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Short communication

Analysis of amphetamines and metabolites in urine with ultra performance liquid chromatography tandem mass spectrometry

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ABSTRACT

A simple, rapid and sensitive ultra performance liquid chromatography tandem mass spectrometry method was developed and fully validated for the quantitative determination of seven amphetamines and metabolites in urine. The method was validated for selectivity, linearity, LOQ, LOD, imprecision, bias, analyte and processed sample stability, matrix effect, recovery, carryover and dilution integrity. A classic liquid–liquid extraction with ethyl acetate was used as sample preparation procedure. The compounds were separated on an Acquity UPLC HSS C_{18} column in 6.8 min. The linear dynamic range was established from 25 to 500 ng/mL. The limit of quantification was fixed to the lowest calibrator level and the limit of detection ranged from 0.125 to 2.5 ng/mL. The method presented an excellent intra- and inter-assay imprecision and bias (<10.7%) at each measured concentration of two external quality controls (QC) and three "in house" QC. No matrix effects were observed and good recoveries (>70%) were obtained for all the compounds. No carryover was observed after the analysis of high concentrated samples (8000 ng/mL). The method was subsequently applied to authentic samples.

1. Introduction

Nowadays, the main objective of toxicology and forensic laboratories is to develop reliable, fast and efficient procedures for performing qualitative and quantitative analyses. High performance liquid chromatography (HPLC) still remains a method of choice, as it is able to separate quite complicated mixtures of low and high molecular weight compounds, as well as different polarities and acid-base properties in various matrices. Unfortunately, conventional HPLC methods must sacrifice either time or resolution.

There is a modern approach in HPLC methods which enable the reduction of analytical time without compromising resolution and separation efficiency: the use of ultra-high pressures using sub-2-microne particle packed columns. Optimal separations are also achieved at higher linear velocities because of the low mass transfer resistance of these supports. These factors have been taken advantage of through the increasingly used technique of ultra performance liquid chromatography (UPLC). Besides that, the combination of UPLC with a tandem mass spectrometer (MS/MS) appears to be a suitable approach that fulfils key requirements in terms of sensitivity and selectivity for the rapid determination of analytes at low concentrations in complex matrices [1–3].

It is common knowledge that poor sample preparation, even when most modern and sophisticated techniques are applied, may negatively affect detection and quantification [4]. Nowadays liquid–liquid extraction (LLE) continues to be widely used in forensic and toxicology laboratories, even with the newly developed UPLC techniques [5].

The consumption of amphetamines, stimulant drugs known for many decades, has increased significantly over the past years due to the easy availability and low cost [6]. Laboratories that follow the Substance Abuse and Mental Health Services Administration (SAMHSA) guidelines or European Laboratory Guidelines for Legally Defensible Workplace Drug testing, etc., for the analysis of amphetamines in urine, first conduct one or more types of immunoassay screens confirmed usually by gas chromatography–MS(/MS) or LC–MS(/MS).

The usefulness of classical LC–MS(/MS) for analysis of amphetamines in biological matrices has been already demonstrated in the past [7–15]. However, to our knowledge, to date, no report has been published dealing the development and validation of a specific method for the analysis of the main amphetamines in urine using UPLC–MS/MS. Apollonio et al. [8] have published a paper concerning the analysis of amphetamines in blood using UPLC–MS to demonstrate the usefulness of this technique for the analysis of biological samples, but no validation of the method was made.

Therefore, the aim of this study was to develop and fully validate a simple, reliable and fast UPLC–MS/MS method for

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Table 1
MRM transitions and conditions for the amphetamines and their deuterated analogues. Underlined transitions were used for quantification.

	Precusor ion (*m*/*z*)	Product ions (*m*/*z*)	Cone voltage (V)	Collision energy (eV)
Amphetamine	136.1	<u>91.1</u> 118.9	15	15 10
Methamphetamine	150.0	<u>91.1</u> 119.1	20	15 10
MDA	180.1	<u>105.0</u> 134.9	15	20 15
MDMA	194.2	<u>105.0</u> <u>163.1</u>	20	25 15
MDEA	208.2	105.0 <u>163.1</u>	20	15 15
PMA	166.1	121.1 <u>149.0</u>	15	18 10
Ephedrine	166.1	<u>133.0</u> 148.3	15	20 10
Amphetamine-d_{11}	147.1	98.0	15	15
Methamphetamine-d_5	155.0	91.9	20	20
MDA-d_5	185.0	167.9	18	10
MDMA-d_5	199.2	165.1	15	15
MDEA-d_6	214.2	165.9	20	12
Ephedrine-d_3	169.1	151.1	20	15

quantification of the main amphetamines in urine. A simple LLE was used as sample clean up procedure and the validated method was applied to authentic urine samples from amphetamine users.

2. Materials and methods

2.1. Reagents

Individual stock solutions of amphetamine, ephedrine, MDA (methylenedioxyamphetamine), MDEA (methylenedioxyethylamphetamine), MDMA (methylenedioxymethamphetamine), methamphetamine and PMA (4-methoxyamphetamine) (all certified at a concentration of 1 mg/mL in methanol), and the internal standards (IS) amphetamine-d_{11}, methamphetamine-d_5, MDA-d_5, MDMA-d_5, MDEA-d_6 and ephedrine-d_3 (certified concentration at 0.1 mg/mL in methanol) were from LGC Promochem (Molsheim, France). Water (HPLC-grade) and methanol (UPLC–MS grade) were purchased from Biosolve (Valskenswaard, The Netherlands). Ammonium formate (powder) and sodium hydroxide 1 M were purchased from Sigma–Aldrich (Steinheim, Germany). Ethyl acetate and hydrogen chloride 1.25 M in 2-propanol were from VWR (Leuven, Belgium).

External quality controls (QC) C1 and C3 were purchased from Bio-Rad Laboratories (Irvine, CA).

2.2. Specimens

Blank urine samples were obtained from drug-free volunteers. Authentic urine samples were obtained from forensic and toxicology cases.

2.3. Preparation of standard solutions for calibrators and QC samples

Separate working solutions of the drugs were prepared in the laboratory at a concentration of 4 mg/L in methanol for tuning, selectivity experiments and the 'in house' QCs. A mixed working solution of non-deuterated compounds at 4 mg/L for all the compounds in methanol was used for the preparation of calibrators. A mixed IS working solution of 1 mg/L for deuterated standards was also prepared in methanol. All working solutions were stored at 4 °C. Freshly dilutions in water were further prepared in each experiment.

The external QCs were prepared following the indications of the manufacturer.

2.4. Experimental

2.4.1. Sample preparation

The extraction procedure was carried out in 10 mL disposable screw top vials of high quality glassware (Chromacol, Herts, UK) using 100 μL of urine. Fifty microliters of the IS working solution (corresponding to a concentration in urine of 50 ng/mL), 400 μL of deionised water and 500 μL of sodium hydroxide 1 M were added. After adding 2.5 mL of ethyl acetate, mechanical shaking was carried out for 10 min. Then, the samples were centrifuged (10 min at 4000 × *g*), the organic phase was transferred to a 5 mL disposable screw top vial (Chromacol) and then evaporated up to 500 μL with a programmed vacuum centrifuge (Jouan, Saint Herblain, France). Due to volatility of the amphetamines in the base form, 100 μL of hydrogen chloride 1.25 M in 2-propanol was then added before the samples were evaporated to dryness. The extract was reconstituted in 500 μL of aqueous mobile phase, filtered with 0.20 μm filters (Millipore, Brussels, Belgium) and an aliquot of 5 μL was injected into the UPLC–MS/MS system.

2.4.2. UPLC–MS/MS

2.4.2.1. Chromatographic conditions. Analytes were separated using an Acquity UPLC HSS C_{18} (2.1 mm × 100 mm, 1.8 μm) (Waters, Milford, MA, US). The column was kept at 30 °C. A gradient elution using two solvents, A and B, was applied. Solvent A consisted of 5 mM ammonium formate buffer containing 0.05% formic acid. Solvent B was methanol. The gradient was carried out starting from 10% B to be linearly increased to 30% B over the first 8 min. At 8.1 min B was set to 95% for 1 min returning then to the initial conditions and equilibrating for 2 min, resulting in a total run time of 11 min. The mobile phase flow was set to 0.3 mL/min during the whole run.

Table 2
Intra-assay (expressed as RSD_r (%)) and inter-assay precision (expressed as RSD_t (%)) and bias of the LOQ and QC urine samples. Intra-assay, inter-assay precision and bias were evaluated by replicate ($n = 2$) analysis of the QC samples performed over eight different days.

	Nominal value (ng/mL)	Average ($n = 16$) (ng/mL)	RSD_r (%) ($n = 2$)	RSD_t (%) ($n = 8$)	Bias (%) ($n = 16$)
Amphetamine					
LOQ	25	23.5	3.3	4.2	−6.2
Q_{low}	40	40.1	2.9	2.6	0.3
QC_{Med}	200	207.9	0.9	1.5	3.9
QC_{High}	400	403.6	2.1	2.8	0.9
C1	100	110.5	1.8	4.1	10.5
C3	600	586.8	1.5	3.3	−2.2
Ephedrine					
LOQ	25	25.6	2.7	2.3	2.5
Q_{low}	40	40.0	2.2	2.5	0.0
QC_{Med}	200	195.2	3.1	3.3	−2.4
QC_{High}	400	405.8	3.3	2.8	1.5
C1	–	–	–	–	–
C3	–	–	–	–	–
MDA					
LOQ	25	25.1	4.0	5.1	0.5
Q_{low}	40	40.2	6.2	5.5	0.5
QC_{Med}	200	193.2	2.0	2.8	−3.4
QC_{High}	400	409.8	3.1	5.5	2.4
C1	100	97.9	2.9	5.5	−2.1
C3	300	310.2	3.7	3.6	3.4
MDEA					
LOQ	25	26.0	1.3	2.4	4.2
Q_{low}	40	40.1	2.5	8.1	0.2
QC_{Med}	200	193.1	1.2	1.8	−3.5
QC_{High}	400	408.6	1.5	1.8	2.1
C1	100	102.8	1.9	4.4	2.8
C3	300	317.5	1.1	2.4	5.9
MDMA					
LOQ	25	26.2	3.2	2.9	4.9
Q_{low}	40	40.1	2.4	3.2	0.3
QC_{Med}	200	194.1	1.8	2.6	−3.0
QC_{High}	400	413.6	1.4	2.5	3.4
C1	100	103.3	2.6	4.6	3.3
C3	300	25.9	2.4	2.6	3.5
Methamphetamine					
LOQ	25	25.9	2.4	2.5	3.5
Q_{low}	40	40.2	1.3	1.4	0.6
QC_{Med}	200	196.2	1.7	2.4	−1.9
QC_{High}	400	409.8	1.1	2.2	2.4
C1	100	106.4	1.5	4.6	6.4
C3	600	645.6	2.6	3.3	7.6
PMA					
LOQ	25	25.5	3.4	4.7	2.1
Q_{low}	40	39.9	2.5	3.3	−0.3
QC_{Med}	200	197.2	3.1	3.3	−1.4
QC_{High}	400	403.0	3.4	2.9	0.7
C1	–	–	–	–	–
C3	–	–	–	–	–

2.4.2.2. Tandem mass spectrometry. A Quattro Premier tandem mass spectrometer (Waters) was used. Ionization was achieved using electrospray in positive ionization mode (ESI+). Nitrogen was applied as nebulisation and desolvation gas at a flow rate of 700 L/h and heated to 350 °C. Capillary voltage and source block temperature were 1 kV and 120 °C, respectively.

In order to establish the appropriate multiple reaction monitoring (MRM) conditions for the individual compounds, solutions of standards (200 ng/mL, in ammonium formate buffer 5 mM (0.05% formic acid):methanol (50:50, v/v)) were infused into the mass spectrometer and the cone voltage (CV) was optimised to maximise the intensity of the protonated molecular species $[M+H]^+$. Collision-induced dissociation (CID) of each protonated molecule was performed. The collision gas (argon) pressure was maintained at 0.35 Pa (3.5×10^{-3} mBar) and the collision energy (eV) adjusted to optimise the signal for the most abundant product ions, which were subsequently used for MRM analysis (Table 1).

3. On-line SPE-LC–MS/MS assay validation

Validation was performed based on the FDA guidelines and publications concerning validation of bioanalytical methods [16].

3.1. Linearity, limit of quantification (LOQ), limit of detection (LOD), precision and bias

Quantification was performed by integration of the area under the specific MRM chromatograms in reference to the integrated area of the deuterated analogue. Freshly prepared working solutions of 1000, 250 and 100 ng/mL in water were used to prepare urine calibrators at a concentration of 500, 250, 125, 50 and 25 ng/mL using HPLC-grade water. Standard curves, freshly prepared with each batch of QC samples and authentic samples, were generated using a least-squares linear regression, with a $1/x$-weighting factor for all compounds.

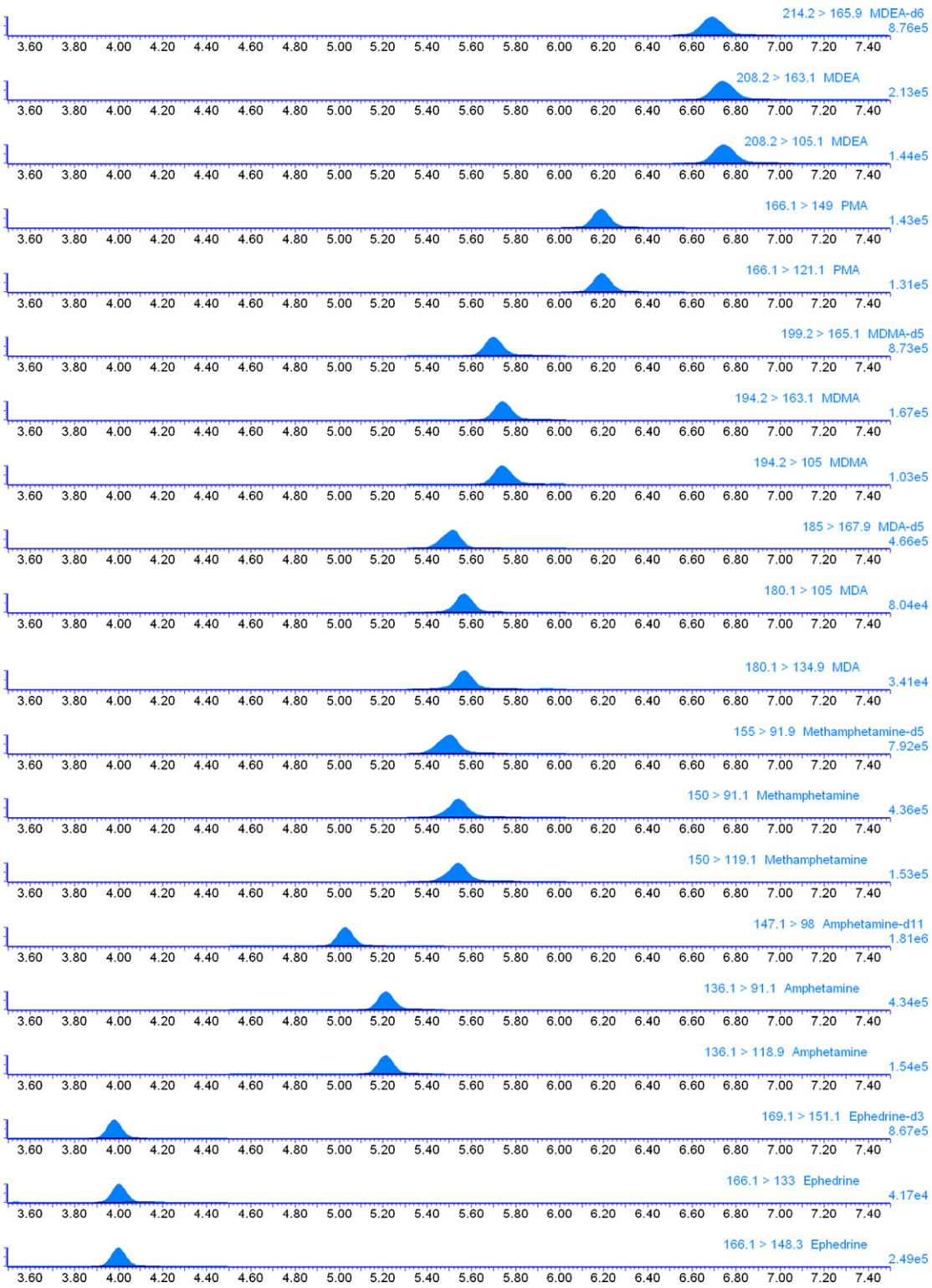

Fig. 1. MRM chromatograms obtained following the analysis of a spiked urine sample with 25 ng/mL (LOQ). Peak intensity is shown in the top right-hand corner of each trace.

The limit of quantification (LOQ) was estimated by replicate analysis ($n = 2$) over eight different days and was defined as the concentration of the lowest calibrator that was calculated within ±20% of the nominal value and with a relative standard deviation (RSD) less than 20%. Imprecision (RSD) was determined by performing the analysis of variance: a 'single factor' ANOVA test (significance level (α) of 0.05).

The limit of detection (LOD) was estimated from blank urine samples, spiked with decreasing concentrations of the analytes. It was defined as the concentration for which the response of the qualitative ion could reliably be differentiated from background noise, i.e. signal-to-noise ratio (S/N) equal to or greater than 3:1. The acceptance criteria for ion ratios equal to or lower than 20% and retention time deviations lower than

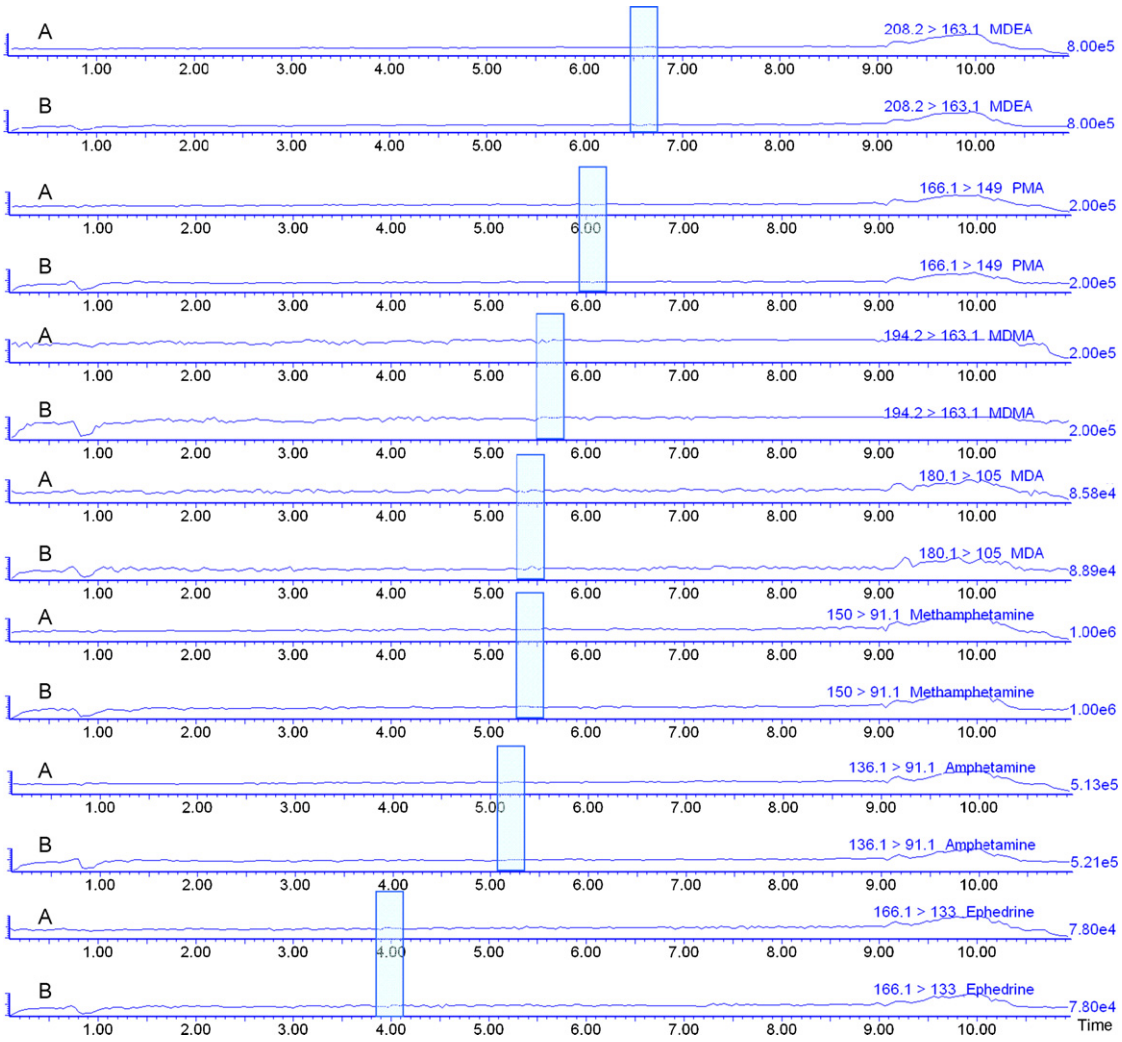

Fig. 2. Evaluation of the matrix effect on amphetamine, ephedrine, methamphetamine, MDA, MDMA, PMA and MDEA by post-column infusion following an injection of mobile phase only control (A) and extracted blank urine (B) for each compound. The dotted areas indicate the elution position of each drug. MRM transitions and peak intensity are shown on the right-hand corner of each trace.

3.5% relative to that of the corresponding control or calibrator.

Five QCs were analyzed: two external QCs, C1 and C3 (Bio-rad Laboratories) containing amphetamine, MDA, MDMA, MDEA and methamphetamine, and three 'in house' QCs.

Intra-assay and inter-assay imprecision was evaluated by replicate ($n=2$) analysis of the QC samples performed over eight different days. Imprecision (expressed as $\%RSD_r$ for intra-assay imprecision and $\%RSD_t$ for inter-assay imprecision) was determined by performing the analysis of variance: a 'single factor' ANOVA test (significance level (α) of 0.05). Bias of the method was determined by comparison of the mean of calculated concentrations of QC samples to their respective nominal values.

3.2. Selectivity and specificity

The selectivity and specificity of the method against endogenous interferences was verified by examination of the chromatograms obtained after the extraction of eight different blank urine samples from healthy volunteers spiked with the IS, eight blank urines not spiked with the IS, and after the analysis of authentic urine samples from cannabis, cocaine, and opiates users. Moreover, a blank urine sample spiked at 2000 ng/mL with several over the counter drugs (opiates, cocaine, cannabinoids and hallucinogens) was also analyzed to check for interferences.

3.3. Stability

The autosampler stability of processed samples at concentrations of 400, 200 and 40 ng/mL ($n=6$ at each concentration) was monitored as follows; one pool of samples were determined immediately, while another pool of samples was analyzed after remaining in the autosampler at $6\pm2\,^{\circ}C$ for 72 h. All samples were spiked with the IS just before analysis (to prevent from the IS instability and to properly calculate the ratios).

Stability of compounds in the matrix was determined through spiked blank urine samples with concentrations of 400, 200 and 40 ng/mL ($n=6$ at each concentration). Stability was checked after three freeze/thaw cycles.

All the stability experiments were tested against a lower percentage limit corresponding to 85–115% of the ratio (mean value of stability samples/mean value control samples) with a 90% of the confidence interval of the stability samples between 80 and 120% of the mean of the control samples.

Table 3
Extraction recoveries and matrix effects (% effects and RSD%) at three concentration levels ($n = 6$).

	Recovery (%)			Matrix effect					
	40 ng/mL ($n = 6$)	200 ng/mL ($n = 6$)	400 ng/mL ($n = 6$)	40 ng/mL		200 ng/mL		400 ng/mL	
				%Effect ($n = 6$)	RSD (%) ($n = 6$)	% Effect ($n = 6$)	RSD (%) ($n = 6$)	% Effect ($n = 6$)	RSD (%) ($n = 6$)
Amphetamine	96.1	81.9	89.8	3.7	1.5	−3.0	4.6	−4.8	1.8
Ephedrine	85.7	70.3	77.4	4.2	2.3	−0.7	1.7	−1.2	1.9
MDA	100.7	98.9	99.8	7.2	2.0	−1.1	4.3	−0.2	2.1
MDEA	111.3	102.4	100.7	9.6	3.0	2.4	3.4	0.7	2.0
MDMA	114.1	101.9	101.2	8.0	2.3	1.9	1.8	1.2	1.6
Methamphetamine	108.4	81.9	88.7	4.8	2.5	−0.6	2.6	1.5	2.0
PMA	97.2	80.8	86.0	6.7	2.4	−1.7	2.5	−2.6	2.0

3.4. Assessment of matrix effects

To assess any potential suppression or enhancement of ionization due to the sample matrix, two different experiments were carried out.

The first one involved a post-column infusion experiment [17] ($n = 6$) and the second experiment consisted of a comparison between the peak responses of amphetamines spiked to a blank urine sample at concentrations of 400, 200 and 40 ng/mL ($n = 6$, for each concentration) with those obtained after being spiked in the mobile phase at the same concentration levels [18].

3.5. Recovery

Extraction recoveries were estimated by comparing the ratio of the peak areas of the non-deuterated compounds to the peak areas of the IS (i.e. responses) of blank urine samples spiked at 400, 200 and 40 ng/mL ($n = 6$, for each concentration) when the non-deuterated compounds were added before the extraction step with those obtained when the non-deuterated analytes were added after sample extraction (e.g. "non-deuterated area (compounds added before extraction)/IS area" versus "non-deuterated area (compounds added after extraction)/IS area"). In both cases, the deuterated analogues were added after the extraction.

3.6. Carryover

Carryover was evaluated by the analysis of blank urine samples spiked with the IS after the analysis of the upper calibrator (500 ng/mL, $n = 8$), after the analysis of authentic urine samples from amphetamine users ($n = 16$) and after the analysis of highly concentrated samples (8000 ng/mL, $n = 3$).

3.7. Dilution integrity

Spiked blank urine samples at 8000 ng/mL ($n = 3$) were rediluted 1:20 (v/v) with blank urine and analyzed to evaluate the dilution integrity.

4. Results and discussion

The method was validated for selectivity, linearity, LOQ, LOD, imprecision, bias, analyte and processed sample stability, matrix effect, recovery, carryover and dilution integrity.

The applied chromatographic method ensured the elution of all the compounds within 6.7 min and produced peaks of acceptable symmetry. Selectivity of the method was achieved by a combination of retention time, precursor and two product ions (Table 1). For the corresponding deuterated analogue, only one transition was monitored. The following ion ratios (quantifier/qualifer) were obtained: amphetamine 2.9, ephedrine 6.3, MDA 2.3, MDEA 1.5, MDMA 1.5, methamphetamine 3.0 and PMA 1.1.

Fig. 1 shows the MRM chromatograms obtained following the analysis of the urine lowest calibrator (25 ng/mL). No interferences were observed after the analysis of blank urine samples and the blank urine spiked with over the counter drugs, ensuring the selectivity of the method.

During pre-validation experiments, the linearity was tested up to 1000 ng/mL and an $r^2 > 0.99$ for all the compounds was observed. However, due to the recommended cut-off established in our laboratory for the confirmation of amphetamines in urine is fixed at 200 ng/mL (following the European Laboratory Guidelines for Legally Defensible Workplace Drug Testing [19]), the quantification range was limited to 25–500 ng/mL, for practical considerations in future toxicology routine application of the method in the laboratory. Correlation coefficients of the weighed ($1/x$) linear regressions

for the selected range were also higher than $r^2 > 0.99$ in the eight calibration curves carried out during eight different days.

The LOQ was fixed at the lowest calibrator at 25 ng/mL for all the compounds as an S/N >10:1 was observed for the qualifiers and the criteria for LOQ were satisfied. LOD was determined as follows: amphetamine 2.5 ng/mL, ephedrine 2.5 ng/mL, MDA 2.5 ng/mL, MDEA 0.125 ng/mL, MDMA 0.5 ng/mL, methamphetamine 0.25 ng/mL and PMA 2.5 ng/mL.

The intra- and inter-assay imprecision for the LOQ, 'in house QCs' and external QCs, C1 and C3, were satisfactory, with all RSDs lower than 8.2% (Table 2). The results indicated that the bias of the assay was lower than 10.6% for all the compounds. Although the concentration of amphetamine and methamphetamine of the external QC C3 was higher than the upper calibrator (600 ng/mL) (not diluted), the intra- and inter-assay precision were excellent which demonstrates the good linearity of the method.

Stability of the processed samples in the autosampler (at 2–6 °C) was monitored after 72 h. No instability was observed during this period of time. Moreover, the compounds spiked to blank urine samples were also stable after the three freeze/thaw cycles.

Post-column infusion experiments were performed to provide information of the matrix effect throughout the course of the elution time for the analyte and its IS. No significant changes in response were observed. Fig. 2 shows the evaluation of the effect of the matrix on amphetamines response of an injection of a mobile phase control (A) and an extracted urine sample (B). The second experiment performed to assess matrix effects compared the peak area responses, obtained when the compound was spiked into blank urine samples, with the responses obtained when the compounds were added to mobile phase at the same concentration. The results of matrix effects and the extraction recovery study are presented in Table 3. Very high and reproducible recoveries were obtained with this LLE procedure for all analytes (>86% for all the compounds except for ephedrine which was >70%).

No carryover was observed in the analysis of a blank urine sample injected after the analysis of the upper calibrator (500 ng/mL), neither after the analysis of authentic urine samples or after a highly concentrated sample (8000 ng/mL).

Moreover, the dilution integrity test, the dilution 1/20 of high concentrated samples (8000 ng/mL) ($n = 3$), demonstrated a bias <11% and an RSD (%) < 9% for the diluted blank urine samples.

4.1. Samples

Authentic amphetamine positive urines ($n = 19$) were analyzed for the final evaluation of this method and amphetamine was the most common drug found. No positive cases for MDEA, ephedrine and PMA were detected. Those samples with concentrations above the upper calibrator were diluted at least 1/20 with blank urine and reanalyzed. The mean, minimum and maximum ranges were as follows (in ng/mL): amphetamine [1227 (49–18,522)] ($n = 17$), methamphetamine [7073 (68–2062)] ($n = 4$), MDMA [3448 (2576–4825)] ($n = 4$) and MDA [1348 (347–1364)].

5. Conclusions

A rapid and sensitive method was fully validated for the determination of amphetamine, ephedrine, MDA, MDEA, MDMA, methamphetamine and PMA in urine by UPLC–MS/MS after a simple LLE. Very good precision, bias and recovery were obtained and no significant matrix effects and carryover were observed. The method was successfully applied to authentic samples from amphetamines users.

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