Journal of Chromatography B, 878 (2010) 2905–2910

Contents lists available at ScienceDirect

Journal of Chromatography B

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

Short communication

Determination of 3,4-methylenedioxymethamphetamine and its five main metabolites in rat urine by solid-phase extraction and high performance liquid chromatography with on line mass spectrometry

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ARTICLE INFO

Article history:
Received 14 April 2010
Accepted 14 August 2010
Available online 21 August 2010

Keywords:
3,4-Methylenedioxymethamphetamine
Metabolites
Rat urine
Solid-phase extraction
Liquid chromatography/electrospray ionization/mass spectrometry
Validation

ABSTRACT

The consumption of psychostimulant amphetamine-like drugs has increased significantly in recent years. Some MDMA metabolites are probably involved in the neurotoxicity and neurodegeneration caused by prolonged use rather than MDMA itself. We recently developed a method to analyze MDMA and its five main metabolites in rat plasma [7]. We have now fully validated this method to the quantification of these drugs in rat urine. We extracted MDMA and its metabolites with Oasis WCX cartridges, separated them on a Nucleodur C18 analytical column and quantified them by ion-trap mass spectrometry. Linearity was excellent: 12.5–1250 ng/mL urine for HMA, HMMA, MDA and MDMA, 25–2500 ng/mL for HHMA, and 150–7500 ng/mL for HHA ($r^2 > 0.993$ for all analytes). The lower limits of quantification were 12.5 ng/mL urine for MDMA, MDA, HMA and HMMA, 25 ng/mL for HHMA and 150 ng/mL for HHA. Reproducibility was good (intra-assay precision = 1.7–6.1%; inter-assay precision = 0.6–5.7%), as was accuracy (intra-assay deviation = 0.1–4.8%; inter-assay deviation = 0.7–7.9%). Average recoveries were around 85.0%, except for HHMA (66.2%) and HHA (53.0%) (CV < 8.3%). We also checked the stability of stock solutions and the internal standards after freeze-thawing and in the autosampler. Lastly, we measured the MDMA, MDA, HHMA, HHA, HMMA and HMA in urine samples taken over 24 h from rats given subcutaneous MDMA.

1. Introduction

The consumption of psychostimulant amphetamine-like drugs like 3,4-methylenedioxy-methamphetamine (MDMA, ecstasy) has increased significantly in recent years. Whereas the use of MDMA as a recreational drug is believed to be without risk, several clinical studies have shown that this drug and more particularly its metabolites are toxic, causing various disorders [1]. MDMA is rapidly metabolized by two phase I metabolic pathways: [1] *O*-demethylenation (major pathway in the humans) leads to the formation of 3,4-dihydroxymethamphetamine (HHMA); and [2] *N*-demethylation, (primary pathway in the rats) leads to the formation of 3,4-methylenedioxyamphetamine (MDA); it is followed by *O*-demethylenation leading to 3,4-dihydroxyamphetamine (HHA). The MDMA catechol metabolites, HHMA and HHA, can undergo catechol-*O*-methyltransferase (COMT)-catalyzed methylation to form 4-hydroxy-3-methoxymethamphetamine (HMMA) and 4-hydroxy-3-methoxyamphetamine (HMA). HHMA, HHA, HMMA, and HMA are subsequently conjugated by phase II enzymes (sulfotranferases and glucuronosyltransferases) [1].

The investigation of the pharmacokinetics/toxicokinetics of MDMA depends on the availability of the methods that can simultaneously analyse, mixtures of both hydrophobic (MDMA and MDA), less hydrophobic (HMMA and HMA) and hydrophilic (HHA and HHMA) compounds (Table 1), and protect MDMA *O*-diphenolic metabolites, HHA and HHMA, from oxidization into their corresponding quinones at pH ≥ 7.4 [2]. Consequently, the analytical methods presently available, using liquid chromatography (LC) [3,4] or gas chromatography (GC) [5,6] coupled to mass spectrometry (MS), are generally limited to analysing MDMA and a limited number of its metabolites in biological fluids. We have recently developed and partially validated a new selective LC/positive-ion electrospray ionization (ESI)/MS method in order to simultaneously determine MDMA and its five main metabolites, MDA, HHA, HHMA,

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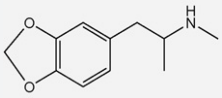
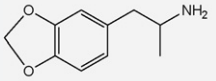
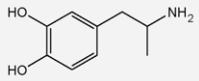
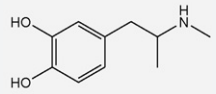
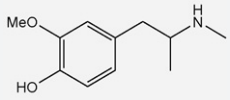
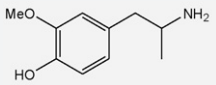
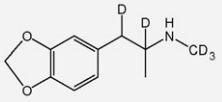
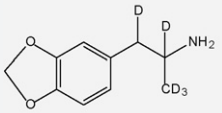
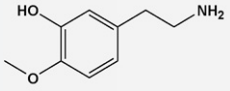
doi:10.1016/j.jchromb.2010.08.018

Table 1
Chemical structures and some physico-chemical properties of MDMA, its five main metabolites and the internal standards for these drugs.

Compound	Chemical structure	Molecular weight	log *P* octanol/water	p*Ka* values
3,4-Methylenedioxymethamphetamine (MDMA)		193.24	1.806	10.32
3,4-Methylenedioxyamphetamine (MDA)		179.22	1.667	9.94
3,4-Dihydroxyamphetamine (HHA)		167.21	0.467	9.70 and 10.05
3,4-Dihydroxymethamphetamine (HHMA)		181.23	0.606	9.70 and 10.34
4-Hydroxy-3-methoxymethamphetamine (HMMA)		195.26	0.913	9.84 and 10.63
4-Hydroxy-3-methoxyamphetamine (HMA)		181.23	0.774	10.50 and 9.61
d5-3,4-Methylenedioxymethamphetamine (d5-MDMA)		198.21	*n.d.*	*n.d.*
d5-3,4-Methylenedioxyamphetamine (d5-MDA)		184.18	*n.d.*	*n.d.*
3-Hydroxy-4-methoxyphenethylamine (HMP)		167.21	0.427	9.38 and 10.30

P: octanol–water partition coefficient; *Ka*: acid dissociation constant; *n.d.*: not determined.

HMMA and HMA, in rat plasma [7]. In spite of rather low limits of quantification, this method left HHMA and HHA undetected in the plasma of MDMA-treated rats.

We have now extended this LC–ESI-MS method to analyse MDMA and its five main metabolites in rat urine. After the optimization of the solid-phase extraction of MDMA and its five metabolites, we fully validated the LC–ESI-MS method to determine MDMA, MDA, HHA, HHMA, HMMA and HMA in urines of rat treated by MDMA, according to internationally accepted recommendations [8].

2. Experimental

2.1. Chemicals and materials

Reference standards of 3,4-methylenedioxyamphetamine (MDA) and 3-hydroxy-4-methoxyphenylethylamine (HMP) were procured from Sigma–Aldrich (St-Quentin-Fallavier, France). Reference standards of 3,4-methylenedioxymethamphetamine (MDMA), 3,4-dihydroxyamphetamine (HHA), 4-hydroxy-3-methoxyamphetamine (HMA) and 4-hydroxy-3-methoxymethamphetamine (HMMA) were kindly provided by Prof. H. Galons (INSERM U648, Université Paris Descartes, Paris, France), and 3,4-dihydroxymethamphetamine (HHMA) by Dr. Largeron and Dr. Neudörffer (CNRS UMR8638, Université Paris Descartes, Paris, France). The purity of MDMA, HHMA, HHA, HMMA and HMA was better than 95%, as determined by NMR. d5-3,4-methylenedioxymethamphetamine (d5-MDMA) and d5-3,4-methylenedioxyamphetamine (d5-MDA) were obtained from Cerilliant (LGC Promochem, Molsheim, France). All other chemicals and the solvent used were of analytical grade and chromatographic grade respectively and provided from local suppliers. Oasis WCX (weak cation exchange) extraction cartridges (1 mL/30 mg) were obtained from Waters (St-Quentin-en-Yvelines, France). Nucleodur Pyramid C18 column (250 mm length × 2.1 mm inner diameter/particle size 5 μm) was from Macherey-Nagel (Hoerdt, France). All water was prepared with the Milli-Q water purification system (Millipore, Molsheim, France).

2.2. Stock solutions of calibration standards and internal standards

Initial standard stock solutions (1 mg/mL) of MDMA, MDA, HHMA, HHA, HMMA and HMA were prepared in aqueous TFA

Table 2
Calibration curves and lower limits of quantification for MDMA, MDA, HMMA, HMA, HHMA and HHA in rat urine.

Analyte	Standard curve			Lower limit of quantification (LLOQ)		
	Linearity range (ng drug/mL urine)	Regression equation (%CV_s) (%CV_i)	r^2 (%CV)	ng drug/mL urine	Precision (%CV)	Accuracy (%DEV)
MDMA	12.5–1250	$y = 1.7378x + 0.0235$ (3.1) (4.3)	0.9998 (0.02)	12.5	7.8	12.8
MDA	12.5–1250	$y = 1.9359x + 0.0069$ (1.6) (8.9)	0.9998 (0.02)	12.5	11.5	10.7
HMMA	12.5–1250	$y = 1.4332x + 0.0459$ (2.2) ((9.4)	0.9998 (0.02)	12.5	12.3	9.7
HMA	12.5–1250	$y = 2.3192x + 0.0081$ (3.5) (6.2)	0.9986 (0.19)	12.5	14.8	8.5
HHMA	25–2500	$y = 1.7033x - 0.0176$ (1.3) (7.4)	0.9997 (0.04)	25	8.5	13.7
HHA	150-7500	$y = 0.2829x + 0.0084$ (2.7) (7.1)	0.9995 (0.05)	150	17.4	5.3

Standard curves were analyzed in blank urine samples (100 μL) containing 12.5, 50, 100, 150, 250, 375, 500, 625 and 1250 ng/mL each of MDMA, MDA, HMA and HMMA; 25, 100, 200, 300, 500, 750, 1000, 1250 and 2500 ng/mL for HHMA; and 150, 300, 600, 900, 1500, 2250, 3000, 3750 and 7500 ng/mL for HHA. Each calibration standard also contained 5 μL of the internal standard combined stock solution to give final amounts of 42.5 ng of HMP and 50 ng each of d5-MDMA and d5-MDA. The linear range for each specific curve is presented along with its regression equation and coefficient of determination (r^2). Mean r^2 values are shown. All data result from six replicates for each calibration standard analyzed on 6 separate days. The precision (%CV) for specific concentrations on the standard curves was 2.4–8.5% for MDMA (average: 4.3%), 0.9–10.1% for MDA (average: 3.5%), 1.2–6.2% for HMMA (average: 3.8%), and 2.0–6.4% for HMA (average: 4.6%). The precision for specific concentrations of HHMA and HHA standards was 1.1–9.9% for HHMA (average: 3.4%), and 1.8–5.1% for HHA (average: 3.7%). The average deviation from theoretical values (%DEV) for MDMA was 3.3% and 2.6% for MDA, with values ranging from 0.2% to 9.3% and from 0.1% to 9.5%. It was 1.9% for HMMA (range: 0.1–5.3%), 3.2% for HMA (range: 0.8–8.3%), 3.4% for HHMA (range: 0.5–11.1%), and 3.7% for HHA (range: 0.1–8.5%). The lower limits of quantification of MDMA and its five main metabolites are shown with associated precisions (%CV) and accuracies (%DEV) ($n = 10$ for each analyte). %CVS: CV slope; %CV_i: CV intercept.

(0.05%, v/v). A stock solution of all six compounds was then prepared in the same solvent (300 μg/mL HHA, 100 μg/mL HHMA, and 50 μg/mL each of MDMA, MDA, HMMA and HMA). The concentrations of the internal standards d5-MDMA and d5-MDA stock solutions were 1 mg/mL in methanol (MeOH). A stock solution of the internal standard HMP (1 mg/mL) was prepared by dissolving in aqueous TFA (0.05%, v/v). The final internal standard stock solution combining all three internal standards (IS) with concentrations of 8.5 μg/mL of HMP and 10 μg/mL each of d5-MDMA and d5-MDA, was prepared in aqueous TFA (0.05%, v/v). All stock solutions of analytes and internal standards were stored at −20 °C in the dark.

2.3. Animals and urine sample collection

Male Sprague–Dawley rats (430–455 g; 10–11 weeks) were obtained from Janvier (Le Genest-St-Isles, France). All animal experiments were carried out in compliance with the European Community Council (86/609/EEC) and French laws (law no. 87-848), with the standard ethical guidelines and under control of the Ethical Committee of the Faculty of Pharmacy.

The volume of urine collected from each rat following MDMA injection (5 mg/kg in sterile saline) was 9 ± 2 mL (mean ± S.D.; $n = 5$). The volume of drug-free samples collected from a second group of rats was 13 ± 4 mL (mean ± S.D.; $n = 4$). Urine samples were collected and stored according the procedure described in Ref. [7].

2.4. Determination of MDMA and its metabolites

The compounds of interest were subjected to an acidic hydrolysis and the solid-phase extraction by using Oasis WCX cartridge (Waters, Saint-Quentin en Yvelines, France) described in Ref. [7]. Low pH used during the hydrolysis and extraction steps ensure stability of HHA [2]. Molecules were separated on a Nucleodur Pyramid C18 column connected to a ThermoFinnigan "Surveyor" high performance liquid chromatograph (HPLC) coupled to a diode array detector and LCQ Advantage ion-trap mass spectrometer (ThermoFinnigan, Courtaboeuf, France). The mobile phase was 0.05% (V/V) TFA in water and acetronitrile in gradient mode. The parameters of the electrospray interface were optimized to provide a maximum of $[M+H]^+$ ions for all the analytes and internal standards. The MS was operated in positive ionization mode and single ion monitoring (SIM). ThermoFinnigan Xcalibur® software for LCQ Advantage LC–MS (Version 1.3 SR1) was used to quantify the analytes.

2.5. Bio-analytical method validation

We carried out a thorough, complete validation of the assay method for MDMA and its metabolites in rat urine [8]. Calibration standards and quality control (QC) samples were prepared by adding stock analyte solution or a dilution to blank urine samples on each validation day. The concentrations of analytes in QC samples were calculated using calibration curves prepared the same day.

The linearity of the method was determined by analyzing blank urine samples (100 μL) containing nine non-zero concentrations of the analytes (Table 2). After sample extraction and their analysis by LC-ESI-MS, the response (peak area) ratio for each analyte to its internal standard was used for calculations. Least-square regression analysis for a linear model was used to calculate all calibration curves A coefficient of determination (r^2) > 0.99 was desirable for all the calibration curves. The lowest standard on the calibration curve was accepted as the lower limit of quantification (LLOQ) if the analyte concentrations of the LLOQ sample was reproducible with a precision (%CV) not worse than 20.0% and accuracy (%DEV) of 80.0–120.0%. The deviation of standards other than LLOQ from the nominal concentration should not be more than ±15.0%, and the precision should not exceed 15.0%.

Six replicates containing different concentrations of analytes in blank urine (100 μL) were used to determine the intra- and inter-assay variations (Table 3). Inter-assay variation was assessed on 6 separate experimental days. The precision of the method was determined by calculating the percent coefficient of variation (%CV) for each specific target concentration, and the accuracy by calculating the percent deviation (%DEV) at each concentration from the nominal target concentration.

Matrix ion suppression/enhancement effects on the LC–ESI-MS sensitivity were evaluated as follows: extract of 100 μL of blank urine (without analyte or internal standard) was dissolved in 100 μL of TFA (0.05%) in water, and analytes were added (six replicates per concentration) (Table 4). The ionization yields of each analyte were determined by comparing the peak area ratios of each analyte of the test samples to those for the same analyte in control samples.

We determined the absolute recovery (extraction and ionization efficiency) of MDMA and metabolites using blank urine samples (100 μL) containing two concentrations of analytes (six replicates per concentration) plus internal standards (Table 4). Each spiked sample was extracted and the absolute recovery was obtained by calculating the peak area ratio of each analyte in the urine samples to that of the same analyte in control samples.

Table 3
Intra- and inter-assay precision and accuracy for determining MDMA, MDA, HMMA, HMA, HHMA and HHA in rat urine.

Analyte	QC sample	Intra-assay		Inter-assay	
	Nominal concentration (ng drug/mL urine)	Precision (%CV)	Accuracy (%DEV)	Precision (%CV)	Accuracy (%DEV)
MDMA	37.5	*n.d.*	*n.d.*	4.9	4.0
	125	3.8	3.1	4.3	4.2
	300	3.7	3.4	2.2	1.7
	550	2.3	2.4	1.3	1
MDA	37.5	*n.d.*	*n.d.*	2.1	0.7
	125	3.5	1.7	3.1	2.5
	300	3.8	1.5	3.5	3.5
	550	3.3	2.3	0.6	2.6
HMMA	37.5	*n.d.*	*n.d.*	3.9	7.9
	125	3.1	4.8	2.6	3
	300	4.6	1.7	2.2	1.7
	550	2.2	0.5	1.9	2.8
HMA	37.5	*n.d.*	*n.d.*	3.8	5.4
	125	3.0	1.2	5.7	4.2
	300	3.3	0.7	2.6	2.7
	550	5	2.8	2.6	2.5
HHMA	75	*n.d.*	*n.d.*	3.3	5.7
	250	4.8	0.3	1.9	3.4
	600	5.7	1	1.4	2.3
	1100	1.7	0.2	2.2	3.9
HHA	225	*n.d.*	*n.d.*	2.6	0.7
	750	3.6	1	2.2	3.2
	1800	6.1	1.5	4.5	3.5
	3300	4.2	0.1	3.2	3.5

Intra-assay precision and accuracy were determined by replicate analyses ($n = 6$) of urine samples (100 μL) containing all the analytes on the same day. Inter-assay precision and accuracy were analyzed at four concentrations of each analyte (six replicates at each concentration). Three concentrations were the same as those used to determine the intra-assay variation, the fourth was a low QC sample. *n.d.*: not determined.

Control samples were prepared by adding the same amounts of reference substances and internal standards to 100 μL of TFA (0.05%) in water (six replicates for each analyte concentration).

2.6. Stability studies

Combined stock solutions of the analytes and the internal standards (in 0.05% aqueous TFA) were checked for freeze–thaw stability by two cycles of freezing (at −20 °C for 1 week) and thawing (without warming) at room temperature. The results were evaluated by measuring the area response of analyte or internal standard in stability test samples against those of freshly prepared combined solutions of analytes or internal standards at identical concentrations. The solutions were considered to be stable if the deviation from the nominal value was within ±15.0%. For assessment of long-term-stability, low and high QC samples were stored at −20 °C for 4 months, with six replicates for each mixture. The samples were considered to be stable if the deviation from the mean calculated concentration of freshly prepared quality control samples (six replicates for each mixture) was within ±15%. Autosampler stability was checked using low and high QC samples. Each dry extract of each QC was reconstituted in TFA (0.05%) in water. All extract of each concentration was pooled and transferred to autosampler vials. Aliquots (20 μL) of the pool were injected 20 times under the conditions used for an analytical run. The absolute response area of each analyte and internal standard measured after each run (34 min) was plotted against the run time (over 680 min), and the relationship analyzed by least-square linear regression. The analytes and internal standards were considered to be stable during LC–MS analysis if the slopes of the regression curves did not differ significantly from zero, using Student's *t*-test. Statistical significance was set at $p < 0.05$.

Table 4
Matrix ion-suppression/enhancement effect and analytical recovery for determining MDMA, MDA, HMMA, HMA, HHMA and HHA in rat urine.

Analyte	Nominal concentrations (ng drug/mL urine)	Matrix effect		Analytical recovery	
		Ionization yield (%)	%CV	Recovery (%)	%CV
MDMA	125	104.2	3.7	86.9	6.2
	500	99.0	4.7	87.0	5.3
MDA	125	98.3	5.3	83.1	6.9
	500	92.9	6.6	85.9	5
HMMA	125	92.1	8.9	79.7	9.6
	500	93.0	4.1	85.7	8.1
HMA	125	88.6	6.2	86.8	7.4
	500	84.5	7.1	83.2	6
HHMA	250	61.2	7.9	63.3	8.3
	1000	88.5	7.5	69.0	6.7
HHA	750	86.4	9.3	51.9	7.2
	3000	78.7	8.9	54.0	7.4
HMP	425	96.5	9.4	83.0	6.3
d5-MDMA	500	*n.d.*	*n.d.*	95.2	5.6
d5-MDA	500	*n.d.*	*n.d.*	93.4	5.1

Ionization yield and recovery were analyzed at two concentrations of each analyte: 125 and 500 ng/mL urine each of MDMA, MDA, HMMA and HMA, 250 and 1000 ng/mL of HHMA, and 750 and 3000 ng/mL of HHA. The ionization yield and analytical recovery of the internal standard, HMP, are also shown, as well as the recoveries of d5-MDMA and d5-MDA. The data result from six replicates and are given as means plus their coefficients of variation. *n.d.*: not determined.

3. Results and discussion

3.1. Method development

3.1.1. Solid-phase extraction

We used SPE because liquid–liquid extraction is not suitable to extract hydrophilic compounds like HHA and HHMA. We tested four sorbents by using 100-μL aliquots of blank urine containing 500 ng/mL each of MDMA, MDA, HMA and HMMA, 1000 ng/mL HHMA, and 3000 ng/mL HHA. The extraction on Bond Elut C18 columns (3 mL/500 mg, Macherey Nagel) was performed according to Katagi et al. [9]. The conditions used for extraction on Chromabond SCX (Strong Cation Exchange) columns (SA, 3 mL/200 mg, Macherey Nagel) were those described in Ref. [3]. The procedure recommended by the manufacturer was followed for the extraction on BEC (Bond Elut Certify) cartridges (3 mL/130 mg, from Varian, Les Ulis, France) [10], and the extraction on Oasis WCX columns was as described in Ref. [7]. The extracted samples were then analyzed by LC–ESI-MS. We have determined the extraction yields for each compound and each extraction procedure.

Briefly, HHMA and HHA were poorly retained on Bond Elut C18 columns (recoveries lower than 20%) although the other analytes were retained more strongly (recoveries higher than 75%). The Chromabond SCX columns retained all the analytes tested, but the extraction recoveries were relatively low and were similar (~50% for all molecules). Retention on BEC columns was better than 80% for the most hydrophobic MDMA and MDA, ~60% for HMMA and HMA, but lower than 5% for the most hydrophilic HHMA and HHA. This suggests that the amount of SCX moieties compared to that of C18 moieties in the BEC cartridge is too low to efficiently retain hydrophilic compounds like HHA and HHMA by ionic interactions. The Oasis WCX columns gave the best extractions of MDMA and its five metabolites (see Table 4 for detailed values). This is not surprising as all three types of interaction are involved in the retention of compounds by these columns: hydrophobic and hydrophilic interactions with the N-vinylpyrrolidone polymer, and ionic interactions with the carboxylate groups on the polymer.

3.1.2. LC–MS

Fig. 1 shows the LC–ESI-MS chromatograms obtained after chromatography on Nucleodur Pyramid C18 columns of an extract of blank urine (100 μL) (Fig. 1A) and an extract of urine of rat treated by MDMA (100 μL) containing MDMA, MDA, HMMA, HMA, HHMA and HHA, plus HMP, d5-MDMA and d5-MDA internal standards (Fig. 1B). The blank urine contained no peaks to interfere with MDMA and its metabolites. The two deuterated IS were eluted with their non-deuterated analogues (Fig. 1B). The low TFA concentration (0.05%) in the mobile phase allows a good retention of the most hydrophilic compounds and is suitable for ESI mode ionization. MDMA and d5-MDMA, and MDA and d5-MDA were resolved under selected ion monitoring mode: the *m/z* ions of the deuterated and non-deuterated compounds were recorded on separate channels so that they could be visualized and integrated separately.

3.2. Method validation

3.2.1. Linearity and lower limit of quantification (LLOQ)

Data for the linearity of the method and the LLOQ are shown in Table 2. All six standard calibration curves analyzed were linear. A straight-line was fitted to the data points by least-square regression analysis. Table 2 gathers the linearity range, regression equations, coefficients of determination (r^2) and their coefficient of variation, the precision (%CV) for specific concentrations of MDMA and its metabolites on the standard curves and the average deviation from theoretical values (%DEV). The lower limit of quantification (LLOQ)

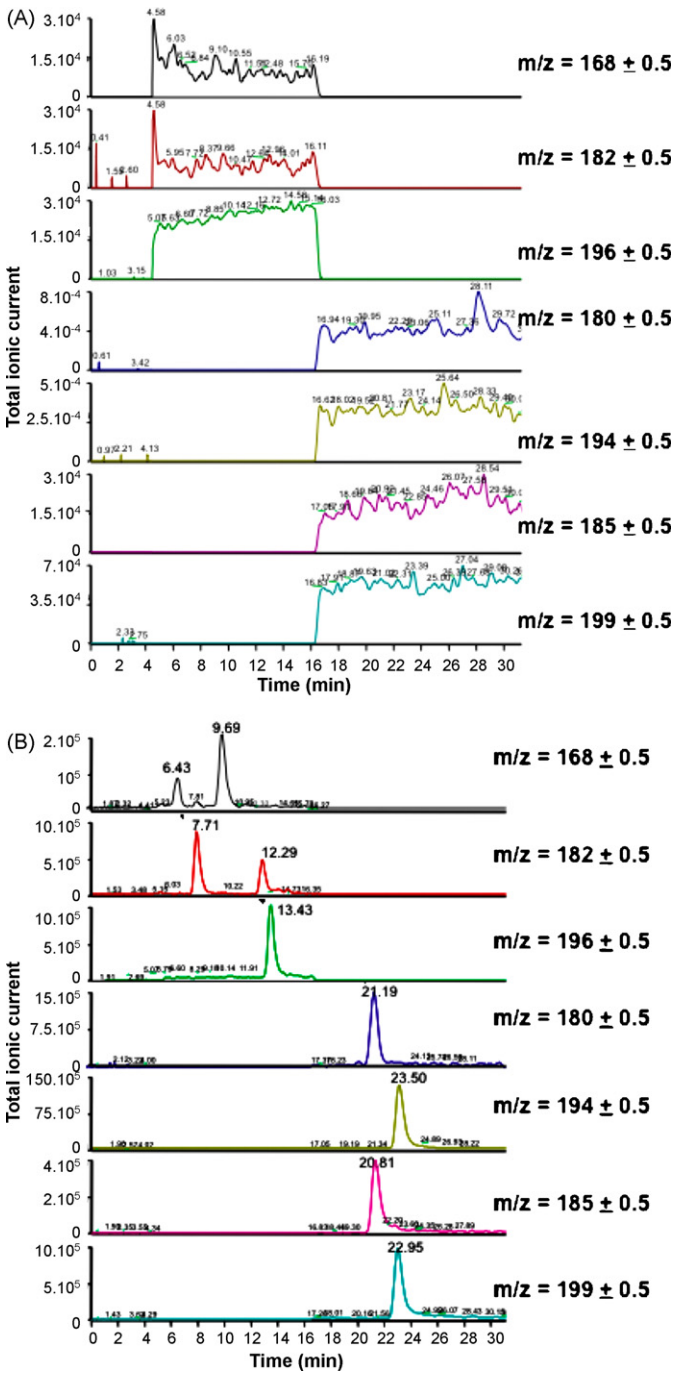


Fig. 1. LC–ESI-MS chromatograms after chromatography on Nucleodur Pyramid C18 columns of (A) a blank urine extract (100 μL) and (B) an extract of urine (100 μL) collected for 24 h from rats given a subcutaneous injection of MDMA (5 mg/kg). Retention times are indicated on the figure. The samples were extracted on WCX Oasis columns prior to chromatography. The MS was operated in positive ionization mode and single ion monitoring (SIM); pseudomolecular ions were detected at *m/z* 168.0 ± 0.5 for HHA and HMP, 182 ± 0.5 for HHMA and HMA, 196 ± 0.5 for HMMA, 180 ± 0.5 for MDA, 185 ± 0.5 for d5-MDA, 194 ± 0.5 for MDMA and 199 ± 0.5 for d5-MDMA. The injection loop (20 μL) and the HPLC column were both kept at 20 °C. The mobile phase was 0.05% (v/v) TFA in water (solvent A) and ACN (solvent B). Analytes were eluted (flow-rate: 200 μL/min) as follows: 2 min with 95% solvent A; linear decrease from 95% to 83% solvent A for 19 min; 2 min at 83% solvent A; linear increase from 83% to 95% solvent A for 1 min; 10 min at 95% solvent A for re-equilibration. The concentrations of HHA (690 ng/mL), HHMA (1590 ng/mL), HMA (950 ng/mL) and HMMA (1210 ng/mL) were determined with an undiluted sample, while the concentration of MDA (5710 ng/mL) was determined using urine diluted 1:8 with blank urine, and that of MDMA (19560 ng/mL) using urine diluted 1:20. Extract of real urine (100 μL) contains the internal standards (42.5 ng HMP and 50 ng of both d5-MDMA and d5-MDA).

of each analyte is shown in Table 2 with associated accuracy and precision.

3.2.2. Intra- and inter-assay variations

These results for intra- and inter-assay variations of all QC samples (Table 3) satisfy the current criteria for bio-analytical methods [8], as the precision (%CV) was <15.0%, and the deviation from nominal concentration (%DEV) was within ±15%.

3.2.3. Matrix ion-suppression effect and recovery

We checked the effect of the matrix on analyte quantification with respect to consistency in signal suppression/enhancement. The matrix slightly inhibited the ionization of HHMA. The average recoveries were 53.0% for HHA, 66.2% for HHMA and over 80% for all the other analytes (Table 4). The relatively high ionization yields for HHA and HHMA plus the relatively low analytical recoveries of these two drugs indicate that the most hydrophilic analytes were not completely retained by the WCX columns. Nevertheless, the low coefficients of variation of the results indicate that the recoveries were highly reproducible.

3.2.4. Stability

Criteria were fulfilled for all analytes.

3.3. Proof of applicability

We measured the concentrations of MDMA and its five main metabolites in the urine of rats for 24 h after they had been given 5 mg/kg MDMA by subcutaneous (sc) injection [7]. The example of a chromatogram obtained thanks to the LC–ESI-MS analysis of urine samples collected for 24 h after MDMA treatment is shown in Fig. 1B.

4. Conclusion

To our knowledge, the work described above turn out to be the first full validation of a LC–ESI-MS procedure capable of simultaneously determining the concentrations of MDMA and its main metabolites (including the catechol-like HHA and HHMA) in urine samples. These urine data provide a useful supplement to the plasma concentrations of MDMA and its metabolites measured during pharmacokinetic and toxicokinetic studies. This method may also be suitable to identify and assay MDMA and its metabolites in human urine, after appropriate validation.

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