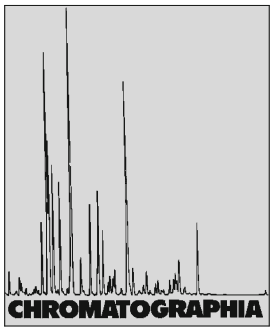
High-Throughput Screening of Drugs of Abuse in Urine by Supported Liquid–Liquid Extraction and UHPLC Coupled to Tandem MS



2009, *70*, 1373–1380

Aubert Maquille[1,2], Davy Guillarme[1], Serge Rudaz[1], Jean-Luc Veuthey[1,✉]

[1] School of Pharmaceutical Sciences, University of Geneva, University of Lausanne, Boulevard d'Yvoy 20, 1211 Geneva 4, Switzerland; E-Mail: jean-luc.veuthey@unige.ch
[2] *Present Address*: Laboratory of Chemical and Physico-Chemical Analysis of Drugs (CHAM), Louvain Drug Research Institute, Université Catholique de Louvain, Avenue E. Mounier, 72, bte 72.30, 1200 Brussels, Belgium

Received: 18 May 2009 / Revised: 17 August 2009 / Accepted: 19 August 2009
Online publication: 9 October 2009

Abstract

A qualitative method, involving supported liquid–liquid extraction (SLE) and ultra high pressure liquid chromatography coupled to tandem mass spectrometry (UHPLC-MS–MS), was developed for the rapid tentative identification of various drugs of abuse in urine. In this study, 28 drugs and metabolites were covered by the screening procedure. Before analysis, urine samples were extracted by SLE and good extraction recoveries were obtained for most investigated compounds. The UHPLC strategy was then selected for the rapid separation of amphetamines, cocaine, opiates and related compounds in urine. Using columns packed with sub-2 μm particles, analysis time was reduced down to 2 min, while maintaining acceptable performance. Finally, the detection was by tandem MS operating in the single reaction monitoring (SRM) mode. The most intense transition was selected for the different drugs and SRM dwell times set at 5 ms, to maintain sufficient data points across the narrow UHPLC peaks. The tentative identification of the drugs of interest, including amphetamines, opiates and cocaine, was based on both, retention times and mass spectrometry information. With the proposed method, limits of detection were estimated at about 1 ng mL^{-1} and the applicability was assessed by successfully analyzing several samples of drug abusers. Finally, this study demonstrates the potential of UHPLC coupled to tandem MS for the rapid screening of drugs of abuse in urine.

Keywords

Supported liquid–liquid extraction
Ultra high pressure liquid chromatography
Tandem mass spectrometry
Drugs of abuse in urine

Introduction

The determination of the abuse of several drugs and illicit substances is frequently performed in urine since large volumes of sample is available, and its collection is easy and non-invasive [1–3]. In addition, urine testing provides a relatively long detection window for drugs. However, the latter are mostly excreted as metabolites, parent drugs being generally found in trace amounts so that very sensitive methods are needed for their analysis. Usually, drugs of abuse in urine are screened by immunochemical techniques and positive samples are further confirmed by hyphenated techniques such as LC-MS or GC-MS.

Because no pre-derivatization step is required, there is an increased use of liquid chromatography coupled to MS operating in the single or tandem mode (LC-MS or LC-MS–MS) for the analysis of drugs in toxicological and forensic analyses [4, 5]. Indeed, such an approach provides more specific information on substance identity, with lower detection limits and less interference, compared to immunoassays.

Recently, some improvements were brought to conventional LC to speed up

the analytical process. It appears that columns packed with sub-2 μm particles and working under ultra high pressure (UHPLC) have emerged as an attractive approach for high throughput screening, providing faster separations without compromising chromatographic resolution or MS sensitivity [6, 7]. Therefore, UHPLC-MS–MS strategy is often employed, particularly in the fields of bioanalysis (determination of various drugs and metabolites in biological fluids) [8–10] or multi-residue screening (determination of numerous contaminants such as pesticides, drugs or doping agents in complex matrices) [11–13].

Particularly, several authors reported the determination of drugs of abuse by UHPLC-MS–MS. For instance, Lurie et al. [14] demonstrated the applicability of UHPLC-MS–MS for the profiling of fentanyl and 16 of its homologues or heroin and several of its by-products and precursors [15], but in seized exhibits only. In the field of doping analysis, UHPLC-MS–MS strategy was employed for determining between 30 and 130 prohibited substances in urine in a single analytical run [16–18], using a simple "dilute and shoot" as sample preparation. Finally, Berg et al. [19] developed and validated an SPE-UHPLC-MS–MS method allowing the determination of six opiates, cocaine and benzoylecgonine in less than 6 min, reequilibration included. They also mentioned that their method has been routinely used in more than 2,000 urine samples with two replicates of each sample.

In the present work, the UHPLC-MS–MS set-up was evaluated for the rapid screening of several drug of abuse in urine. Three different classes of drugs were tested: amphetamines and related substances, opiates including some of their metabolites, and cocaine and its major metabolites. Since urine is a complex matrix with an important amount of electrolytes, sample preparation is often mandatory to minimize matrix effects, and concentrate the analytes of interest [20, 21]. Due to the physico-chemical properties (i.e. polarity and ionization state) of the investigated drugs, liquid–liquid extraction (LLE) should be selected [22]. Then, to automate the sample preparation procedure and make it coherent with the high throughput afforded by UHPLC, urine samples were extracted by supported liquid–liquid extraction (SLE), a promising technique appeared in 1997 [23–25], which can be easily automated in a 96-well plate format. For the first time, we report the evaluation of SLE as sample preparation, prior to UHPLC-MS–MS analysis.

Experimental

Chemicals and Reagents

Methamphetamine, 3,4-methylenedioxyamphetamine (MDA), 4-methylenedioxymethamphetamine (MDMA, Ecstasy), 3,4-methylenedioxyethylamphetamine (MDEA),2-methylamino-1-(3,4-methylenedioxyphenyl)butane (MBDB), 2-ethylidene-3,3-diphenyl-1,5-dimethylpyrrolidine (EDDP), 6-monoacetylmorphine (6-MAM), ecgoninemethylester (EME), benzoylecgonine (BE), cocaethylene, cocaine and norcocaine were purchased from Lipomed (Arlesheim, Switzerland). Dextromethorphan, tramadol, ketamine, procaine, ephedrine, pseudoephedrine, norephedrine and nalbuphine were from Sigma (St. Louis, MO, USA). Papaverine, pethidine, methadone, morphine, codeine, amphetamine, noscapine and ethylmorphine were provided by Siegfried (Zofingen, Switzerland). Fentanyl was obtained from Sintetica (Mendrisio, Switzerland). The list of the tested drugs is reported in Table 1.

Formic acid and acetonitrile (ACN) were of ULC-MS grade and purchased from Biosolve (Valkenswaard, Netherlands). Ammonium hydroxide was provided by Sigma-Fluka (Buchs, Switzerland). Water was obtained from a Milli-Q Water Purification System from Millipore (Bedford, MA, USA). Ammonia buffer 10 mM was prepared with an adapted volume of ammonium hydroxide and the pH adjusted to 9.0 with formic acid. Finally, pH was measured with a Metrohm pH meter (Herisau, Switzerland). After verification with Phoebus software (Analis, Namur, Belgium), the prepared buffer has a buffer capacity higher than 5 mM pH^{-1} unit.

Urine samples from drug abusers and blank urine samples from healthy volunteers were provided by Dr. Marc Fathi from HUG (Geneva University Hospitals, Switzerland). The samples were stored at −18 °C prior to extraction and analysis.

Extraction Procedure

The extraction of drugs from urine was carried out by supported liquid–liquid extraction (SLE). Before extraction, urine was filtered through a nylon filter 0.45 μm × 47 mm and procaine was added at a concentration of 10 ng mL^{-1}, as internal standard. The SLE procedure was performed on Merck Extrelut NT3 cartridges (Darmstadt, Germany) which contain a modified form of diatomaceous earth. No pre-treatment of the cartridge was necessary. In a first instance, 1 mL of 250 mM borate buffer at pH 9.0 was added into 2 mL of sample urine and vortexed for 30 s. The 3 mL solution was loaded on the SLE cartridge (such volume was suitable for loading, to avoid saturation of the sorbent bed, as recommended by the manufacturer) and equilibrated for 10 min. Elution was with 15 mL ethyl acetate. The sample solution was then evaporated to dryness at 40 °C under a stream of nitrogen. Finally, the dried samples were reconstituted in 200 μL HCl 10^{-4} M prior injection in the UHPLC-MS–MS system.

Matrix effects arising from the SLE-UHPLC-MS–MS procedure were evaluated in terms of process efficiency (PE), according to a procedure originally described by Matuszewski et al. [26] and recently updated by Marchi et al. [27]. It is noteworthy that the determined PE corresponds to both matrix effects arising from sample preparation and electrospray ionization alteration. For its evaluation, a urine sample spiked with all analytes, was extracted several times, using the SLE approach and compared to a neat standard solution (target analytes diluted in pure water) at the target concentration.

UHPLC-MS–MS Instrumentation

Analyses were performed on a Waters Acquity ultra performance liquid chromatograph (UPLC) system hyphenated with a Waters TQD triple quadrupole mass spectrometer fitted with a Z-spray electrospray ionization source (Waters, Milford, MA, USA).

The chromatographic system included a binary solvent manager with a maximum delivery flow rate of 2 mL min^{-1}, a sample manager with an injection loop volume of 2 µL (full loop injection), and a column oven set at 30 °C. The chromatographic column was a Waters Acquity BEH C18 (50 × 2.1 mm I.D., 1.7 µm) and a precolumn Waters Acquity BEH C18 (5 × 2.1 mm I.D., 1.7 µm) was always used with urine samples. Dwell volume (V_d) of the UPLC-MS–MS configuration was estimated at 100 µL with the 2 µL injection loop. Chromatographic conditions for the separation were as follow: the analysis was carried out in the gradient mode at a flow rate of 600 µL min^{-1} (without splitting) and a temperature of 30 °C. Mobile phase consists in a mixture of aqueous ammonia buffer 10 mM at pH 9 (A) and acetonitrile (B). A linear gradient from 10 to 70% B was applied for 2 min. Between analyses, the column was reequilibrated with the initial conditions for 1 min (corresponding to five column dead volumes).

The TQD instrument operated at single mass resolution of m/z 0.7 FWHM (i.e. full width at half maximum), and possesses an upper mass limit of m/z 2000. The ESCi ionization source was used in the ESI positive mode and selected reaction monitoring (SRM) was performed, using the protonated molecular ion of each compound as the precursor and the most intense fragment. Collision energies and cone voltages were tuned by infusing individually each compound at 1 µg mL^{-1} in pH 9 buffer and at a flow rate of 600 µL min^{-1}. Optimal cone voltage and collision energy values were summarized in Table 1 along with the corresponding protonated molecular ions and fragments. Nitrogen was used as the drying gas and argon as collision gas. The capillary voltage and the source extractor voltages were set at +3.5 kV and +3 V, respectively. The source temperature was maintained at 150 °C, the desolvatation gas temperature and flow at 300 °C and 800 L h^{-1}, respectively, and the cone gas flow at 40 L h^{-1}. The collision gas flow was set at 0.2 mL min^{-1} of Argon and entrance and exit potentials, respectively, adjusted to 1 and 0.5 V. Finally, dwell time and inter-channel delay were set to 5 ms, to maintain enough data points across the narrow peaks produced by UHPLC.

Data acquisition, data handling and instrument control were performed by Masslynx v4.1 Software (Waters).

Table 1. Precursor ions, MS–MS transitions, cone voltages and collision energies for the selected drugs along with the retention times from the UHPLC-MS–MS method

Compound	$[M + H]^+$	MS2	R.T. (min)	Cone voltage (V)	Collision energy (eV)
Amphetamines and related compounds					
Amphetamine	136	119	0.98	20	12
Ephedrine	166	148	0.82	20	20
Ketamine	238	125	1.55	25	30
MBDB	208	135	1.23	25	18
		177		25	10
MDA	180	163	0.96	20	15
		135		25	18
MDEA	208	163	1.15	20	14
MDMA	194	79	1.03	25	25
Methamphetamine	150	119	1.06	20	15
Norephedrine	152	134	0.70	20	14
Pseudoephedrine	166	148	0.82	20	20
Opioids and metabolites					
6-Monoacetylmorphine	328	58	1.11	40	30
(6-MAM)		165	1.11	40	30
Codeine	300	165	1.09	50	35
Dextromethorphan	272	215	1.86	30	20
EDDP	278	234	1.74	50	25
Ethylmorphine	314	165	1.24	50	35
Fentanyl	337	105	1.95	40	25
Methadone	310	105	2.09	25	35
		157	0.90	40	30
Morphine	286	185	0.85	40	35
Nalbuphine	358	69	1.69	30	35
Noscapine	414	220	1.84	50	25
Papaverine	340	202	1.41	50	25
Pethidine	248	70	1.56	45	30
Tramadol	264	58	1.54	25	20
Cocaine and metabolites					
Benzoylecgonine (BE)	290	168	0.71	35	19
Cocaethylene	318	82	1.82	35	30
Cocaine	304	182	1.65	35	17
Ecgonine methylester (EME)	200	82	0.60	35	31
		168		35	19
Norcocaine	290	136	1.44	30	25
Internal standard					
Procaine	237	100	1.26	70	10

Results and Discussion

Evaluation of the Extraction Procedure

Direct analysis of urine samples by LC-MS often results in ion suppression due to interference from endogeneous or co-eluting exogeneous compounds. Therefore, a sample preparation is often performed in order to avoid these adverse effects. When dealing with the determination of forensic drugs in biological fluids, liquid–liquid extraction (LLE) is generally the technique of choice for producing clean extracts that can be directly injected into the LC-MS system [28].

Table 2. Physico-chemical properties of the investigated drugs of abuse. The pK_a, log P and log D values were calculated using Advanced Chemistry Development (ACD/Labs) Software V8.14 for Solaris

	p*K*a	log *P*	log *D* (pH 8)	log *D* (pH 9)	log *D* (pH 10)
Amphetamines and related compounds					
Amphetamine	9.94	1.8	−0.11	0.82	1.53
Ephedrine	9.38	1.05	−0.33	0.52	0.96
Ketamine	6.46	2.18	2.17	2.18	2.18
MBDB	10.46	2.33	−0.03	0.87	1.75
MDA	9.94	1.66	−0.25	0.68	1.39
MDEA	10.34	2.33	0.07	0.99	1.84
MDMA	10.32	1.8	−0.45	0.47	1.31
Methamphetamine	10.38	1.94	−0.36	0.55	1.41
Norephedrine	8.47	0.8	0.21	0.68	0.77
Pseudoephedrine	9.38	1.05	−0.33	0.52	0.96
Opiates and metabolites					
6-Monoacetylmorphine (6-MAM)	7.96(b)/9.46(a)	1.32	0.98	1.13	0.64
Codeine	8.25	1.2	0.76	1.13	1.19
Dextromethorphan	9.13	4.11	2.95	3.74	4.06
EDDP	7.71	5.51	5.33	5.49	5.51
Ethylmorphine	8.25	1.73	1.29	1.66	1.72
Fentanyl	9.06	3.89	2.79	3.55	3.84
Methadone	9.05	4.2	3.12	3.87	4.15
Morphine	8.26(b)/9.50(a)	0.43	−0.04	0.23	−0.2
Nalbuphine	7.35(b)/9.39(a)	1.78	1.68	1.63	1.08
Noscapine	6.32	2.82	2.82	2.82	2.82
Papaverine	6.32	3.74	3.73	3.74	3.74
Pethidine	8.58	2.35	1.67	2.21	2.33
Tramadol	9.6	2.51	0.91	1.81	2.36
Cocaine and metabolites					
Benzoylecgonine	10.82(b)/3.35(a)	2.71	0.21	0.21	0.16
Cocaethylene	9.04	3.61	2.53	3.29	3.56
Cocaine	8.97	3.08	2.07	2.79	3.04
Ecgonine methylester	9.57	−0.23	−1.8	−0.9	−0.37
Norcocaine	9.02	2.78	1.72	2.47	2.74
Internal standard					
Procaine	9.24	2.36	1.11	1.93	2.29

Taking into account the well-known drawbacks of LLE (i.e. formation of emulsion, poor phase separation, relatively high solvent consumption, low degree of automation and labour-intensive procedure), the extraction of drugs from urine was carried out, in the present study, by supported liquid–liquid extraction (SLE) [23–25], to simplify and automate the procedure. Basically, SLE consists in the adsorption of the aqueous samples on diatomaceous earth followed by the application of a non-miscible solvent through the cartridge with subsequent extraction and elution of the analytes. Finally, SLE allows faster sample preparation than liquid–liquid extraction, there is no need for phase separation avoiding emulsion problems and is available in 96-well plate format for high throughput sample preparation.

In the case of a multi-component analysis (i.e. 28 compounds in our case), the sample preparation is one of the most critical steps of the whole analytical process, because compounds often possess different physico-chemical properties, as illustrated in Table 2 where dissociation constant and lipophilicity have been indicated for each analyte. The simultaneous extraction of all these compounds from urine should thus be generic which usually involves a compromise in the selection of experimental conditions for satisfactory recoveries of each class of compounds.

In the case of SLE, the most critical parameter for increasing recovery is the pH at which urine is loaded on the SLE cartridge. Similarly to LLE, it should be adjusted to have the highest proportion of un-dissociated analytes in the aqueous extract. In the present study, the pH of urine was set to a compromise value of 9.0. Indeed, as most of the investigated drugs of abuse are basic, with pK_a in the range 6.3–10.8, a basic pH value (beyond pK_a + 2) should be employed to have the analytes of interest under their neutral form. However, there are some exceptions in the set of investigated compounds, and particularly, the amphoteric nature of morphine necessitates careful adjustment of pH within the range 6.9–9.0 [29] to ensure that it was in the appropriate form (as neutral as possible) for further elution with ethyl acetate. A similar problem was also observed with related substances (containing both acidic phenol and basic amine groups) such as 6-MAM and nalbuphine, as example. For these reasons, and in agreement with data given in Table 2, the pH was adjusted to 9.0, as a compromise which is certainly not the optimal value for amphetamines and related compounds.

For each extracted compound, the process efficiencies of SLE were calcu-

lated by comparing peak area measured in urine samples spiked with a known amount of reference substance and that of an un-extracted standard aqueous solution at the same concentration. This experiment was repeated sixfold and data for process efficiencies and corresponding standard deviations have been reported in Table 3. Procaine was chosen as the internal standard as it possesses a pK_a close to that of the investigated analytes but also since it is completely metabolized in humans and cannot be found in urine. For amphetamine and related substances, process efficiencies were in the range 53–65% since at pH 9, only a fraction of these basic compounds was neutral (estimated pK_a are in average equal to 10) and because of their high polarity (calculated log D at pH9 always lower than 1, except ketamine). For opiates, cocaine and their metabolites, higher process efficiencies (between 71 and 92%) were obtained because of the higher hydrophobicity of these compounds (calculated log D at pH 9 were systematically higher than 1, except for a few compounds). Only morphine presented a lower process efficiency, of 65%, which could be attributed to the amphoteric nature of this compound, with very weak acidic properties and its hydrophilicity even at pH 9 (log D of 0.23), compared to other opiates.

Table 3. Mean process efficiencies ($n = 6$) obtained after SLE of the different drugs

	Mean recovery (%) ($n = 6$)	Standard deviation (%)
Amphetamines and related compounds		
Amphetamine	55	1
Ephedrine	59	3
Ketamine	65	2
MBDB	59	3
MDA	53	5
MDEA	63	5
MDMA	62	2
Methamphetamine	60	6
Norephedrine	54	2
Opiates and metabolites		
6-MAM	75	9
Codeine	80	5
Dextromethorphan	89	6
EDDP	86	3
Ethylmorphine	86	9
Fentanyl	89	7
Methadone	80	2
Morphine	65	6
Nalbuphine	92	9
Norcocaine	80	4
Noscapine	92	7
Papaverine	83	10
Pethidine	85	8
Tramadol	81	4
Cocaine and metabolites		
BE	71	7
Cocaethylene	86	9
Cocaine	88	3
EME	81	4
Norcocaine	83	6

Optimization of UHPLC-MS–MS Analysis

Chromatographic Conditions

As discussed previously, the 28 investigated drugs of abuse are mainly basic, with pK_a around 9–10. Therefore, the pH of the UHPLC separation needs to be carefully selected to attain sufficient chromatographic selectivity and retention, without compromising MS sensitivity. Usually, LC-MS is performed in acidic conditions, using 0.1% formic acid as mobile phase additive, while the use of alkaline conditions was only scarcely reported, because it is believed that compounds of interest should be under their ionized form for efficient ionization in ESI mode. It was recently demonstrated that alkaline conditions present some obvious benefits in LC-MS of basic drugs, both from a chromatographic point of view (i.e. higher retention and improved selectivity) and MS sensitivity (i.e. better signal-to-noise because of the improved mobile phase desolvatation in presence of a high percentage of organic modifier) [19, 30]. Therefore, a pH value of 9 was selected as a good compromise between sufficient retention/selectivity and elevated MS sensitivity. In these conditions, interferences with un-retained sample matrix components (i.e., matrix effect) inducing ion suppression or enhancement were also strongly reduced. To ensure method stability at this relatively high pH, the selected chromatographic column (Waters Acquity BEH) was a hybrid material with hydrophobic ethylene groups present throughout the particle backbone, preventing the silica units from dissolution [31]. This material is stable within the range $1 < pH < 12$ and can thus be safely used at pH 9.

To analyze the compounds of interest presenting a wide polarity range, a generic gradient from 10 to 70% ACN was carried out in 2 min. Because the column dead time was around 0.2 min (50 × 2.1 mm column operating at a flow rate of 600 μL min^{-1}), this gradient time corresponds to an average retention factor during gradient elution, k_e around 3, which provides a good compromise between expected resolution and analysis time, as discussed elsewhere [32]. Despite a very short analysis time, all drugs of abuse were eluted during the gradient run, after un-retained sample matrix components. Indeed, taking into account the system dwell time of 0.16 min and column dead time of 0.20 min, the investigated compounds should possess analysis times at least equal to 0.36 min, which is the case since the lowest retained compound, ecgonine methylester

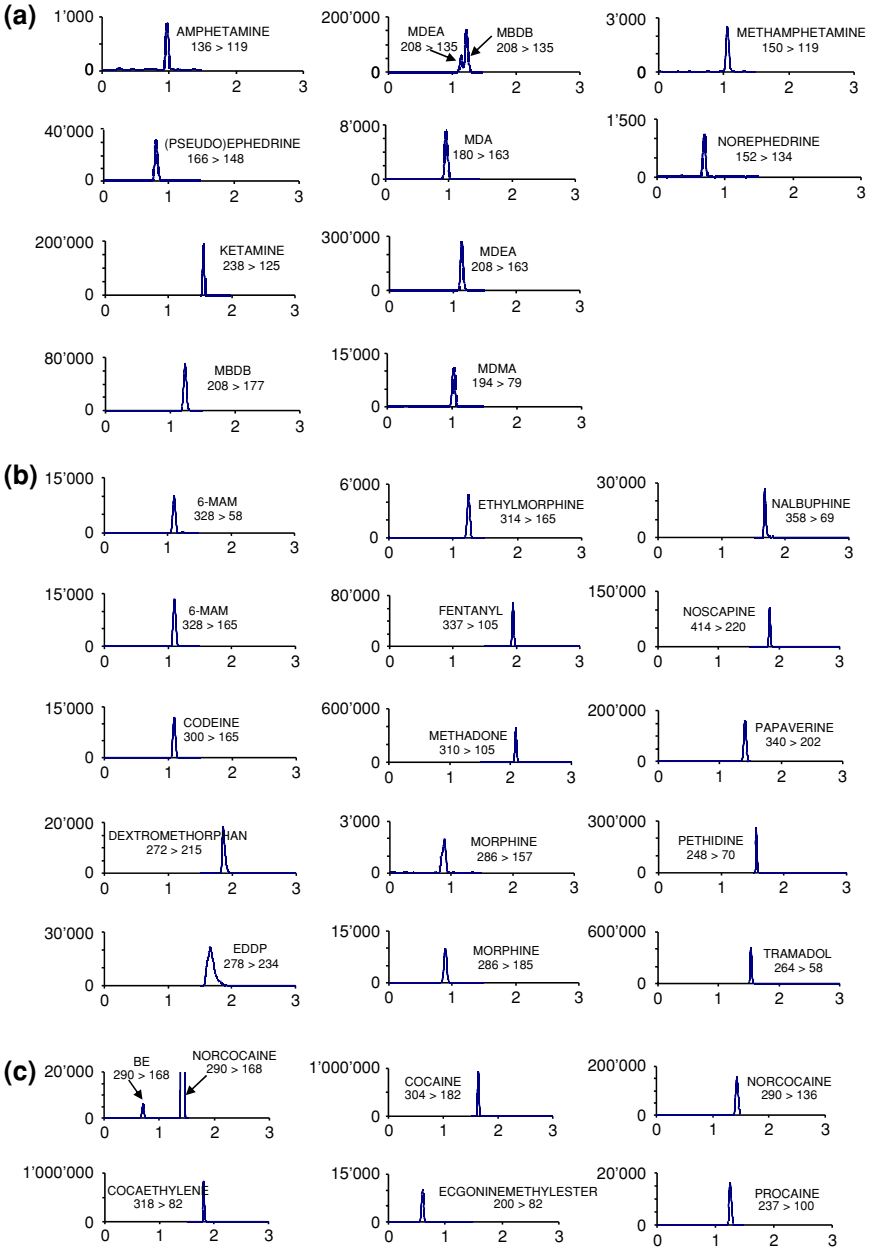


Fig. 1. Selected reaction monitoring chromatograms obtained for extracted spiked urine samples at 10 ng mL^{-1}. **a** Sample containing amphetamine and related substances. **b** Sample containing opiates and some metabolites. **c** Sample containing cocaine and its main metabolites but also the internal standard, procaine. In these figures, *X*-axis is time (min) and *Y*-axis is intensity (counts)

is eluted at 0.60 min (corresponding to an elution composition of ca. 15% ACN).

Detection Conditions

Because of the narrow peaks afforded by fast gradient in UHPLC (i.e. around 2–3 s in our conditions [31]), and the important number of drugs of abuse investigated, it is mandatory to adequately adjust the SRM dwell time and inter-channel delay of the MS–MS instrument. In the present study, the SRM dwell time and inter-channel delay were both set to their minimal value (i.e. 5 ms) to gather maximal information. In addition, to ensure a sufficient acquisition rate providing suitable peak shape, it is possible to define various time-schedule windows containing different SRM channels and time intervals, as it is often reported for multi-residue screening. Therefore, sequential time-schedule windows (i.e. 18 drugs followed in the time range 0–1.5 min and 10 other analytes between 1.5 and 3 min) were implemented to correctly define chromatographic peaks with a SRM dwell time of 5 ms.

For each compound, the most suitable SRM transition was carefully selected. Because no significant adducts were observed, the protonated ion ($[M + H]^+$) was selected as precursor ion and the most abundant collision induced dissociation (CID) fragment monitored. In some particular cases, such as for MDEA, MBDB, morphine and norcocaine, two predominant transitions were followed. For amphetamine and related substances, the fragments of interest generally corresponded to the cleavage of C–N bond. For ephedrine, the major fragments corresponded to the loss of water. Isobaric MBDB and MDEA with mass to charge ratios (m/z) of 208 shared the same m/z 135 fragment but were eluted with different retention times (i.e. 1.15 and 1.23 min), as reported in Table 1. In addition, m/z 163 fragment was specific to MDEA whilst m/z 177 fragment was specific to MBDB. For noscapine, the major fragment was m/z 220, as reported elsewhere [33]. For morphine, fragments 157 and 185 were followed, in agreement with other studies [15]. For fentanyl as well as for methadone, the major fragments corresponded to a phenethyl group (m/z 105) [34]. The fragment m/z 70 of pethidine most probably originated from the fragmentation of the piperidine ring. For tramadol, the fragment m/z 58 corresponded to $C_3H_8N^+$. A similar fragment was observed for 6-MAM, the major metabolite of heroin, along with a m/z 165 fragment that corresponded to $C_{13}H_9^+$ [35]. The same fragment was also monitored for ethylmorphine and codeine. The major fragment of EDDP, with m/z 234, was due to the consecutive losses of a methyl and an ethyl group [36]. For papaverine, the m/z 202 fragment was obtained after the loss of dimethoxybenzene moiety [37]. For cocaine and its metabolites [38], the

fragments were either m/z 82 ($C_5H_8N^+$) for cocaethylene and EME, or corresponded to the loss of benzoic acid to give fragments with m/z 168 (norcaine and BE) or 182 (cocaine). BE and norcocaine both shared the transition m/z 290 → m/z 168 although an intense fragment with m/z 136 ($C_8H_{10}NO^+$) was only observed for norcocaine. In addition, these two compounds were eluted with different retention times (i.e. 0.71 and 1.44 min), as reported in Table 1.

Evaluation of the proposed SLE-UHPLC-MS–MS Method

The chromatograms of the investigated drugs of abuse are presented in Fig. 1 and retention times listed in Table 1. As shown, all the selected substances could be accurately identified, based on MS data (m/z, fragment) and retention time, except ephedrine and pseudoephedrine which could not be discriminated from each other since they have the same m/z ratio, a similar fragmentation pathway and were co-eluted with the generic gradient conditions.

The method selectivity was evaluated by analyzing different negative urine samples ($n = 6$). Each sample was treated to highlight the presence of potential interfering compounds. For each MRM transition, no interferences were found at the retention times corresponding to tested substances.

In this work, the limits of detection (LOD) were evaluated in spiked urine samples after SLE extraction, for a signal-to-noise ratio (S/N) of about 3. At the lowest tested concentration for spiked urine samples (i.e. 1 ng mL^{-1}), S/N ratios of all compounds remained always superior to 3, allowing the identification of all tested compounds in urine.

Case Study: Application to Real Urine Samples

Ten real case samples were evaluated with the screening method to evaluate its applicability for routine applications. The selected urine samples also included negative urines certified after a routine LC-MS analysis. As example, Fig. 2 presents the results obtained for the analysis of urine declared positive for opiates. The UHPLC-MS–MS profile of this urine confirmed the presence of morphine, codeine, noscapine and 6-MAM which indicated a probable consumption of heroin. In addition, this urine contained an important quantity of methadone, widely used in heroin substitution treatment programme, and its main metabolite, EDDP. Finally, the sample also included fentanyl, which can serve as a direct pharmacological substitute for heroin in opiate dependent individuals and a certain amount of tramadol, an analgesic. This example demonstrated the important number of substances that can be found in case of toxicological issue.

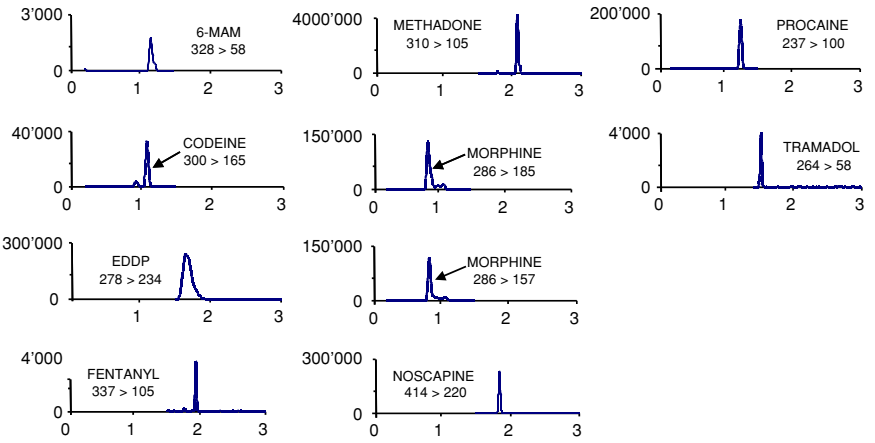


Fig. 2. Selected reaction monitoring chromatogram of real urine sample from drug abuser. In these figures, *X*-axis is time (min) and *Y*-axis is intensity (counts)

Conclusion

A method involving supported liquid–liquid extraction and ultra high pressure liquid chromatography coupled to tandem mass spectrometry was developed for the separation and identification of 28 drugs of abuse in urine samples, including amphetamines, cocaine, opiates and related compounds.

Prior to analysis, urine samples were extracted by SLE, allowing to minimize matrix effects and obtain acceptable process efficiencies for most of the investigated compounds. However, because of important differences in physico-chemical properties of selected drugs, the pH of the urine was carefully adjusted and a value of 9 was selected. Even if not investigated in the present study, the SLE procedure can be easily automated, using a 96-well plate format, to perform a high throughput sample preparation in agreement with the UHPLC-MS–MS procedure.

This screening procedure clearly highlighted the obvious benefits of columns packed with sub-2 μm particles (UHPLC), since the analysis time was reduced down to 2 min, maintaining suitable performance.

Using tandem MS detection operating in the single reaction monitoring mode, sensitivity as well as selectivity of the method were acceptable, and the ng mL^{-1} level was reached for all tested substances.

Finally, the applicability of the method was successfully assessed by analyzing several samples of drug abusers, demonstrating unambiguously the consumption of heroin in some cases.

Acknowledgments

The authors whish to thank Dr. Marc Fathi from HUG (Geneva University Hospitals, Switzerland) for providing urine samples from drug abusers and blank urine samples from healthy volunteers.

References

1. Stanley SMR, Ching Foo H (2006) J Chromatogr B 836:1–14
2. Nordgren HK, Beck O (2004) Ther Drug Monit 26:90–97
3. Deventer K, Pozo OJ, Van Eenoo P, Delbeke FT (2007) Rapid Commun Mass Spectrom 21:3015–3023
4. Fuh MR, Wu TY, Lin TY (2006) Talanta 68:987–991
5. Maurer HA (2007) Anal Bioanal Chem 388:1315–1325
6. Apollonio LG, Pianca DJ, Whittall IR, Maher WA, Kyd JA (2006) J Chromatogr B 836:111–115
7. Guillarme D, Nguyen DTT, Rudaz S, Veuthey JL (2006) J Chromatogr A 1149:20–29
8. Petsalo A, Turpeinen M, Pelkonen O, Tolonen A (2008) J Chromatogr A 1215:107–115
9. Churchwell MI, Twaddle NC, Meeker LR, Doerge DR (2005) J Chromatogr B 825:134–143
10. Pedraglio S, Rozio MG, Misiano P, Reali V, Dondio G, Bigogno C (2007) J Pharm Biomed Anal 44:665–673
11. Kaufmann A, Butcher P, Maden K, Widmer M (2007) Anal Chim Acta 586:13–21
12. Gervais C, Brosillon S, Laplanche A, Helen C (2008) J Chromatogr A 1202:163–172
13. Romero-Gonzalez R, Garrido Frenich A, Martinez Vidal JL (2008) Talanta 76:211–225
14. Lurie IS, Lio R (2009) J Chromatogr A 1216:1515–1519
15. Lurie IS, Toske SG (2008) J Chromatogr A 1188:322–326
16. Thorngren JO, Ostervall F, Garle M (2008) J Mass Spectrom 43:980–992
17. Badoud F, Grata E, Perrenoud L, Avois L, Saugy M, Rudaz S, Veuthey JL (2009) J Chromatogr A 1216:4423–4433
18. Ventura R, Roig M, Monfort N, Saez P, Berges R, Segura J (2008) Eur J Mass Spectrom 14:191–200
19. Berg T, Lundanes E, Christophersen AS, Strand DH (2009) J Chromatogr B 877:421–432
20. Moore C, Coulter C, Crompton K (2007) J Chromatogr B 859:208–212
21. Johansen SS, Bhatia HM (2007) J Chromatogr B 852:338–344
22. Schugerl K (1993) In: Stephanopoulos G (ed) Biotechnology, vol 3, 2nd edn. VCH, Weinheim, pp 557–592
23. Breitenbucher JG, Arienti KL, McClure KJ (2001) J Comb Chem 3:528–533
24. De Korompay A, Hill JC, Carter JF, NicDaeid N, Sleeman R (2008) J Chromatogr A 1178:1–8
25. Venn RF, Merson J, Cole S, Macrae P (2005) J Chromatogr B 817:77–80
26. Matuszewski BK, Constanzer ML, Chavez-Eng CM (2003) Anal Chem 75:3019–3030
27. Marchi I, Rudaz S, Veuthey JL (2009) J Pharm Biomed Anal 49:459–467
28. Bonfiglio R, King RC, Olah TV, Merkle K (1999) Rapid Commun Mass Spectrom 13:1175–1185
29. Lee MR, Yu SC, Hwang BH, Chen CY (2006) Anal Chim Acta 559:25–29
30. Schappler J, Nicoli R, Nguyen DTT, Rudaz S, Veuthey JL, Guillarme D (2009) Talanta 78:377–387
31. Wyndham KD (2003) Anal Chem 75:6781–6788
32. Guillarme D, Nguyen DTT, Rudaz S, Veuthey JL (2008) Eur J Pharm Biopharm 68:430–440
33. Matchett MW, Berberich DW, Johnson JE (2001) J Chromatogr A 927:97–102
34. Thevis M, Geyer H, Bahr D, Schanzer W (2005) Eur J Mass Spectrom 11:419–427
35. Poeaknapo C, Fisinger U, Zenk MH, Schmidt J (2004) Phytochemistry 65:1413–1420
36. Etter ML, George S, Graybiel K, Eichhorst J, Lehotay DC (2005) Clin Biochem 38:1095–1100
37. Wickens JR, Sleeman R, Keely BJ (2006) Rapid Commun Mass Spectrom 20:473–480
38. Wang PP, Bartlett MG (1998) J Mass Spectrom 33:961–968