	0.9995
Phenobarbital	4.6 ± 0.3	6.0	0.01	1.0	-	-
Pentobarbital	62.4 ± 2.0	3.2	0.01	0.1	0.10-20.00	0.9950
MPPH (I.S.)	43.7 ± 1.3	3.0	0.1	0.03	0.05-20.00	0.9993

7.7 Example Chromatograms

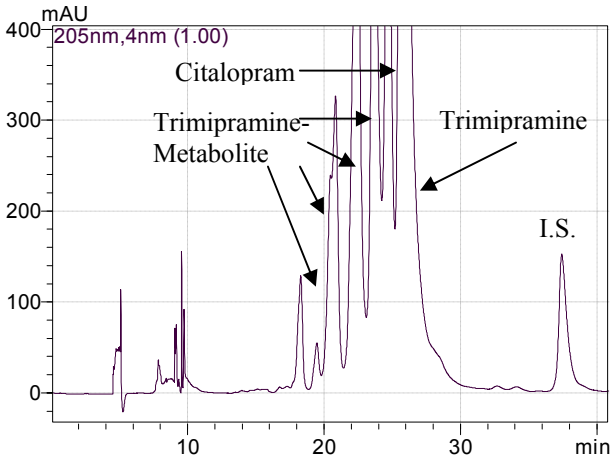
STA: Antidepressants



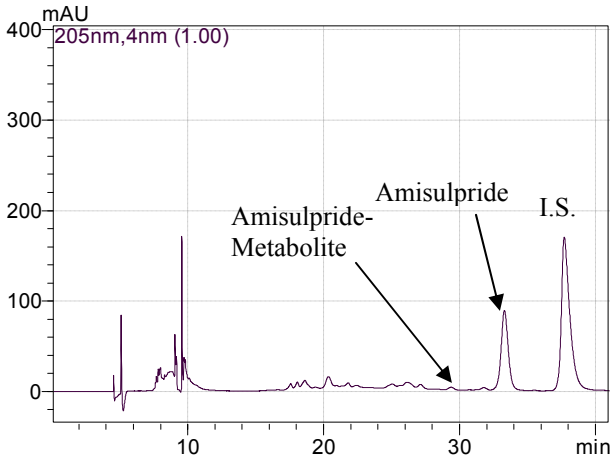
STA: Antidepressants



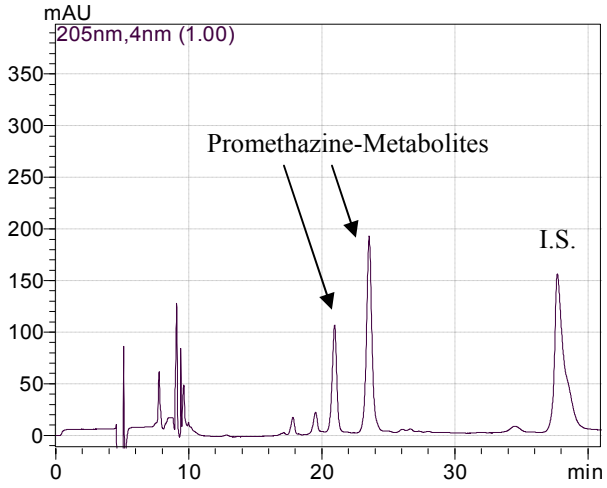
STA: Antidepressants



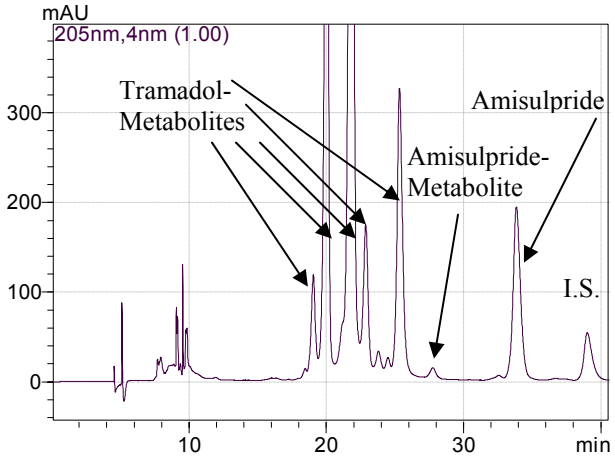
STA: Antidepressants



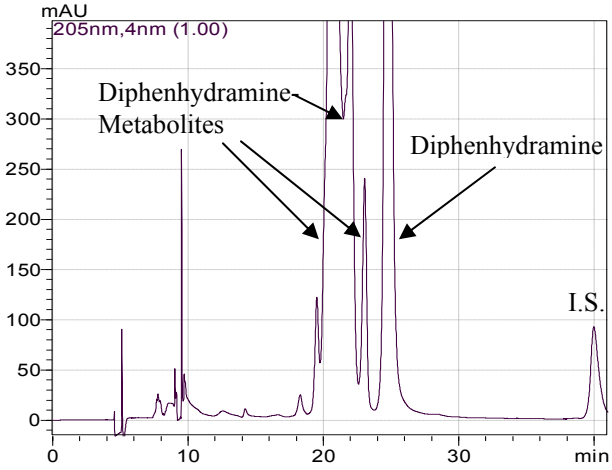
STA: Neuroleptics



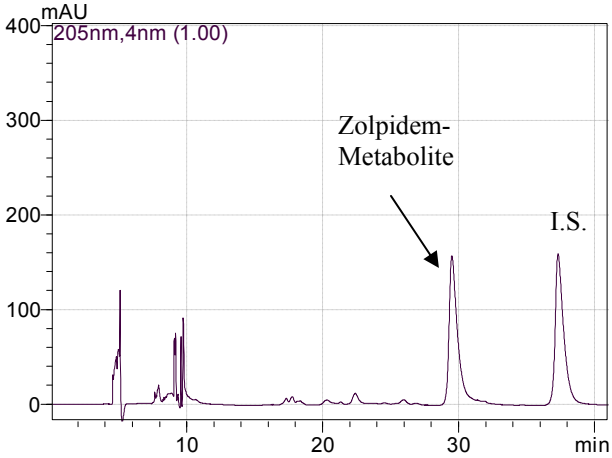
STA: Analgetics



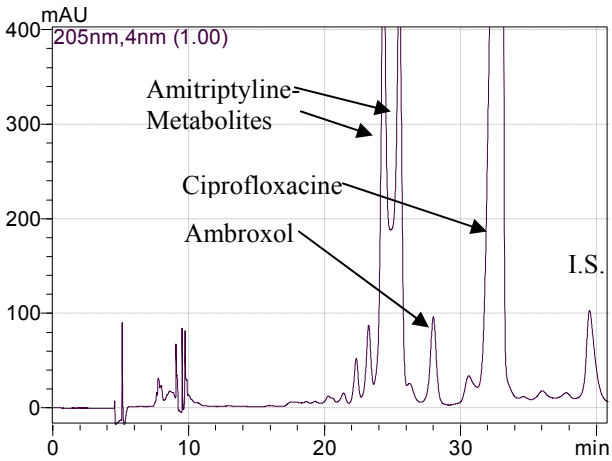
STA: Antihistamines



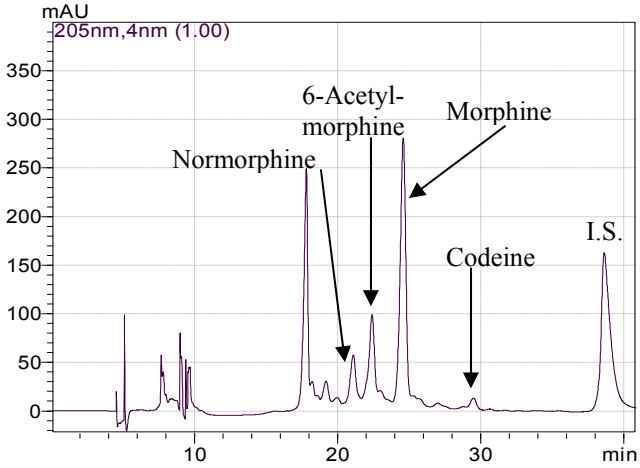
STA: Sleeping medication



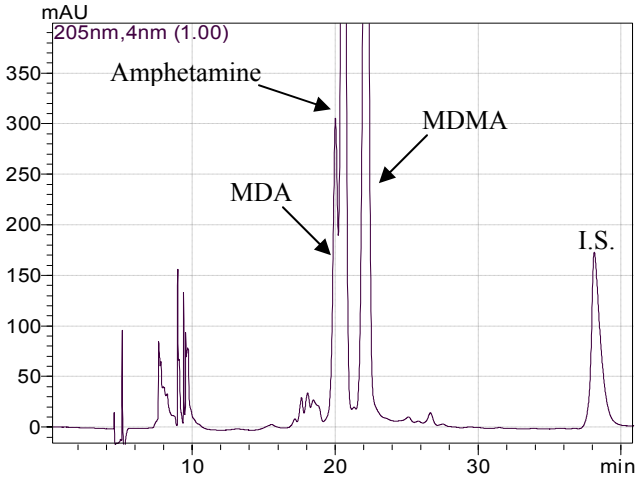
STA: Others



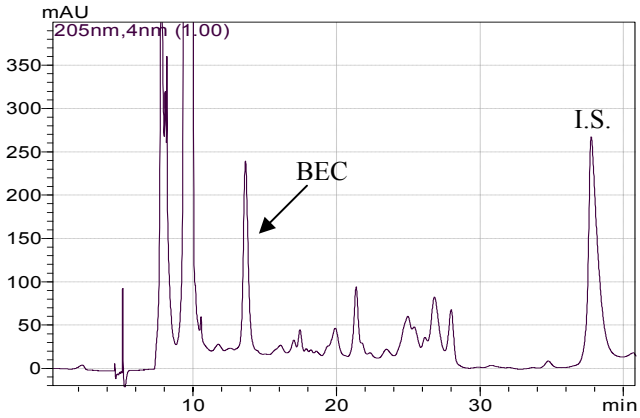
DOA: Opiates



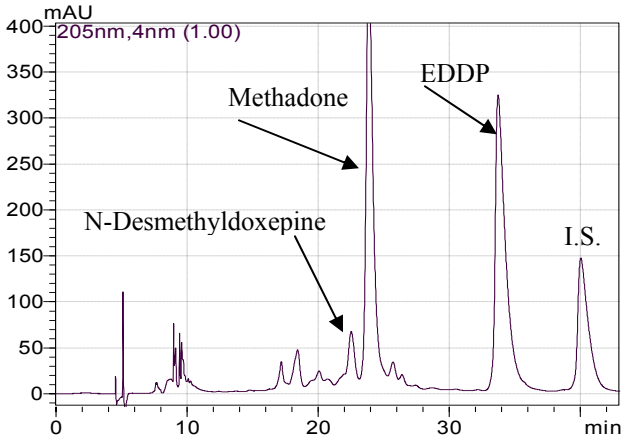
DOA: Amphetamines



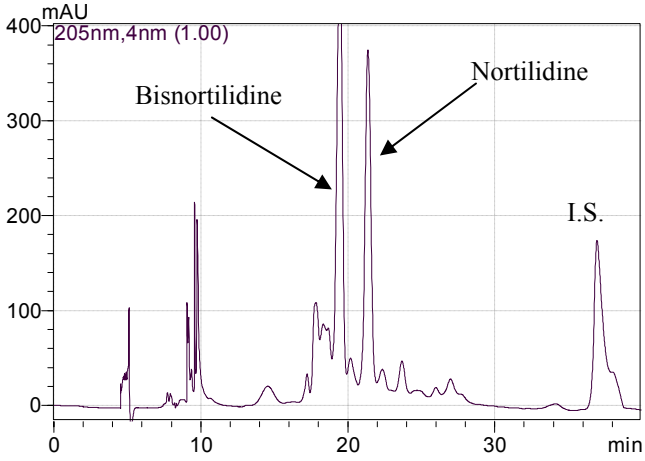
DOA: Cocaine (BEC method)



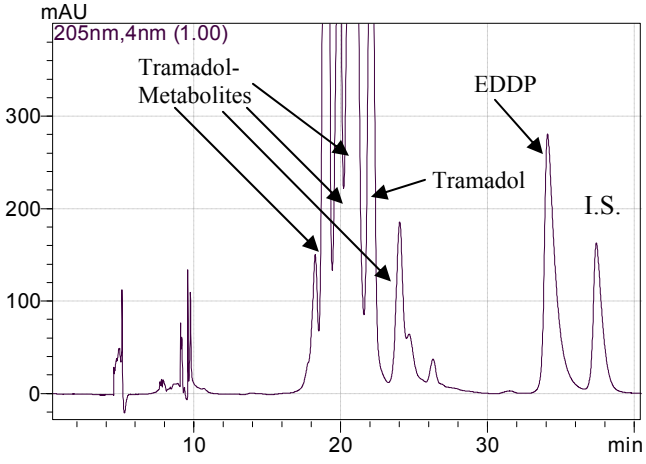
DOA: Methadone substitution



DOA: Opioid abuse



DOA: Opioid abuse



7.8 Evaluation of Benzodiazepine Positive Samples

Immunological result analysed by CEDIA with deglucuronidation reagent*	Detected benzodiazepines
1774	Oxazepam, Temazepam
655	Temazepam
930	-
1867	Hydroxymidazolam
1654	Oxazepam, Temazepam
1654	Overlapped spectra
1690	Oxazepam, Temazepam
1600	Oxazepam, Temazepam
747	Temazepam
1378	Overlapped spectra
1819	Oxazepam
1524	Demoxepam, Oxazepam
1918	Demoxepam, Oxazepam, Nordiazepam, Temazepam
1967	Oxazepam, Temazepam
1813	Oxazepam, Temazepam
1392	Overlapped spectra
1998	Oxazepam, Temazepam
1375	Oxazepam, Temazepam
287	-
297	-
234	Oxazepam, Temazepam
244	-
285	-
291	Oxazepam, Temazepam
308	-
314	-
502	Lorazepam, Oxazepam, Temazepam
526	-
530	-
649	-
794	-
830	-
937	-
1043	Oxazepam, Temazepam
1051	-
1363	Flurazepam
1378	Temazepam
1404	Temazepam
1480	Oxazepam, Temazepam
1521	Hydroxymidazolam
1640	Temazepam
1640	Oxazepam
1708	Oxazepam, Temazepam
1721	Oxazepam, Temazepam
1751	Oxazepam, Temazepam
1801	Temazepam
1857	Oxazepam, Nordiazepam, Temazepam
1861	Oxazepam, Temazepam
1876	Oxazepam, Temazepam
1927	Oxazepam, Temazepam
1928	Oxazepam, Temazepam
1929	Oxazepam, Nordiazepam, Temazepam
1940	Oxazepam, Temazepam
1997	Oxazepam, Temazepam

Immunological result analysed by CEDIA with deglucuronidation reagent*	Detected benzodiazepines
2182	Oxazepam, Temazepam
2258	Oxazepam, Temazepam
2297	Oxazepam, Nordiazepam, Temazepam
2312	Nordiazepam, Temazepam
2338	Oxazepam, Temazepam
2345	Oxazepam, Temazepam
2362	Temazepam
2404	Oxazepam, Temazepam
2451	Oxazepam, Nordiazepam, Temazepam
2490	Oxazepam, Temazepam
2505	Oxazepam, Nordiazepam, Temazepam
2521	Oxazepam, Temazepam
2121	Temazepam
2157	Tetrazepam
234	Oxazepam, Temazepam
244	-

**investigated within DOA confirmation screening, creatinine ranged from 0.6-3.8 g/L*

7.9 Negative List

Compound	Compound
Allopurinol	Nelfinavir
Amantidine	Nicotine
Amodiaquine	Nifluminic acid
Aripiprazol	Nilvadipine
Atovaquone	Nimodipine
Bromocryptine	Omethoat
Caffeine	Paracetamol
Candesartan	Perazine
Cantharidine	Pipamperone
Ceftriaxone	Pregabaline
Cidofovir	Propylthiouracil
Cisapride	Salicylic acid
Clopenthixol	Sulfadiazine
Delavirdine	Sultiam
Diazoxid	Theophylline
Dimethoat	Zidovudine
Doxylamine	Ziprasidon
Enalapril	Zotepin
Ethenzamide	Zuclopentixol
Felbamat	
Felodipine	
Fenoterol	
Ganciclovir	
Gliborunide	
Glipizid	
Gliquidone	
Hydroxychloroquine	
Ibuprofen	
Indinavir	
Iomperol	
Itraconazol	
Lamivudine	
Lisinopril	
Lonazelac	
Loperamide	
Loratadine	
Mebendazol	
Memantine	
Meprobamat	
Mesoridazine	
Metronidazol	
Naproxene	

7.10 Comparison of Column Shelf-Lives: Developed System versus Remedi^{TM}-HS

Developed system		**Remedi^{TM}-HS**	
Column type	**Shelf-life**	**Column type**	**Shelf-life**
C18 (Pre-(guard)cartridge	50 injections	Purification cartridge	250 injections*
Extraction column	≥ 300 injections	Extraction cartridge	250 injections*
SCX (Pre-(guard)cartridge	≥ 300 injections	Separation cartridge 1	250 injections*
Analytical columns	500-1000 injections per column	Separation cartridge 2	250 injections*
		Saturator cartridge	250 injections*
		Filter	250 injections**

** guaranteed shelf-life by manufacturer*
*** exchanged together with the saturator cartridge*

Curriculum Vitae

Name	Lena Schönberg
Date of birth	08. September 1976
Place of birth	Tauberbischofsheim, Germany
Nationality	German

Education and Qualifications

Since 09/2003	Doctoral thesis under the supervision of Prof. Dr. Charlotte Kloft, Department Clinical Pharmacy, Freie Universität Berlin and Martin-Luther-Universität Halle-Wittenberg and PD. Dr. Dagmar Lampe, Institute of Toxicology, Clinical Toxicology and Poison Control Centre Berlin
05/2003	Registration as a pharmacist in Germany
10/1997-12/2001	Undergraduate studies in pharmacy, Freie Universität Berlin, Degree in Pharmacy (2. Staatsexamen)
08/1996-06/1997	Muswell Hill School of English, London and Barnet College, London (Cambridge Certificate in Advanced English)
08/1985-06/1996	High school diploma (Allgemeine Hochschulreife) Freie Waldorfschule Hildesheim

Professional Experience

Since 06/2003	Employment as pharmacist Oberbaum Apotheke and Berlin Apotheke, Berlin, Germany
01/2003-02/2003	Scientific Internship Institute of Pharmaceutical Technology and Biopharmacy, University of Vienna
01/2002-12/2001	Pre-registration pharmacist Pfauen Apotheke Berlin, Germany

Publications

Original Papers

L. Schönberg, T. Grobosch, D. Lampe, C. Kloft.
Identification of alkaloids by automated on-line extraction-high performance liquid chromatography-diode array detection in two intoxication cases.
Biomed. Chromatogr., submitted (2007).

L. Schönberg, T. Grobosch, D. Lampe, C. Kloft.
Toxicological screening in urine: Comparison of two automated HPLC screening systems (Toxicological Identification System (TOX.I.S.*) versus REMEDI^{TM}-HS).
J. Anal. Toxicol., **31:** 321-327 (2007).

L. Schönberg, T. Grobosch, D. Lampe, C. Kloft.
Analysis of basic compounds in urine by on-line extraction HPLC-DAD.
T + K, **74:** 64 (2007).

L. Schönberg, T. Grobosch, D. Lampe, C. Kloft.
New screening method for basic compounds in urine by on-line extraction–high-performance liquid chromatography with photodiode-array detection.
J. Chromatogr. A, **1134:** 177-185 (2006).

T. Grobosch, L. Schönberg, D. Lampe, C. Kloft.
New automated screening system for the determination of basic compounds in urine by on-line extraction-HPLC-DAD.
In preparation.

T. Grobosch, B. Angelow, L. Schönberg, D. Lampe.
Acute bromadiolone intoxication.
J. Anal. Tox., **30:** 281-286 (2006).

T. Grobosch, L. Schönberg, D. Lampe.
Toxicological monitoring of an acute risperidone and lithium intoxication.
Bull. Int. Assoc. Forensic Toxicol., **34:** 55-58 (2004).

T. Grobosch, L. Schönberg, D. Lampe.
Toxikologisches Monitoring von Risperidon und Lithium bei einer akuten Intoxikation.
T+K, **71:** 10-16 (2004).

T. Grobosch, L. Schönberg, D. Lampe.
Schnelle Bestimmung von Risperidon und 9-Hydroxy-Risperidon mit LC-MS nach Festphasenextraktion (BondElute Certify®), Varian Inspirations **3:** 14-15 (2004).

T. Grobosch, L. Schönberg, D. Lampe**.**
Fast determination of risperidone and 9-hydroxyrisperidone with LC-MS after solid-phase extraction with BondElute Certify®, Varian Inspirations **3:** 14-15 (2004).

Oral Presentations

L. Schönberg, T. Grobosch, D. Lampe, C. Kloft.
Development of a fully automated screening system for the determination of basic compounds in urine by on-line extraction – high performance liquid chromatography with photodiode-array detection.
44th International meeting of the International Association of Forensic Toxicologists (TIAFT), Ljubljana, Slovenia, 26.08-01.09.2006, (Abstract book, 44, 2006).

L. Schönberg, T. Grobosch, D. Lampe, B. Erxleben, C. Kloft.
Development of an automated screening system for the determination of substances in urine. Jahrestagung der Deutschen Pharmazeutischen Gesellschaft (DPhG) 2006, Marburg, 04.-07.10.2006, (Abstract book, 75, 2006).

L. Schönberg, C. Kloft, T. Grobosch, U. Lemm-Ahlers, D. Lampe, B. Erxleben.
Development of an analytical method for the determination of substances in urine.
Jahrestagung der Deutschen Pharmazeutischen Gesellschaft (DPhG) 2004, Regensburg, 06.-09.10.2004 (Abstract book, 52, 2004).

Poster Presentations

L. Schönberg, C. Kloft, T. Grobosch, U. Lemm-Ahlers, D. Lampe, B. Erxleben.
Entwicklung eines in der toxikologischen Notfall-Diagnostik einsetzbaren Analysenverfahrens.
Deutsche Pharmazeutischen Gesellschaft (DPhG), Landesgruppe Berlin-Brandenburg-Der wissenschaftliche Nachwuchs stellt sich vor, Berlin, 05.07.2004, (Abstract book, P23, 2004).

T. Grobosch, L. Schönberg, D. Lampe, C. Kloft.
New automated screening system for the determination of basic compounds in urine by on-line extraction-HPLC-DAD.
XV. Mosbacher Symposium der Gesellschaft für Toxikologische und Forensische Chemie (GTFCh), 2007, Mosbach, 18.04.-21.04. 2007, (Abstract book, 2007).

B.-T. Erxleben, L. Schönberg, T. Grobosch, F. Kribbel.
Prominence TOX.I.S., Identification of pharmaceuticals and drugs using online sample preparation and HPLC PDA, 7th Balaton Symposion of the Hungarian Society for Separation Sciences, Siofok, Hungary, 05.09-07.09. 2007.

Conference Abstracts

T. Grobosch, B. Angelow, L. Schönberg, D. Lampe.
An Acute Bromadiolone Intoxication, 43th International meeting of the International Association of Forensic Toxicologists (TIAFT), Seoul, 29.08-02.09.2005, (Abstract book, 2005).

Acknowledgements

I would like to express my sincere gratitude to:

Prof. Dr. Charlotte Kloft for supervising this work, for her continuous and ongoing support and encouragement,

PD Dr. Dagmar Lampe for giving me the opportunity to prepare my thesis in the Institute of Toxicology-Clinical Toxicology and Poison Control Centre Berlin, for the revision of several manuscripts and her helpfulness in any questions,

Dr. Thomas Grobosch for the supervision of the experimental work, for his intensive and constructive criticism, the helpful discussions as well as for his enthusiasm and encouragement,

my colleagues from the Institute of Toxicology-Clinical Toxicology and Poison Control Centre Berlin for their help during my thesis and their effort to accept and use the developed system in the daily toxicological routine,

my colleagues in the Department of Clinical Pharmacy in Berlin and Halle for their help and the friendly atmosphere as well as Uta Zschöckner and all the apprentices for their contributions to this thesis,

my family and friends for their support and advices during the past years and especially Nicola La Rocca, Sven Ehlert and Frauke Kreye for their help with the manuscript and Gregor Morrison for his patience, understanding and his help in all English language questions.

Dr. Björn-Thoralf Erxleben and Shimadzu Europe GmbH are gratefully acknowledged for the cooperation and technical support.

Declaration

Hiermit erkläre ich, dass ich mich mit der vorliegenden Arbeit erstmals um die Erlangung eines Doktorgrades bewerbe.

Ferner erkläre ich, dass ich die vorliegende Arbeit selbständig und ohne fremde Hilfe angefertigt, andere als die von mir angegebenen Quellen und Hilfsmittel nicht benutzt und die den verwendeten Werken wörtlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht habe.

Halle (Saale), 28. Januar 2008