Journal of Chromatography B, 909 (2012) 6–13

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Evaluation of a direct high-capacity target screening approach for urine drug testing using liquid chromatography–time-of-flight mass spectrometry

Aljona Saleh[a], Niclas Nikolai Stephanson[b], Ingrid Granelli[a], Tomas Villén[b], Olof Beck[b,*]

[a] Department of Analytical Chemistry, Stockholm University, Sweden
[b] Department of Medicine, Section of Clinical Pharmacology, Karolinska University Hospital, Sweden

ARTICLE INFO

Article history:
Received 29 July 2012
Accepted 2 October 2012
Available online 9 October 2012

Keywords:
Screening
Drugs of abuse
Urine
Time-of-flight mass spectrometry
Liquid chromatography

ABSTRACT

In this study a rapid liquid chromatography–time-of-flight mass spectrometry method was developed, validated and applied in order to evaluate the potential of this technique for routine urine drug testing. Approximately 800 authentic patient samples were analyzed for amphetamines (amphetamine and methamphetamine), opiates (morphine, morphine-3-glucuronide, morphine-6-glucuronide, codeine and codeine-6-glucuronide) and buprenorphines (buprenorphine and buprenorphine-glucuronide) using immunochemical screening assays and mass spectrometry confirmation methods for comparison. The chromatographic application utilized a rapid gradient with high flow and a reversed phase column with 1.8 μm particles. Total analysis time was 4 min. The mass spectrometer operated with an electrospray interface in positive mode with a resolution power of >10,000 at *m/z* 956. The applied reporting limits were 100 ng/mL for amphetamines and opiates, and 5 ng/mL for buprenorphines, with lower limits of quantification were 2.8–41 ng/mL. Calibration curves showed a linear response with coefficients of correlation of 0.97–0.99. The intra- and interday imprecision in quantification at the reporting limits were <10% for all analytes but for buprenorphines <20%. Method validation data met performance criteria for a qualitative and quantitative method. The liquid chromatography–time-of-flight mass spectrometry method was found to be more selective than the immunochemical method by producing lower rates of false positives (0% for amphetamines and opiates; 3.2% for buprenorphines) and negatives (1.8% for amphetamines; 0.6% for opiates; 0% for buprenorphines). The overall agreement between the two screening methods was between 94.2 and 97.4%. Comparison of data with the confirmation (LC–MS) results for all individual 9 analytes showed that most deviating results were produced in samples with low levels of analytes. False negatives were mainly related to failure of detected peak to meet mass accuracy criteria (±20 mDa). False positives was related to presence of interfering peaks meeting mass accuracy and retention time criteria and occurred mainly at low levels. It is concluded that liquid chromatography–time-of-flight mass spectrometry has potential both as a complement and as replacement of immunochemical screening assays.

1. Introduction

Screening for drugs of abuse in urine is commonly performed by using immunochemical assays, which offer cost-effective and high throughput analytical applications. Screening using mass spectrometry methods has been done either as a complement to immunochemical assays in clinical testing or as the method of choice in forensic, clinical toxicology and doping control applications [1–6]. The main limitations in using immunochemical screening methods for urine drug testing are limited number of analytes covered, predefined cutoff limits, and a high prevalence of false positive results.

The limitation of immunochemical assays that the cutoff limit cannot be easily adjusted to provide an optimal detection time and is set more of technical limitations leads to the occurrence of "false" negative results because of limited analytical performance. For example, for amphetamines the common cutoff limits of 500 or 1000 ng/mL using antibodies selective for the D-enantiomers of amphetamine and methamphetamine results in a limited detection of positive samples. Lastly, the limited selectivity of some immunoassay assays (e.g. amphetamines and opiates) make the fraction of false positives high hindering the information to be used even in clinical testing that not always require immediate analytical confirmation. Consequently, there is a need for alternative cost-effective and high throughput techniques with better meet clinical needs of analytical performance.

In order for LC–MS methods to meet these above mentioned requirements it is necessary to simplify sample preparation to

* Corresponding author. Tel.: +46858581046; fax: +46858581070.
E-mail address: olof.beck@karolinska.se (O. Beck).

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http://dx.doi.org/10.1016/j.jchromb.2012.10.006

a dilution step with needed internal standards and to use rapid chromatography. This approach has been successful for multi-component screening using LC–tandem mass spectrometry in selected reaction monitoring mode [1,2,5,7,8]. This analytical approach is also viable for confirmation methods [1,2]. One drawback is that the tandem mass spectrometry is only suitable for a rather limited number of target compounds, which needs optimization for each individual compound and that reference material is available.

In recent time the possible use of liquid chromatography–time-of-flight mass spectrometry (LC–TOFMS) in multi-targeted screening has been demonstrated [6,9–11]. Most applications so far have concerned forensic toxicology and doping control [11]. The potential of this technique to also cover analytes for which reference material are still lacking, e.g. internet drugs, makes it attractive for application also in clinical screening for drugs of abuse.

The aim of this study was to evaluate the performance of an LC–TOFMS method for screening of drugs of abuse in authentic patient urine samples and compare it with existing immunoassay methods and with mass spectrometry confirmation methods. As compared to previous work this study was not directed to demonstrating the possibility to cover large number of analytes or to producing reliable identifications but rather to compare a high-capacity design with immunochemical screening methods. The study comprised nine selected analytes from three drug classes: morphine, morphine-3-glucuronide (M3G), morphine-6-glucuronide (M6G), codeine, codeine-6-glucuronide (C6G), amphetamine, methamphetamine, buprenorphine and buprenorphine-glucuronide (BG).

2. Experimental

2.1. Chemicals, reagents and urine samples

Morphine, M3G, M6G, codeine, C6G, amphetamine, methamphetamine, buprenorphine, BG, and internal standards 3,4-methylenedioxy-N-methylamphetamine-d_5 (MDMA-d_5), amphetamine-d_5 and buprenorphine-d4, were obtained as stock solutions (1.0 mg/mL except BG that was 100 μg/mL) from Cerilliant Co. (Round Rock, TX, USA). Acetonitrile and methanol were of LC–MS grade (Thermo-Fisher Scientific, Loughborough, UK). Leucine enkephaline was obtained from Waters (Manchester, UK). All other chemicals were of analytical grade and ultra-pure water (>18 MΩ/cm) was used.

Randomly selected urine specimens were obtained from left-over and decoded patient samples sent to the laboratory for routine drug testing. Blank urine was collected from healthy volunteers. The urine specimens were stored at +4 °C until analysis (maximum storage time, 2 months) or at −20 °C if stored longer.

2.2. Instrumentation

The LC–TOFMS system consisted of a Waters Acquity UPLC (ultra-performance liquid chromatograph) with a vacuum degasser, binary pump, and sample manager at ambient temperature connected to Xevo quadropoule time-of-flight mass spectrometer with MassLynx™/Target Lynx™/ChromaLynx™ Software Version 4.1 (Waters Co., Milford, MA, USA). The electrospray (ES) interface was used with the instrument operating in the positive ion mode. Nitrogen was used as nebulizer, desolvation and cone gas, and argon as collision gas. The following conditions were used in the mass spectrometer: capillary voltage, 0.9 kV; sampling cone voltage, 20 V; extraction cone voltage, 3 V; source temperature, 130 °C; desolvation gas temperature, 650 °C; desolvation gas flow, 1200 L/h; cone gas flow, 50 L/h; collision energy, 6 eV. The micro-channel plate (MCP) detector was operated at 2250 V. Mass accuracy was maintained by a single lock mass with an exact mass of 556.2771 Da using a leucine enkephalin solution (500 pg/μL), infused post-column through a lock spray capillary at a flow rate of 50 μL/min and scanned every 10th second with a scan time of 0.5 s. The mass resolution specification was >10,000 at m/z 956. All data were evaluated in centroid mode. Mass spectrometric detection was performed using scan mode in the range m/z 100–1000 and scan rate was set at 0.08 s/scan with interscan delay at 0.025 s. Every second scan was performed in dynamic range enhancement (DRE) mode, giving a total cycle time of 0.2 s and 9–12 data points per peak. Sodium formate was used for daily calibration of the TOF instrument and was prepared from sodium hydroxide (0.1 mol/L) dissolved in a mixture of formic acid (10%), acetonitrile and water (50:50). Leucine enkephaline (3 mg) used as a lock mass was dissolved in formic acid (0.1%) and acetonitrile:water (50:50) to a concentration of 500 pg/μL.

The liquid chromatography system was operated in a gradient mode with a flow rate of 1000 μL/min. Chromatography was performed using a 1.8 μm 50 mm × 2.1 mm (inner diameter) high-strength silica tri-functional C_{18} (HSS T3) (Waters Co.) column, preceded by a 0.2 μm column filter (Waters Co.). Solvent A consisted of 0.1% (26.5 mmol/L) formic acid (pH 2.85) and solvent B was 100% acetonitrile. A linear gradient was programmed as following: 0.0 min 2% B; 0.2 min 3% B; 0.21 min 7% B; 0.75 min 10% B; 1.25 min 7% B; 2.5 min 55% B; 2.8 min 80% B; 3 min 95% B; 3.0–3.2 min 95% B; 3.21–4.0 min 2% B. The injection volume was 2 μL and the column oven temperature 60 °C. The total run time of the method was 4.0 min.

2.3. Analytical procedure

A 50 μL aliquot of each urine specimen was added to autosampler vials together with 200 μL of a working solution of internal standards containing 125 ng/mL of amphetamine-d_5 and MDMA-d_5. Working solution of both amphetamine-d_5 (used as internal standard for M3G, M6G, morphine, C6G, codeine, amphetamine and methamphetamine) and MDMA-d_5 (used as internal standard for buprenorphine and BG) internal standards was prepared in 0.1% formic acid and stored at +4 °C until use (maximum storage time, 2 months). The vials were capped, vortexed for ∼30 s, and loaded onto the sample manager.

Calibration curve covering 50–10,000 ng/mL for M3G, M6G, morphine, C6G, codeine, amphetamine and methamphetamine, and 5–1000 ng/mL for buprenorphine and BG were prepared by serial dilution of stock solutions with blank urine. Quality control (QC) samples were prepared from the different stock solutions at three concentrations levels: low – 90 ng/mL, middle – 300 ng/mL and high – 5000 ng/mL for M3G, M6G, morphine, C6G, codeine, amphetamine and methamphetamine. The concentrations were 10 times lower for buprenorphine and BG. Calibration samples and controls were stored at −20 °C until analysis (maximum storage time was 2 months) and the samples were kept at +4 °C for not more than 4 weeks.

Eight calibration levels (0, 50, 100, 500, 1500, 3000, 7500 and 10000 ng/mL for opiates, amphetamines and 10 times lower concentrations for buprenorphine and BG) were routinely used. Calculation of calibration curves was performed using linear regression excluding the zero level. The analytes concentrations of unknown samples were determined from the peak area ratio by reference to the calibration curve between analyte and its internal standard. QC samples were inserted after every 20th sample in a sample sequence.

ChromaLynx™ (Waters, Manchester, UK) was used for identification of analytes in samples with the following identification

Table 1
Mass spectrometric and chromatographic data.

Compound	Formula $[M+H]^+$	Exact mass (Da)	Resolution power	Retention time[a] (min)
Morphine-3-glucuronide	$C_{23}H_{28}NO_9$	462.1764	4835	0.32
Morphine-6-glucuronide	$C_{23}H_{28}NO_9$	462.1764	4835	0.46
Morphine	$C_{17}H_{20}NO_3$	286.1443	2993	0.47
Codeine-6-glucuronide	$C_{24}H_{30}NO_9$	476.1921	4981	0.68
Codeine	$C_{18}H_{22}NO_3$	300.1600	3140	0.75
Amphetamine	$C_9H_{14}N$	136.1126	1424	0.78
Methamphetamine	$C_{10}H_{16}N$	150.1283	1570	0.90
Buprenorphine-glucuronide	$C_{35}H_{50}NO_{10}$	644.3435	6740	1.98
Buprenorphine	$C_{29}H_{42}NO_4$	468.3114	4899	2.19

[a] The void volume eluted at 0.12 min.

criteria: absolute mass accuracy tolerance ±10 mDa and absolute retention time tolerance ±0.05 min. Intensity threshold value of the analytes protonated masses was set approximately to the level corresponding a signal-to-noise ratio of >10 for the less intense compound. The exact protonated molecule masses of nine analytes and their retention times are shown in Table 1. As target analysis was applied in this study a home-made database was created. The database included compound names, empiric formulas and retention times. Absolute retention times were taken from the calibration standards and updated for each batch.

2.4. Immunochemical screening method

Urine samples were analyzed for presence of opiates, amphetamines and buprenorphine using CEDIA reagents (Microgenics, Passau, Germany). Assays were performed on an Olympus AU 640 (Beckman, Sweden) according to the manufacturers instructions with cut-off limit of 300 ng/mL for opiates (calibrator – morphine), 500 ng/mL for amphetamines (calibrator – D-amphetamine) and 5 ng/mL for buprenorphine. Calibrated ranges were 0–2000 ng/mL for opiates, 0–5000 ng/mL for amphetamines and 0–50 ng/mL for buprenorphine. Detection limits were: 74 ng/mL for opiates, 41 ng/mL for amphetamines and 1.3 ng/mL for buprenorphine.

Quality controls were CEDIA Specialty Control Set (Microgenics, Passau, Germany). The inter-assay CV for opiates assay at the 225 and 375 ng/mL morphine controls levels were <6% (n = 100); for amphetamines assay at the 375 and 625 ng/mL D-amphetamine levels were <4.5% (n = 100) and for buprenorphine assay at the 2.1 and 6.5 ng/mL levels were <12.5% (n = 100).

2.5. LC–MS confirmation method for buprenorphine

Sample preparation involved hydrolysis of conjugates by addition of 50 μL β-glucuronidase (Helix Pomatia) to 1 mL urine sample aliquot followed by heating at 50 °C for 90 min. After incubation, 50 μL of internal standard (1 μg/mL buprenorphine-d_4) and 0.2 mL 1 M K_2CO_3 were added. Buprenorphine was then extracted with 4 mL of hexane/isopropanol (98:2) mixture. The organic phase was transferred to a new glass test-tube followed by addition of 0.25 mL of 50 mmol/L formic acid. After extraction the water phase was transferred to a 500 μL glass autosampler vial for analysis by LC–MS.

A volume of 5 μL was injected into an Agilent 1100 MSD LC–MS system (Agilent Technologies, CA, USA). The system was equipped with an ES interface, a dual LC pump, degasser, column thermostat and an autosampler. A 100 mm × 2.1 mm C18 HyPurity Aquastar column, particle size 3 μm (ThermoFisher Scientific) was used. The mobile phase was pumped at a flow of 300 μL/min and a linear binary gradient of 0–100% B was used, where A consisted of 20% acetonitrile in 25 mmol/L formic acid and B of 80% acetonitrile in 25 mmol/L formic acid. The instrument operated in the positive SIM mode with a fragmentor voltage of 70 V for m/z 468.3 (buprenorphine), m/z 472.4 (buprenorphine-d4) and 250 V for m/z 396.2 (buprenorphine fragment), m/z 165.2 (buprenorphine fragment), m/z 414.3 (buprenorphine-d4 fragment). The dwell time was 197 ms, drying gas flow rate 10 L/min, drying gas temperature 350 °C, and nebulizer gas pressure was 25 psi (172 kPa).

The measuring range of the LC–MS method was 2–1000 ng/mL buprenorphine and the intra- and inter-assay coefficients of variation (CV) were <12% at levels of 10 ng/mL (n = 15) and of 100 ng/mL (n = 15), respectively. The limit of detection (LOD) of the method was 0.3 ng/mL (signal-to-noise ratio of 3). This method was in routine use and approved by SWEDAC (www.swedac.se) for accreditation according to ISO 17025 and by CAP (College of American Pathologists, www.cap.org) for FUDT. The applied reporting limit was 5 ng/mL of buprenorphine.

2.6. LC–MS/MS confirmation method for opiates and amphetamines

The LC–MS/MS system consisted of a Waters Acquity UPLC (ultra-performance liquid chromatograph) with a vacuum degasser, binary pump, and sample manager at ambient temperature connected to a Quattro Premier XE tandem mass spectrometer with MassLynx™/Target Lynx™ Software Version 4.1 (Waters Co., Milford, MA, USA). The liquid chromatography system was operated with a flow rate of 200 μL/min using mobile phase 0.1% (26.5 mmol/L) formic acid and a gradient of acetonitrile for amphetamines and methanol for opiates as organic modifiers. Chromatography was performed using a 1.8 μm 100 mm × 2.1 mm (inner diameter) high strength silica (HSS) C_{18} column (Waters Co.), preceded by a 0.2 μm column filter (Waters Co.) for opiates and a 1.7 μm 100 mm × 1.0 mm (inner diameter) bridged ethylene hybrid BEH C_{18} column (Waters Co.) preceded by a 0.2 μm column filter (Waters Co.) for amphetamines. Sample preparation involved urine sample dilution with deuterium labelled internal standards: 1:5 for opiates and 1:10 for amphetamines. The injection volume was 2 μL for opiates and 1 μL for amphetamines. The column oven temperature was 60 °C and the total run time of the methods was 4.5 min.

The ES interface was used with the instrument operating in the positive ion mode. Nitrogen was used as nebulizer, desolvation and cone gas, and argon as collision gas. Data was acquired in selected reaction monitoring using two product ions per compound. The following transitions were monitored: m/z 462.2/286.2, m/z 462.2/201.1 for both M3G and M6G; m/z 286.3/201, m/z 286.3/165 for morphine; m/z 476.3/300.3, m/z 476.3/282.3 for C6G; m/z 300.3/215.0, m/z 300.3/165.0 for codeine; m/z 135.9/119.0, m/z 135.9/91.0 for amphetamine and m/z 150.0/119.0, m/z 150.0/91.0 for methamphetamine. Linearity range for amphetamines and opiate methods ranged from 150 to 50,000 ng/mL. The limit of detection (signal-to-noise ratio of 3) was 0.9 ng/mL for M3G, 1.3 ng/mL for M6G, 1.2 ng/mL for morphine, 0.4 ng/mL for C6G, 1.5 ng/mL for codeine, 10 ng/mL for amphetamine and 5 ng/mL

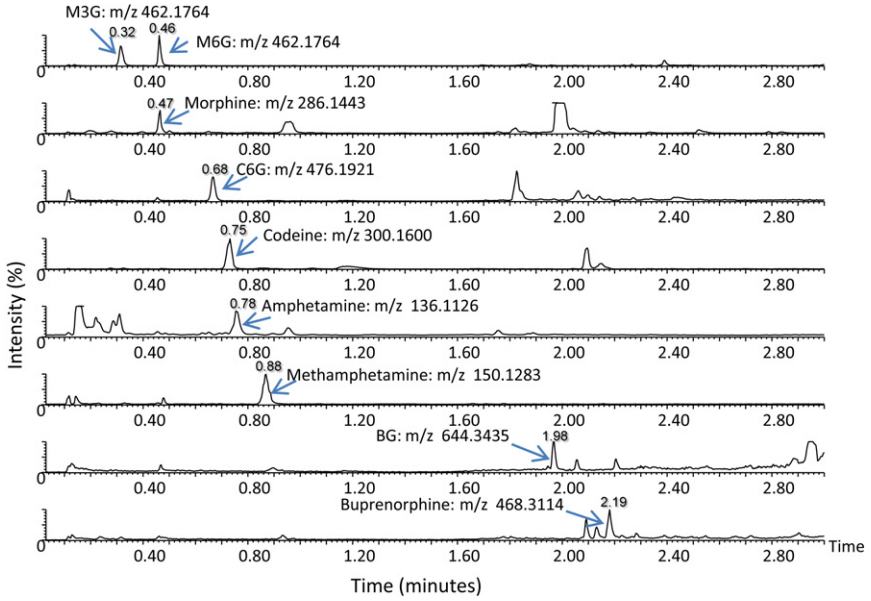

Fig. 1. LC–TOF chromatograms showing the monitoring of the target analytes in a urine calibrator sample. The concentrations were 50 ng/mL for buprenorphine and BG, and 500 ng/mL for the other. The data was evaluated in the centroid mode using a 10 mDa mass tolerance.

for methamphetamine. The intra- and inter-assay coefficients of variation (CV) for amphetamines were <5% at levels of 300 ng/mL (n = 16) and of 8000 ng/mL (n = 16), respectively. The intra- and inter-assay coefficients of variation (CV) for opiates were <7% at levels of 200 ng/mL (n = 43) and of 30,000 ng/mL (n = 43), respectively. These methods were in routine use and approved by SWEDAC (www.swedac.se) for accreditation according to ISO 17025 and by CAP (College of American Pathologists, www.cap.org) for FUDT. The applied reporting limit for the present study was 100 ng/mL for all compounds.

2.7. Validation of LC–TOFMS method

The linearity was studied for each analyte by generating 10 calibration curves. The limit of detection (LOD, S/N = 3) and limit of quantification (LOQ, S/N = 10) were determined from four standard samples at four different concentrations (125 ng/mL, 62.5 ng/mL, 31.25 ng/mL and 15.6 ng/mL for amphetamines and opiates, and at 10 times lower concentrations for buprenorphines). Precision as well as accuracy of the method were determined from quality control samples at three levels (low: 90 ng/mL; middle: 300 ng/mL; high: 5000 ng/mL for amphetamines and opiates, and 10 times less in concentration for buprenorphines) in triplicates at 5 different occasions. The test of selectivity was performed by using urine samples from 10 healthy volunteers and controlled for the nine studied analytes. The same 10 blank urines were fortified (5000 ng/mL) with 19 other most common drugs of abuse for selectivity study. In addition, the selectivity of the LC–TOFMS method was ensured of co-eluted M6G and morphine at different concentration levels.

The robustness of the method has been carried out by preparing new mobile phases and using a column of another batch. Carry-over was studied for samples at concentration 50 μg/mL followed by four negative urine samples. Matrix effect was studied by post-column infusion of 1000 ng/mL solution of M3G and M6G with a flow rate of 20 μL/min and simultaneous injection of a diluted blank urine.

2.8. Experimental work on study samples

A total of 812 unknown patient samples were analyzed with the immunochemical screening method. All samples were investigated for amphetamines, 796 for buprenorphine and 779 for opiates. All 812 urine samples were also screened with the LC–TOFMS method. Positive samples obtained with any of the two screening methods were also analyzed by the confirmation methods. Samples found to be negative in both immunochemical and LC–TOFMS screenings were not further investigated. Evaluation of LC–TOFMS data was performed using 100 ng/mL reporting limits for amphetamines and opiates, and 5 ng/mL for buprenorphines.

3. Results

3.1. LC–TOF method design

A chromatographic method was developed with a gradient that was suited for analysis of a large number of analytes covering a polarity span from gamma-hydroxybutyric acid to tetrahydrocannabinol carboxylic acid. Due to the large number of samples analyzed only three classes of drugs (amphetamines, opiates and buprenorphine) were chosen for full evaluation of the LC–TOFMS screening method. Total analysis time of the developed method for separation of the nine analytes (M3G, M6G, morphine, C6G, codeine, amphetamine, methamphetamine, buprenorphine and BG) was 4 min. Elution time ranged from 0.32 min for the earliest (M3G) to 2.19 min for the last eluting compound (buprenorphine), with the void eluting at 0.12 min. M6G (0.46 min) and morphine (0.47 min) were not chromatographically separated. The conversion of M6G to morphine in the ion source was <0.02%. The number of data points per peak was about 12. Table 1 shows exact masses and retention times of analytes, while chromatographic performance of the nine analytes is presented in Fig. 1. Columns can be used for over 3000 injections using this design.

Identification of the analytes in urine using the LC–TOFMS method was based on two criteria: accurate mass of the monoisotopic species and correct retention time. A key task for unambiguous identification was definition of accurate mass error tolerance. Evaluation of standard samples showed that mass accuracy in the range of ±10 mDa and retention time tolerance of ±0.05 min gave no false results. Peaks with mass accuracy range between 10 and 20 mDa and the correct retention time (±0.05 min) were reported as tentative by the software to call for manual reviewing. Stable retention times of analytes in the

Table 2
Validation data for the LC–TOFMS method.

Substance	Target conc. (ng/mL)	Observed conc. (ng/mL)	Intraday assay RSD (%)	Interday assay RSD (%)	LOD (ng/mL) (RSD)	LOQ (ng/mL) (RSD)	Linearity range (ng/mL)	Mean r	RSD[a] for r[b] (%)	n
Morphine-3-glucuronide	90	105	3.0	2.9	6.7 (1.2%)	22 (4.0%)	50–10,000	0.9930	0.60	10
	300	284	7.4	3.6						
	5000	4908	5.3	5.3						
Morphine-6-glucuronide	90	100	3.5	7.4	<6	<16	50–10,000	0.9946	0.40	10
	300	314	2.6	8	(0.60%)	(2.2%)				
	5000	5268	7.7	5.3						
Morphine	90	98	3.5	3.2	<6 (0.85%)	17 (2.9%)	50–10,000	0.9959	0.39	10
	300	341	6.3	2.5						
	5000	5334	7.9	6						
Codeine-6-glucuronide	90	106	2.3	9.5	9.4 (1.4%)	31 (4.7%)	50–10,000	0.9941	0.51	10
	300	292	6.0	4.8						
	5000	5145	3.5	5.4						
Codeine	90	92	5.3	8.5	6.1 (1.2%)	20 (3.7%)	50–10,000	0.9974	0.20	10
	300	352	6.9	4.6						
	5000	4537	6.9	3.7						
Amphetamine	90	94	2.4	3.1	12 (1.6%)	41 (5.6%)	50–10,000	0.9985	0.19	10
	300	340	5.4	3.4						
	5000	5552	5.9	2.6						
Methamphetamine	90	93	1.9	3.3	<6 (0.10%)	<16 (0.32%)	50–10,000	0.9915	0.86	10
	300	321	2.5	3.8						
	5000	5707	5.1	3.6						
Buprenorphine-glucuronide	9	10	12	14	2.9 (9.5%)	9.4 (7.1%)	5–1000	0.9920	0.66	10
	30	35	13	8.0						
	500	613	20	4.9						
Buprenorphine	9	10	15	16	2.8 (13%)	9.0 (8.2%)	5–1000	0.9938	0.50	10
	30	36	2.9	5.9						
	500	617	7.5	8.7						

[a] Relative standard deviation.
[b] Correlation coefficient.

chromatographic system are essential for proper identification. In this study the relative standard deviation (RSD) of the retention times (t_r) was <1% for all analytes. Reduction of the retention time window to ±0.02 min was tested but samples with high concentrations of amphetamine showed that the peak could become outside the criteria (±0.02 min) due to overloading, but were still within ±0.05 min.

3.2. Validation of the LC–TOFMS method

The developed method showed linear response within the measuring ranges for all analytes. The mean coefficient of correlation (r) varied from 0.97 to 0.99 as determined from 10 calibration curves for all of the nine analytes (Table 2).

The intra- and interday precision in quantification was studied at three levels and expressed in terms of relative standard deviation (RSD). The precision was <10% for M3G, M6G, morphine, C6G, codeine, amphetamine, methamphetamine and ≤20% for buprenorphine and BG (Table 2). The accuracy of the method ranged between 90.7% and 123% for the different analytes and concentration levels. The accuracy was acceptable for opiates and amphetamines but outside acceptance criteria for buprenorphines at the highest level (500 ng/mL).

Limit of detection (LOD, S/N = 3) varied between 0.85 and 9.4 ng/mL and limit of quantification (LOQ, S/N = 10) varied between 2.8 and 41 ng/mL depending on the analyte. The results are presented in Table 2.

Carry-over was tested for all analytes and the results showed a carry-over degree of 0.021% for buprenorphine and BG, 0.023% for morphine and <0.01% for M3G, M6G, C6G, codeine, amphetamine and methamphetamine. Therefore positive findings following a sample with a high concentration was routinely re-injected in order to reveal erroneous results.

Selectivity was ensured since no interfering peaks of endogenous substances were detected at the retention times of the analytes in 10 blank urine samples and in urine samples after addition of other drugs of abuse with exact masses varying between 106.1216 and 344.2226 Da. The selectivity of the developed method was further studied regarding the co-eluting morphine and M6G at different concentrations. The identification based on accurate mass was not being affected.

Robustness of the method was tested regarding new mobile phase and chromatographic column. The results showed that retention times were stable with RSD <1% when replacing mobile phase. Small variations in retention times appeared when the column was replaced.

The matrix effect was apparent directly after elution of the void volume and gave a 99% suppression of signal. The suppression recovered quickly and was 10% at the elution of the first eluting analyte M3G.

3.3. Method comparison

The LC–TOFMS method showed a higher positive rate for amphetamines but somewhat lower for opiates and buprenorphine as compared with the immunoassay screening (Table 3). For all three classes the rate of false positive results was lower for the LC–TOFMS method (Table 3). The rate of false negative results was lower for LC–TOFMS. The rate of agreement between methods ranged from 94.2% to 97.4% (Table 3).

The comparison of LC–TOFMS results with the confirmation method is presented in Table 4 for all analytes. Both false positive and negative results occurred but differed between analytes. False positive results were predominantly due to presence of interfering peaks at low concentration levels, but in one instance for codeine

Table 3
Comparison of performance for CEDIA immunoassay and LC–TOF screening.

	Amphetamines (n = 812)		Opiates (n = 779)		Buprenorphines (n = 796)	
	CEDIA	LC–TOF	CEDIA	LC–TOF	CEDIA	LC–TOF
Positive rate	26.8%	28.4%	26.6%	26.1%	28.1%	27.3%
False positive rate	7.3%	0%[a]	5.8%	0%[b]	6.7%	3.2%
False negative rate	4.4%	1.8%	12.9%	0.6%	0.5%	0%
Agreement	94.2%		97.4%		97.4%	

[a] All samples contained amphetamine.
[b] All samples contained M3G and/or CG.

at a high level (5600 ng/mL). False negative results were mainly due to deviation from mass accuracy criteria (±20 mDa) but also because of shift in retention time outside the acceptance criteria (±0.05 min). In one case both of this occurred in a sample with a high concentration (>50,000 ng/mL of amphetamine) causing a false negative result. Example chromatograms from authentic patient samples are shown in Fig. 2a–c.

4. Discussion

The aim of this study was to investigate if the LC–TOFMS technology could have a potential for routine urine drug testing, either as a complement or as replacement of the immunochemical screening. The LC–TOFMS method was therefore designed for allowing a future high-capacity and multi-target screening application covering a broad spectrum of analytes. The total analysis time of this method of 4 min corresponds to a maximal capacity of 360 injections per 24 h on one instrument. Assuming that 250 unknowns is possible to run in this time period this approach may well meet economical requirements for routine application for urine drug testing laboratories. The results obtained were supporting the potential of LC–TOFMS for urine drug testing and demonstrated that the LC–TOFMS screening method performed even better than the immunochemical assay (Table 3). The LC–TOFMS method proved to be more selective and gave lower frequency of false positive results. This may be an important feature since unconfirmed positive results are being used clinically in many instances. In addition, mainly due to lower reporting limit a higher rate of true positives was obtained for amphetamines. Also the observed rate of false negatives was favourable for the LC–TOFMS method (Table 3).

As compared with the confirmation methods the LC–TOFMS gave both false positive and negative results (Table 4). False negative results were due to presence of a peak but with accurate mass assignment outside acceptance criteria. This occurred mainly at concentrations close to the reporting limit. Since data were evaluated in centroid mode this might have occurred as a result of an interfering compound being close in exact mass. Another drawback of concern is detector saturation in samples with high concentrations. Detector saturation may result in false negative results due to incorrect assignment of both retention time and accurate mass. Although only one such example was observed in this study this requires special attention when designing instruments for drug screening application since high concentrated samples are frequent and must not be reported false negative. False positive results were mainly caused by an interfering peak at a low level. These problems might be solved simply by increasing mass resolution power since that will increase selectivity and reduce interference from matrix compounds. More new TOFMS instruments already available have much increased resolution power as compared to instrument used in this study [11]. A need for resolving compounds 3 mDa (3 ppm) close in exact mass has been proposed to be needed based on theoretical considerations [9]. This resolution power is now available (corresponds to 45,000 at m/z 956) but was not obtained from the instrument used in the present study (10,000).

One reason for the need to find new screening techniques alternative to immunoassays is that in recent years the use of “new” psychoactive substances (“designer drugs, legal highs, etc.”) has spread as a consequence of internet trading [12]. These new compounds are easily available as they are not yet regulated as narcotics and are most often not detected in immunoassay drug screening. The advent of the product “Spice” is a good example of this phenomenon. This product rapidly became popular among drug users and was sold on internet as herbal marijuana alternatives. Following the identification of chemicals in “Spice” products being synthetic cannabinoid receptor agonists, and classification of these as narcotics the producers were able to simply switch to use other analog substances not yet regulated [13]. The detection of these substances in body fluids requires mass spectrometry methods [14]. In addition, several new therapeutic drugs for pain treatment, sedatives, hypnotics and antiepileptics have become of interest for urine drug testing.

The use of TOFMS for screening applications in the clinical and forensic toxicology field has gained increasing attention in recent years [3,6,9,11,15]. The potential and application of accurate mass measurement in this field was originally proposed as a solution for identification of analytes for which reference substance was lacking [16]. By obtaining elemental formula specific data on unknowns search in databases would provide valuable information in the identification of unknowns. Additional strategies to

Table 4
Performance of the LC–TOF screening compared with confirmation results.

	False positives		Detected false negatives
	Relative rate	Number of samples	Number of samples
M3G	0%	0	0
M6G	6.6%	8	6
Morphine	4.8%	3	1
CG	0%	0	2
Codeine	2.9%	2	1
Amphetamine	0%	0	1
Methamphetamine	8.0%	7	1
Buprenorphine	1.8%	4	0
BG	2.3%	5	0

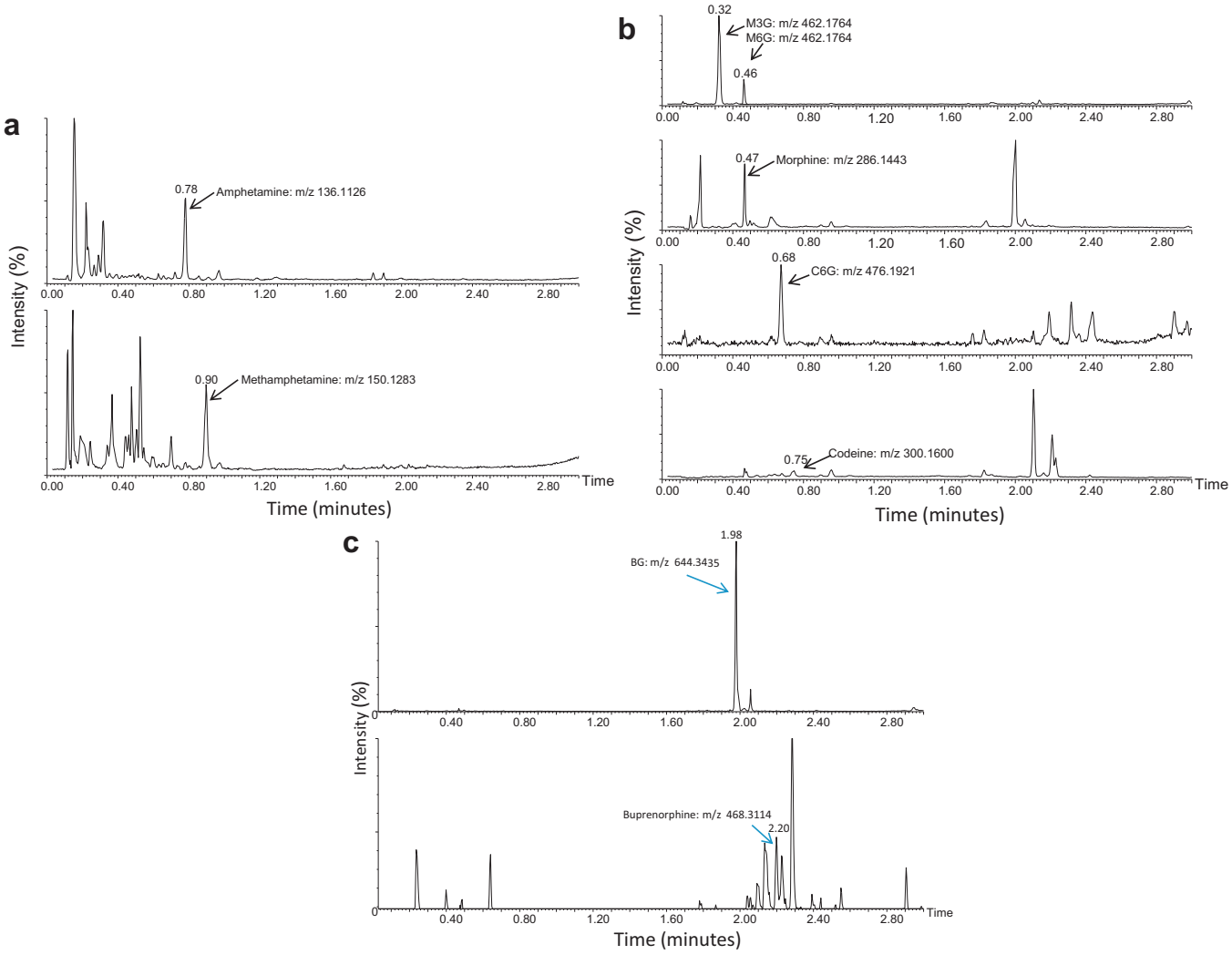

Fig. 2. Chromatograms from the analysis of three patient samples containing (a) amphetamine (535 ng/mL) and methamphetamine (689 ng/mL), (b) morphine (3600 ng/mL), M3G (28700 ng/mL), M6G (6700 ng/mL), codeine (300 ng/mL), and C6G (2040 ng/mL), and (c) buprenorphine (43 ng/mL) and BG (1720 ng/mL). The data was evaluated in the centroid mode using a 10 mDa mass tolerance. Note that for each extracted chromatogram normalization was done on the largest peak present. Data for the whole analysis time is shown to allow a presentation of the selectivity achieved.

aid identification has involved search for potential metabolites in specially designed database [4], use of fragmentation data [3] and MS/MS data [12]. Most work has been directed more toward secure identification in clinical and forensic toxicology rather than high-capacity clinical applications [15]. Consequently, sample preparation by liquid/liquid extraction or solid phase extraction, and chromatography with long total analysis times have often been used [3,6,12]. In LC–MS/MS applications direct injection of urine after dilution, with or without enzymatic hydrolysis, has been shown to be possible [15]. The present study combined the concept of direct injection of urine, rapid high efficient chromatography and the selective detection of TOFMS for finding a more universal, sensitive and selective analytical solution for urine drug screening. This new approach emphasized rapid chromatography, sensitivity and simple (possibly automated) decision making in the method design. Although both false positive and negative results were produced they were of lower prevalence as compared with immunochemical screening. A low rate of false positives is acceptable as long as positives are being subjected to confirmation. It has been shown that use of LC–TOF may produce safe confirmation when including a fragment ion and criteria for relative ratio [17].

In conclusion, the potential of using LC–TOFMS as a complementary and alternative technology for screening in urine drug testing has been demonstrated. However, it should be pointed out that both sensitivity and mass resolution power [18] still needs improvement. The potential contribution of LC–TOFMS is to provide an analytical platform that will be able to include all needed analytes at selected reporting limits. Another important future development is to provide software for automated data analysis and user friendly reporting. In this study a great deal of manual reviewing of data was needed.

References

[1] H.H. Maurer, Ther. Drug Monit. 32 (2010) 324.
[2] J.J. Pitt, Clin. Biochem. Rev. 30 (2009) 19.
[3] H.K. Lee, C.S. Ho, Y.P.H. Iu, P.S.J. Lai, C.C. Shek, Y.C. Lo, H.B. Klinke, M. Wood, Anal. Chim. Acta 649 (2009) 80.
[4] A. Polettini, R. Gottardo, J.P. Pascali, F. Tagliaro, Anal. Chem. 80 (2008) 3050.
[5] J.O. Thörngren, F. Östervall, M. Garle, J. Mass Spectrom. 43 (2008) 980.
[6] M. Kolmonen, A. Leinonen, A. Pelander, I. Ojanpera, Anal. Chim. Acta 585 (2007) 94.
[7] H.K. Nordgren, O. Beck, Ther. Drug Monitor. 26 (2004) 90.
[8] J.C. Eichhorst, M.L. Etter, N. Rousseaux, D.C. Lehotay, Clin. Biochem. 42 (2009) 1531.
[9] S. Broecker, S. Herre, B. Wüst, J. Zweigenbaum, F. Pragst, Anal. Bioanal. Chem. 400 (2011) 101.
[10] M.E. Touber, M.C. van Engelen, C. Georgakopoulus, J.A. van Rhijn, M.W.F. Nielen, Anal. Chim. Acta 586 (2007) 137.

[11] I. Ojanpera, M. Kolmonen, A. Pelander, Anal. Bioanal. Chem. 403 (2012) 1203.
[12] F.I. Carroll, A.H. Lewin, W. Mascarella, H.H. Seltzman, P.A. Reddy, Ann. N.Y. Acad. Sci. 1248 (2012) 18.
[13] C.D. Rosenbaum, S.P. Carreiro, K.M. Babu, J. Med. Toxicol. 8 (2012) 15.
[14] H.A. Spiller, M.L. Ryan, R.G. Weston, J. Jansen, Clin. Toxicol. 49 (2011) 499.
[15] F.T. Peters, Clin. Biochem. 44 (2011) 54.
[16] I. Ojanpera, A. Pelander, S. Laks, M. Gergov, E. Vuori, M. Witt, J. Anal. Toxicol. 29 (2005) 34.
[17] M.H. Blokland, P.W. Zoontjes, S.S. Sterk, R.W. Stephany, J. Zweigenbaum, L.A. van Ginkel, Anal. Chim. Acta 618 (2008) 86.
[18] A. Kaufmann, P. Butcher, Rapid Commun. Mass Spectrom. 20 (2006) 3566.