Analysis of basic compounds in urine by on-line extraction-HPLC-DAD

Lena Schönberg a,b, Thomas Grobosch a, Dagmar Lampe a and Charlotte Kloft b,c

a Berliner Betrieb fuer Zentrale Gesundheitliche Aufgaben (BBGes), Institute of Clinical Toxicology – Clinical Toxicology and Poison Control Centre, Oranienburgerstr. 285, D-13437 Berlin, Germany

b Department of Clinical Pharmacy, Institute of Pharmacy, Freie Universitaet Berlin, Kelchstr. 31, D-12169 Berlin, Germany

c Department of Clinical Pharmacy, Faculty of Pharmacy, Martin-Luther-Universitaet Halle-Wittenberg, Wolfgang-Langenbeck-Strasse 4, D-06120 Halle-Wittenberg, Germany

Abstract
An automated, qualitative screening HPLC method for the identification of basic compounds in urine has been established. A 1-ml volume of urine was extracted by on-line extraction and separated on two coupled strong cation-exchange columns under isocratic conditions. The use of the photodiode-array detector (DAD, λ = 190-370 nm) gave access to a library of > 2600 toxicologically relevant compounds. The validated method is reliable, simple and in addition successfully proven with the analysis of real biological specimen for the routine use.

1. Introduction
Systematic toxicological analysis (STA) based on GC, HPLC and immunological methods is usually performed in plasma/serum and urine. However, some compounds such as psilocin, scopolamine and morphine with very short half-lives in blood, are difficult to detect with common STA screening methods and require specialised analytical methods. The Remedi®-HS (Bio-Rad, Munich, Germany) presents such a specialised system for the analysis of basic compounds (e.g. alkaloids). However, it will be taken out of service at the end of 2008. The aim of this study was to develop a chromatographic screening method for toxicological analysis in urine with main focus on 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 (DOA) analysis, it was proved if the method is suitable for this field of application.

A HPLC-system with a DAD was chosen to access a commercially available spectra library with >2600 spectra [1] and to allow the identification of toxicologically relevant metabolites by comparing their spectra to those of the parent compound. In addition, all chromatographic data of investigated compounds including metabolites that are not available as drug standards were stored in an additional library for spectra and relative retention time comparison (method specific library). To hold sample pre-treatment and costs to a minimum, the developed method was characterised by fully automated on-line extraction and common HPLC equipment (columns, solvents). With the developed method specialised methods such as the Remedi®-HS (analysis of alkaloids, drugs of abuse analysis) should be replaced. The utility of the developed method for STA and DOA is discussed in the following and illustrated with example chromatograms of both, the developed system and the Remedi®-HS.

2. Material and methods

Urine samples
Urine samples were sent to our laboratory from hospital emergency rooms, psychiatric units and substance abuse clinics for analysis. The samples were delivered in monovettes and stored at 5-8°C until they were analysed.
Sample preparation
The urine samples were centrifuged for 5 min at 15 000 x g. 1.0 mL of each sample was transferred to a 2.0-mL polypropylene cup, diluted with 500 µL internal standard 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.

Extraction and analytical procedure
Following sample preparation, the sample was applied with 0.01 \( M \) phosphate buffer pH 6 to the on-line extraction column (StrataX-CW\textsuperscript{TM}). On the weak cation-exchange material of the on-line extraction column, basic target analytes were retained and the urine matrix was washed into the waste. After two subsequent wash steps in the forward (acetonitrile/water) and back flush mode (water), respectively. The analytes were eluted to the analytical columns (2 x LunaSCX (150 x 4.6 mm)) with mobile phase (31.5% ACN/H\textsubscript{2}O (90/10, v/v) and 68.5% 0.05 \( M \) phosphate buffer pH 2.3). Separation was carried out under isocratic conditions at a flow rate of 1.2 mL/min. The time required for the analytical procedure including on-line extraction was 41 min.

Validation
The method was validated using an exemplary performance control test consisting of six different analytes which represented the following groups of interest: alkaloids (scopolamine), amphetamine derivatives (methylenedioxyamphetamine (MDA)) opiates (codeine and morphine), the methadone metabolite 2-ethyliden-1,5-dimethyl-3,3-diphenylpyrrolidene (EDDP) and the internal standard neostigmine bromide (IS). Recovery of the performance control test analytes was > 73-97%. The results for the intra-assay precision ranged from 0.4-7.2% (n = 6), inter-assay precision was < 8% and linearity for the analytes was obtained from 0.1-15.0 µg/mL (\( R^2 > 0.995 \)) for codeine, EDDP and morphine, 0.1-5.0 µg/mL for MDA (\( R^2 = 0.993 \)) and 0.25-15.0 µg/mL for scopolamine and IS (\( R^2 > 0.993 \)), respectively. The method showed sufficient selectivity/specificity and the lower limit of detection was 0.1 µg/mL (S/N >3) and 0.25 µg/mL (S/N >3) for scopolamine. All stock solutions showed stability over a time period of 28 days. The detailed method and validation data has been published elsewhere [2].

3. Results
To prove the utility of the developed method for toxicological screening of urine, authentically clinical samples, were analysed. The results were compared to results achieved by the Remedi\textsuperscript{TM}-HS-system [3]. The evaluation of the data of 405 samples demonstrated, that the developed analytical database represents a reliable method for the identification of basic substances. A detailed report will be given subsequently [4]. In the following figures example chromatograms of two intoxication cases (Fig. 1 and 2) and two drugs of abuse confirmation cases (DOA, Fig. 3 and 4) are shown, which were analysed with the developed system (left) and the Remedi\textsuperscript{TM}-HS (right).

As can be seen from the Figures 1-4, both compared methods showed the same analysis results and thus can be used for the same fields of application. In cases of STA, the HPLC-UV method should be used as a complementary method to other methods within the rational chemical-analytical approach of general unknown screening in order to identify as many xenobiotics as possible. According to N. Sadeg et al. who described a 12 months’ experience of toxicological screening with the Remedi\textsuperscript{TM}-HS in a general hospital in France [6], it can be stated for the developed method as well, that it presents a valuable tool for additional urine screening within STA.
Fig. 1. STA: Psilocin intoxication, female patient, date of birth 1998, creatinine value 0.98 g/L. Psilocin analysis was performed after glucuronide hydrolysis with β-glucuronidase from E. coli (140 units/mg, Roche, Mannheim) for 1.5 h at 45 °C.

Fig. 2. STA: Amitriptyline intoxication, female, date of birth 1977, creatinine value 0.22 g/L.

Fig. 3. DOA: Confirmation screening, male, date of birth not given, creatinine 0.15 g/L.
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In cases of DOA, immunological pre-screening should be performed. If the immunoassay result cannot be verified by the HPLC-UV method, a more sensitive method must be considered. Therefore GC-MS remains the “gold standard” for DOA confirmation screening in urine [5].

With the developed method a broad screening of basic analytes from urine was possible for the analysis of acute intoxications as well as for confirming the intake of drugs of abuse within the given limit of detection. The method is simple, automated and does not require special sample pre-treatment such as derivatisation. The retention and relative retention times in addition to the characteristic spectra allowed individual compounds to be identified from the complex components contained in human urine. The function of the analytical system concerning extraction, recovery and retention time was monitored by a validated performance control sample.

4. Conclusion

An automated method for the qualitative determination of basic drugs from urine was established and validated. 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. 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 authentically toxicological samples proved the utility for toxicological applications, as was illustrated by four example chromatograms and will be reported more detailed subsequently [4]. The validation data met the criteria set in international guidelines for bioanalytical methods [7] and confirmed the reliability of the method. Time required for the complete analysis was 41 min.
In conclusion, the developed on-line extraction-HPLC-DAD method allowed simple and reliable determination of basic drugs in urine and is suitable for the routine use as initial results of authentically sample analyses showed.

References


