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Journal of Chromatography B, 861 (2008) 22–28
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Direct injection LC–MS/MS method for identification and quantification of amphetamine, methamphetamine, 3,4-methylenedioxyamphetamine and 3,4-methylenedioxymethamphetamine in urine drug testing

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Received 8 July 2007; accepted 21 November 2007

Abstract

A method based on direct injection of diluted urine for the identification and quantification of amphetamine, methamphetamine, 3,4-methylenedioxymetamphetamine and 3,4-methylenedioxyamphetamine in human urine by electrospray ionisation liquid chromatography-tandem mass spectrometry was validated for use as a confirmation procedure in urine drug testing. Two deuterium labelled analogues, amphetamine-D5 and 3,4-methylenedioxymetamphetamine-D5, were used as internal standards. Twenty microliter aliquots of urine were mixed with 80 μL internal standard solution in autosampler vials and 10 μL was injected. The chromatographic system consisted of a 2.0 mm × 100 mm C18 column and the gradient elution buffers used acetonitrile and 25 mmol/L formic acid. Two product ions produced from the protonated molecules were monitored in the selected reaction monitoring mode. The intra- and inter-assay variability (coefficient of variation) was between 5 and 16% for all analytes at 200 and 6000 ng/mL levels. Ion suppression occurred early after injection but did not affect the identification and quantification of the analytes in authentic urine samples. The method was further validated by comparison with a reference gas chromatographic–mass spectrometric method using 479 authentic urine samples. The two methods agreed almost completely (99.8%) regarding identified analytes when applying a 150 ng/mL reporting limit. Four deviating results were observed for 3,4-methylenedioxymethamphetamine and this was due to uncertainty in quantification around the reporting limit. For the quantitative results the slope of the regression lines were between 0.9769 and 1.0146, with correlation coefficients >0.9339. We conclude that the presented liquid chromatographic-tandem mass spectrometric method is robust and reliable, and suitable for use as a confirmation method in urine drug testing for amphetamines.
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Keywords: Urine drug testing; Amphetamine; Methamphetamine; 3,4-Methylenedioxymethamphetamine; 3,4-Methylenedioxyamphetamine; LC–MS/MS

1. Introduction

The liquid chromatography–mass spectrometry (LC–MS) technique has great potential in urine drug testing by offering methods capable of replacing existing gas chromatographic–mass spectrometric (GC–MS) methods used for confirmation of preliminary positive results from the immunochemical screening [1–3]. The main benefits offered are a simplified sample preparation and direct measurement of hydrophilic analytes eliminating hydrolysis and derivatization procedures. This may potentially lead to shorter turn-around times and increased robustness in routine applications.

The usefulness of LC–MS for analysis of illicit drugs including the amphetamines in urine was first demonstrated using a combination of solid-phase extraction and thermospray ionisation [4]. With the advent of increased selectivity when using LC–MS/MS the possibility arise to omit the sample preparation step and simply use direct injection of urine and this was proposed for cocaine and benzoylecgonine using electrospray LC–MS/MS [5]. However, the proposed method was not validated using authentic urine samples and in a later published method the same authors included on-line solid-phase extraction to minimize influences from matrix [6]. More recently the usefulness of direct injection of urine in combination with LC–MS/MS has been demonstrated more convincingly for opiates [7,8]. The robustness of this was confirmed by using method

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doi:10.1016/j.jchromb.2007.11.025

comparison with a reference GC–MS method in a large number of authentic urine samples [9].

In addition, to confirmation in urine drug testing the LC–MS/MS technique can also be used for multi-component screening purposes [3,10]. Work on multi-component screening of amphetamine related substances have shown that direct injection of urine is viable with regard to robustness issues [11]. In this work the confirmation of positive results was performed by including solid-phase extraction. The procedure used for screening [11] was adopted for use as confirmation method in a study comparing different immunoassays for the amphetamine class of compounds [12]. However, no validation data of the confirmation application was presented [12]. Other applications of LC–MS/MS for confirmation of amphetamines include sample pre-treatment with on-line and off-line solid-phase extraction, and liquid–liquid extraction [13–15].

The aim of the present study was to investigate if direct injection of urine in combination with electrospray LC–MS/MS is a possible and robust approach for confirmation of the amphetamine class of substances, i.e. amphetamine (AMPH), methamphetamine (MetAMPH), 3,4-methylenedioxymethamphetamine (MDMA) and 3,4-methylenedioxyamphetamine (MDA), by comparison with an established GC–MS method using authentic patient urine samples.

2. Material and methods

2.1. Chemicals

AMPH, cathinone HCl, 2-methylamino-1-(3,4-methylenedioxyphenyl)butane HCl (MBDB), MDA, MDMA, 3,4-methylenedioxyethylamphetamine (MDEA), MetAMPH, methcathinone HCl, methylphenidate HCl, amphetamine-D5, MDMA-D5 were obtained from LGC Promochem (Teddington, UK). Beta-methylphenylethylamine (MPEA) was from ElSohly Lab. Inc. (Oxford, MS, USA). Benzylpiperazine, ephedrine HCl, fenfluramine HCl, phenylethylamine HCl, phentermine HCl, were obtained from Sigma–Aldrich (St Louis, MO, USA). Phenmetrazine HCl and ritalinic acid was from USP (USA). Acetonitrile (HPLC purity) was from J.T. Baker (Deventer, Holland); formic acid (pro analysis quality) and ammonium acetate (HPLC purity) were from Merck (Darmstadt, Germany); propylchlorofomate (98%) was from Sigma–Aldrich. Ultra-pure water was used.

2.2. Urine samples

Anonymous surplus urine samples were randomly selected from the routine flow of clinical samples sent to the laboratory for the analysis of amphetamines. The screening were preformed on a Hitachi 911 (Roche, Mannheim, Germany) using CEDIA reagents (Microgenics, Passau, Germany), according to the manufactures instructions with a cutoff of 500 ng/mL for D-AMPH (calibrator). Screening was performed the day of sample reception and confirmation was performed within 3 days after sample reception and thereafter stored in a refrigerator (4–8 °C) for 1 month.

2.3. LC–MS/MS method

The samples were prepared for analysis by fivefold dilution with ultra-pure water containing 1000 ng/mL of internal standards (AMPH-D5, MDMA-D5) using the following procedure: 20 μL urine and 80 μL internal standard solution were mixed by vortexing the capped vial for about 10 s.

The LC system consisted of a vacuum degasser, a series 200 autosampler, a series 200 quaternary pump from PerkinElmer (Norwalk, CT, USA) and a column oven (Kontron, Zűrich, Switzerland). The analytical column used was a Luna (Phenomenex; Torrance, CA, USA) C18 column (100 mm × 2.0 mm, 3 μm). It was kept at 40 °C and combined with a guard column (10 mm × 2.1 mm). A gradient elution using two solvents, A and B, were used. Solvent A consisted of 25 mM formic acid containing 1% acetonitrile. Solvent B consisted of 25 mM formic acid containing 90% acetonitrile. The gradient was started with 9% of solvent B that was increased to 20% in 4 min, and then further increased to 100% in 3 min. The column was equilibrated at 9% B for 5 min. The mobile phase flow was set to 0.3 mL/min. An aliquot of 10 μL of the prepared extract was injected.

The MS used was a triple quadrupole instrument (Sciex API 2000 from Applied Biosystems, MDS Sciex, Concord, Ontario, Canada) connected to the LC-system via an electrospray ionisation interface (ESI) operating in positive mode using selected reaction monitoring (SRM). The following conditions were used; capillary voltage 4500 V; curtain gas pressure 10 psi; collision gas pressure was 9 psi; nebulizer gas pressure 10 psi, auxiliary gas pressure and temperature were 40 psi and 400 °C, respectively. The acquisition parameters are shown in Table 1.

Quantification was performed using calibration graphs constructed from peak area ratios between target SRM transition for each analyte and the respective internal standard (Table 1). Calibrators were prepared in blank urine from stock solutions in methanol or acetonitrile in 8 levels ranging from 0 to 10,000 ng/mL for the analytes. Controls were prepared in the same manner at two levels (200 and 6000 ng/mL) for each analyte. Prepared calibrators and controls were stored at −20 °C. Identification of an analyte in unknown sample was based on the correct relative retention time of both ions of the analyte and the internal standard (±0.5%) and the relative intensity between the qualifier and target SRM transitions ±20% of expected value.

2.4. GC–MS method

The analytical procedure was a modification of the method published by Meatherall [16]. Aliquots of urine (2.0 mL) were pipetted into glass test-tubes containing 150 μL of internal standard solution (20 μg/mL of MPEA in water) and 0.7 mL of 100 mmol/L of sodium phosphate buffer, pH 6.0. Extracts were prepared by automated solid-phase extraction (SPE) using an Gilson ASPEC XL4 instrument (Gilson Inc., Middleton, WI, USA). The SPE cartridges (30 mg/3 mL SPEC-VC-MP-1 obtained from ANSYS (ANSYS Techn. Inc., Lake Forest,

Table 1
Transitions and acquisition parameters used in the SRM mode of the LC–MS/MS method

Analyte	Mol. weight	Target SRM transition (*m/z*)	Qualifier SRM transition (*m/z*)	Ion ratio (%)	Dwell time (s)	Acq. Time (min)
AMPH	135.2	136.1 → 119	136.1 → 91	105	0.17	0–8.0
MetAMPH	149.2	150.1 → 91	150.1 → 119	98	0.17	
AMPH-D5	140.2	141.2 → 124			0.17	
MDMA	193.2	194.1 → 105	194.1 → 163	97	0.17	
MDA	179.2	180.2 → 163	180.2 → 105	98	0.17	
MDMA-D5	198.2	199.2 → 165			0.17	

CA, USA) were activated by rinsing consecutively with 0.5 mL methanol and 0.5 mL of 100 mmol/L of sodium phosphate buffer, pH 6.0. Following application of the urine extract the cartridges were washed with 0.5 mL of 1.0 mol/L of acetic acid and 0.5 mL of methanol, consecutively. Finally, the cartridges were eluted with 0.3 mL of freshly prepared methylenechloride/isopropanol (80:20, v/v) with 2% of 25% ammonia. The eluates were mixed with 100 μL of 1.0 mol/L potassium carbonate buffer, pH 10.8, and 100 μL of freshly prepared 10% propylchloroformate in chloroform. The extracts were vortex-mixed for 2 min and centrifuged for 5 min. Aliquots of 75 μL of the organic (lower) phase were transferred to autosampler glass-vials.

The instrument used was an Agilent 6890N/5973 GC–MS with a 7683 autosampler. Split injection (1:20) was performed using 1 μL injection volume with injector temperature at 200 °C. The chromatographic column was a DB-1701 (30 m × 0.25 mm × 0.25 μm film thickness). The oven temperature started at 150 °C and was increased to 192 °C at a rate of 5 °C/min. Thereafter, the temperature was increased to 260 °C at a rate of 30 °C/min and held at that temperature for 2 min; total time was 12.7 min. Electron ionisation (70 eV) was used and ions (*m/z*) monitored in the SIM mode were (target, qualifier 1, qualifier 2): 130.0, 44.0, 162.0 for AMPH; 144.0, 58.0, 102.0 for MetAMPH; 144.0, 102.0, 279.0 for MDMA; 135.0, 162.0, 265.0 for MDA. The LOD:s were 0.6 ng/mL for AMPH, 0.2 ng/mL MetAMPH and 1.2 ng/mL MDMA, and the inter-assay CV:s (coefficient of variation) were below 8%. For MDA the LOD was 0.9 ng/mL and the intra-day CV:s below 8% at the 150 and 4000 ng/mL levels. This method was in routine use and approved by SWEDAC for accreditation according to ISO 17025 and by CAP for FUDT. The applied reporting limit was 150 ng/mL. Calibration was achieved using prepared urine matrix standards at 0, 200, 1500, 3000 ng/mL. Quality control (QC) samples at 150 and 4000 ng/mL were used.

3. Results

Chromatograms obtained from analysis of urine calibrator are shown in Fig. 1. The capacity factor (k') was 2.8 for AMPH, 3.1 for MetAMPH, 3.2 for MDA and 3.5 for MDMA. The analytes were separated from each other and eluted as symmetrical peaks. In blank urine no interfering background peaks were observed (Fig. 2). Representative chromatograms from one

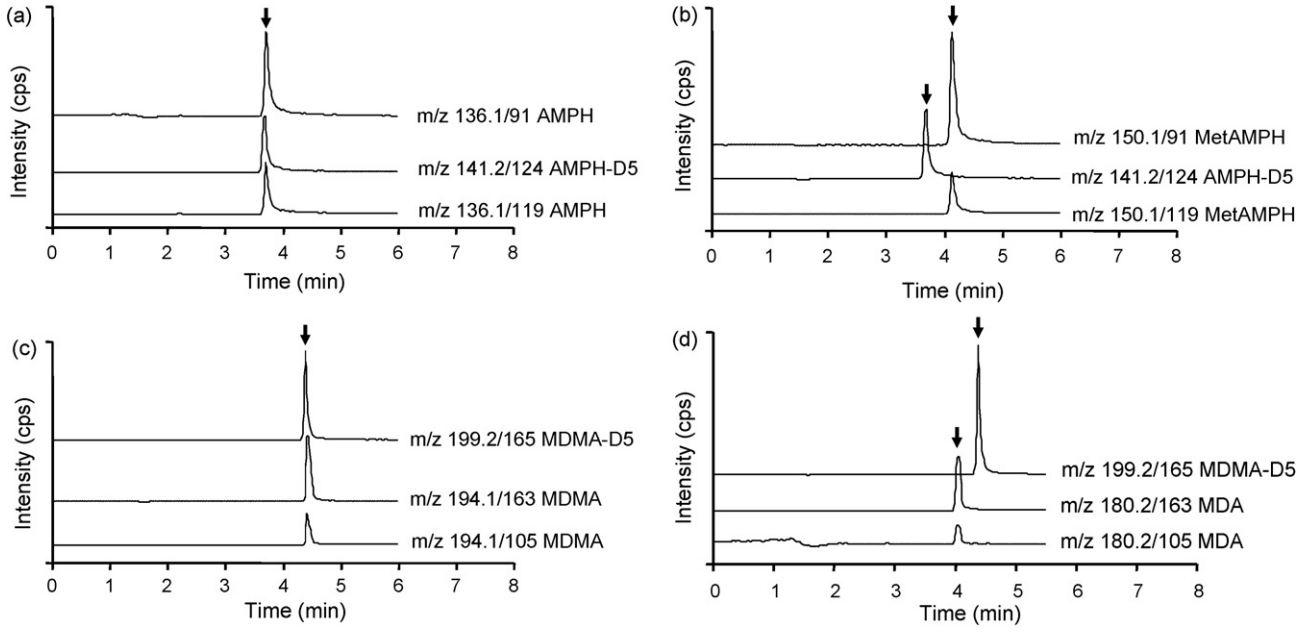

Fig. 1. Chromatograms obtained from the electrospray LC–MS/MS analysis of a urine matrix calibrator containing 4000 ng/mL each of AMPH, MetAMPH, MDMA, MDA and internal standards. Each analyte (retention time indicated with arrow) was monitored at two channels in the positive selected reaction monitoring mode; (a) AMPH, (b) MetAMPH, (c) MDMA, (d) MDA.

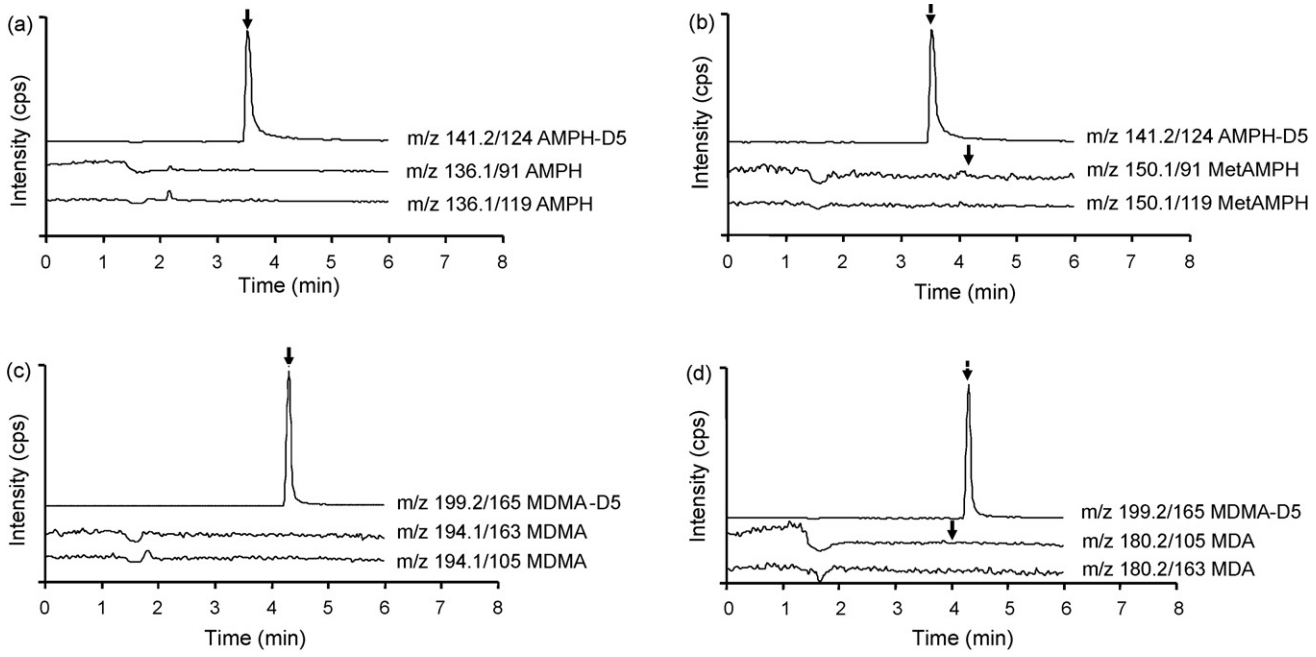

Fig. 2. Chromatograms obtained from the electrospray LC–MS/MS analysis of a blank urine containing internal standards. Each analyte (retention time indicated with arrow) was monitored at two channels in the positive selected reaction monitoring mode; (a) AMPH, (b) MetAMPH, (c) MDMA, (d) MDA.

authentic patient urine sample containing AMPH, MDMA and MDA are shown in Fig. 3. Chromatographic interferences were not observed in patient samples. In routine use the time between injections was 12 min allowing for 5 samples to be analysed per hour.

The limit of detection (LOD, s/n = 3) and lower limit of quantification (LLOQ, s/n = 10) was estimated by measuring the signal-to-noise ratio of blank urine spiked with 200 ng/mL of each analyte. The highest LOD was for AMPH and the lowest for MDA (Table 2). The analytical range was set to 150–10,000 ng/mL for all four analytes.

Linear relationships were observed between peak area ratio of analyte to internal standard and concentration of calibrators in the range of 0–10,000 ng/mL for all analytes ($r^2 > 0.997$). The between-day variability in quantification at the level of applied reporting limit (200 g/mL) ranged between 10.4 and

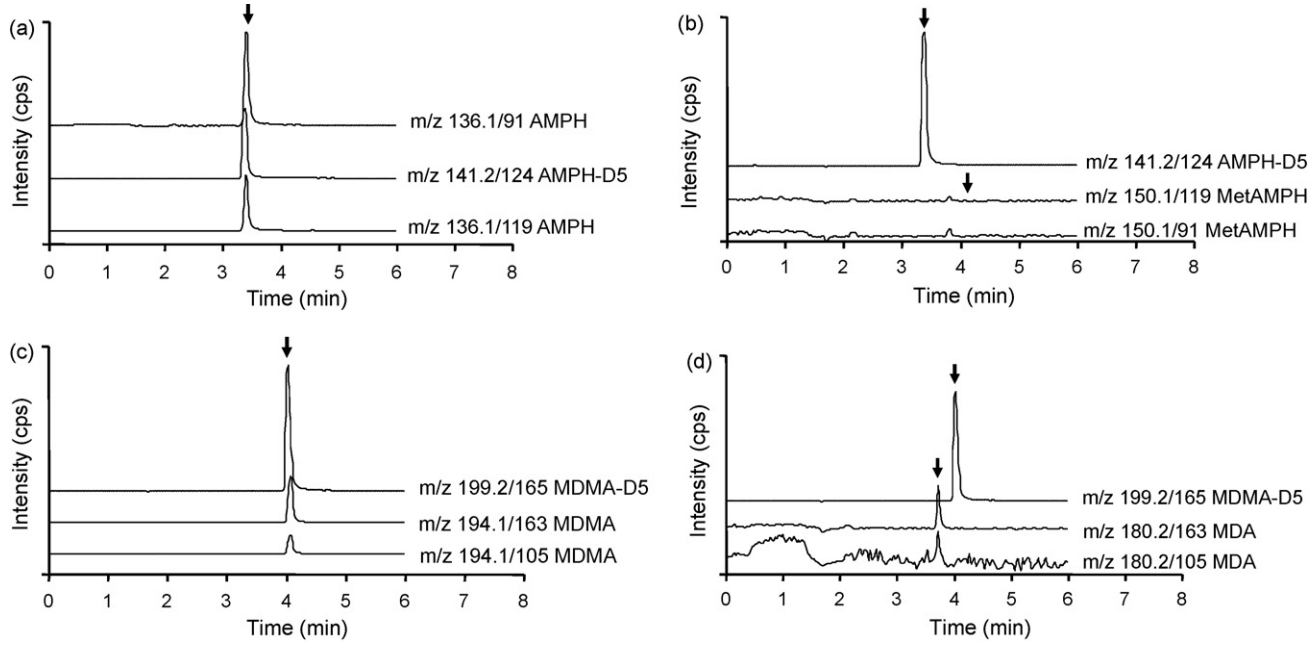

Fig. 3. Chromatograms obtained from the electrospray LC–MS/MS analysis of patient urine sample found to contain 2940 ng/mL of AMPH, 1420 ng/mL of MDMA and 220 ng/mL of MDA. Each analyte (retention time indicated with arrow) was monitored at two channels in the positive selected reaction monitoring mode; (a) AMPH, (b) MetAMPH, (c) MDMA, (d) MDA. Identification of each analyte was based on the presence of peaks at the expected retention with a peak area ratio within ±20% of the value obtained from the calibrators for the two monitored product ions.

Table 2
Data on the limit of detection (LOD) and limit of quantification (LOQ) for the analytes in the LC–MS/MS method

Analyte	LOD, s/n = 3 (ng/mL)	LOQ, s/n = 10 (ng/mL)
AMPH	43	143
MetAMPH	8	28
MDMA	8	26
MDA	2	7

12.6% (Table 3), and tended to be somewhat lower at the higher concentration (6000 ng/mL).

Twenty urine samples that appeared to be "blank" in the CEDIA screening assay for amphetamines were analysed and did not produce response at any mass number for any of the analytes.

Chromatographic interference did not to occur from any of the following tested compounds (concentration 5000 ng/mL): benzylpiperazine, cathinone, ephedrine, fenfluramine, MBDB, MDEA, methcathinone, methylphenidate, phenmetrazine, phenylethylamine, phentermine and ritalinic acid. Interference from other commonly abused drugs can be excluded since these were present in the 479 authentic urine samples analysed.

Ion suppression from injection of diluted urine was observed for the signal from the infused AMPH (Fig. 4). The response was suppressed maximally about 25-fold. The time expressed as capacity factor k' to 90% recovered signal was 2.3, which was well before the elution time of AMPH.

A comparison with the reference GC–MS method was done using 479 authentic patient samples, of which 311 were positive in the immunochemical screening assay. Out of these 311 samples 52 (17%) were not confirmed positive with any of the two methods. The comparison was based on the same reporting limit for both methods (150 ng/mL). The qualitative result using a reporting limit of 150 ng/mL showed an overall 99.8% agreement (Table 4). The four deviating results for MDMA were simply due to the application of reporting limits and were explained by uncertainty in measuring quantities around the reporting limit.

Table 3
Data on within-day and between-day variability in the quantification of the analytes for the LC–MS/MS method

Compound	Concentration added (ng/mL)	Within-day			Between-day		
		Mean	CV (%)	N	Mean	CV (%)	N
AMPH	200	140	5.0	5	173	12.6	24
	6000	5914	6.5	5	5653	7.7	24
MetAMPH	200	199	11.0	5	186	10.4	24
	6000	6398	9.1	5	6143	10.8	24
MDMA	200	155	15.5	5	174	11.6	24
	6000	5708	6.1	5	5675	5.6	24
MDA	200	178	4.3	5	187	12.2	24
	6000	5566	3.9	5	6375	10.3	24

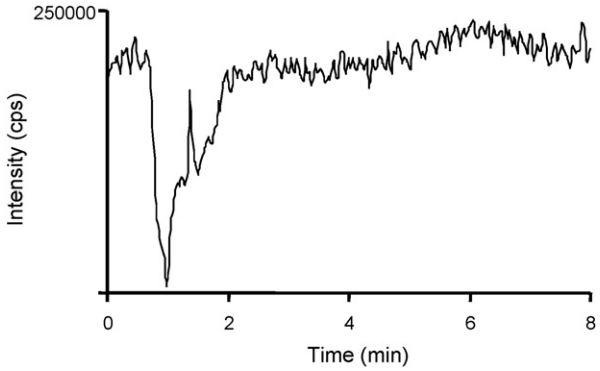

Fig. 4. Ion suppression was studied by monitoring the signal for transition 136.1 → 91 (AMPH) during post-column infusion of 200 ng/min of AMPH. At time zero 10 μL of a blank urine extract was injected. The suppression of signal was at maximum 25-fold.

Table 4
Comparison of qualitative results between the LC–MS/MS and the reference GC–MS methods in 479 patient urine samples

Parameter	LC–MS/MS	GC–MS		Notes
		NEG	POS	
AMPH	NEG	272	0	
	POS	0	207	
MetAMPH	NEG	13	0	
	POS	0	466	
MDMA	NEG	51	2*	*LC–MS/MS: 139; 141 ng/mL,
	POS	2**	424	**GC–MS: 112; 123 ng/mL
MDA	NEG	11	0	
	POS	0	468	

Results from the quantitative comparison of methods are shown in Fig. 5. The slope of the regression line was between 0.8769 and 1.0146 with correlation coefficients between 0.9339 and 0.9963 indicating that the same quantitative results are obtained with LC–MS/MS as with GC–MS (Fig. 5).

An additional method comparison was made with an existing in-house LC–MS method, which was based on direct injection of dilute urine, electrospray ionisation, and selected ion monitoring of the protonated molecules with one qualifying ion each for AMPH, MetAMPH and MDMA. When applying a 300 ng/mL reporting limit the agreement between the methods regarding qualitative results was 100%.

4. Discussion

This study demonstrates that direct injection of urine in combination with electrospray LC–MS/MS is a possible analytical strategy for achieving reliable confirmation of preliminary positive results from the immunochemical screening of the amphetamine class of compounds in urine drug testing. This confirms earlier work focussed on the opiate group of compounds [7–9]. This strategy offers great potential for routine urine drug testing as the time for sample preparation can be shortened since no extraction and derivatization is needed. Recently published methods for identification and quantification

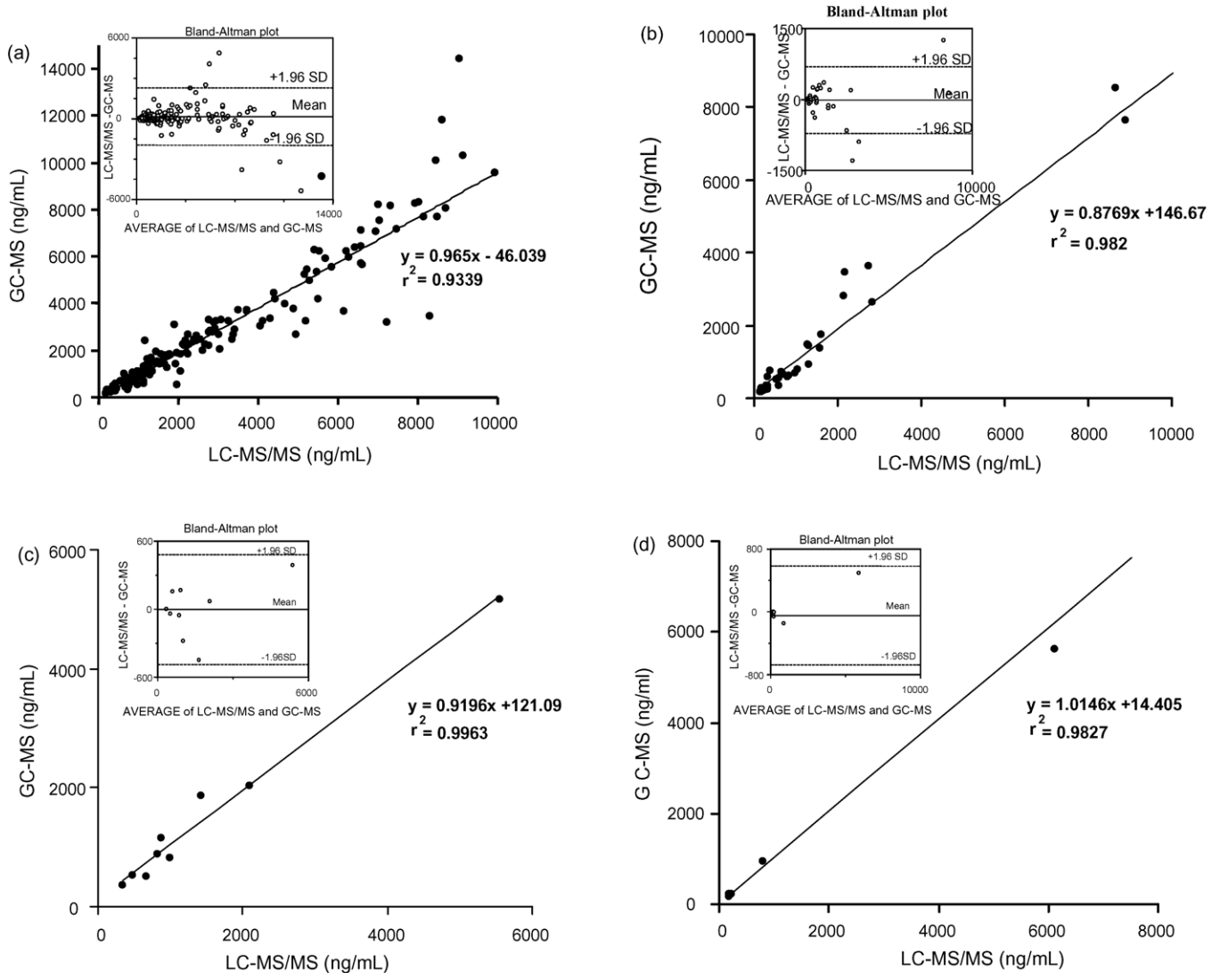

Fig. 5. The relation of quantitative results between the LC–MS/MS and the reference GC–MS methods for AMPH ($n = 164$). The results from the regression analysis is for all data.

of amphetamines in urine comprise both gas chromatography and liquid chromatography in combination with mass spectrometry [13–15,17–22]. Two significant developments using GC–MS are the use of fast gas chromatography technology to reduce analysis time to about half [22] and the single step sample preparation procedure reducing the time for sample preparation [21]. Although both these developments are significant the potential of LC–MS/MS for increased speed and simplicity is greater. Published methods employing LC–MS have used automated or on-line solid-phase extraction [13,15,17]. The use of solid-phase microextraction allowed for omitting the chromatographic system when combined with high-field asymmetric waveform ion mobility spectrometry [18]. Finally, a new ionisation technique, surface-activated chemical ionisation, was used on diluted urine without chromatographic separation [20]. Although these last two methods have potential for very rapid sample processing they have not been validated for routine use in urine drug testing and it is therefore unknown if they meet requirements of reliability. Therefore, the method using direct injection of urine in combination with LC–MS/MS validated in the present study appears to be a very attractive analytical solution by being rapid and robust in addition to being reliable.

The identification of analytes based on a correct ratio between two product ions using MRM in LC–MS/MS is meeting forensic requirements [2]. This study confirms that the proposed requirements of monitoring two product ions and setting a criterion for the ratio is viable in routine use. One interesting observation of this study was that a correct identification was also obtained using single LC–MS detection with only one qualifier ion in combination with direct injection. Although this may not be possible to use in forensic applications, it may be of potential interest for clinical laboratories and underscores the reliability of identifications made by using MS/MS. However, it should be remembered considering MS identification criteria that false identifications have been reported to occur using two product ions in MS/MS when a chromatographic system with low-resolution power has been employed [23,24]. Therefore, the

power of a method in separating the analyte from matrix components must not be forgotten when considering identification criteria.

One critical issue for the strategy of using direct injection of urine is the influence of matrix components to affect the ionisation [25]. Accordingly, it was shown in the present study that a considerable (25-fold) suppression occurs after elution of the column void volume. This is in agreement with our previous observations of direct injection of urine [9,26]. For the first eluting analyte AMPH a deuterated analogue was used as internal standard. It has been shown that a co-eluting isotope labelled analogue will compensate for ion suppression effects for early eluting analytes [27]. In addition, at the time AMPH eluted no ion suppression remained according to the infusion experiment (Fig. 4). A further documentation of the accuracy of the qualitative and quantitative results was obtained by the method comparison with the reference GC–MS method. Taken together our study conclusively shows that the combination of direct injection of urine with electrospray ionisation can offer a robust analytical procedure.

In conclusion, a fully validated analytical procedure for use as a confirmatory method in urine drug testing of AMPH, MetAMPH, MDMA and MDA was presented, which has the potential of replacing existing GC–MS methods by offering shorter turn-around times.

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