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Gas chromatography–ion trap mass spectrometry method for the simultaneous measurement of MDMA (ecstasy) and its metabolites, MDA, HMA, and HMMA in plasma and urine

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ABSTRACT

The investigation of 3,4-methylenedioxymethamphetamine (MDMA; ecstasy) abuse requires very robust methods with high sensitivity and wide linearity ranges for the quantification of this drug of abuse and its main metabolites in body fluids. An optimized gas chromatography–ion trap mass spectrometry (GC–IT/MS) methodology with electron impact ionization addressing these issues is presented. The sample preparation involves an enzymatic hydrolysis of urine and plasma for conjugate cleavage, a SPE extraction, and a derivatization process. The method was fully validated in rat plasma and urine. Linearity for a wide concentration range was achieved for MDMA, and the metabolites 3,4-methylenedioxyamphetamine (MDA), 4-hydroxy-3-methoxyamphetamine (HMA) and 4-hydroxy-3-methoxymethamphetamine (HMMA). Limits of quantification were 2 ng/mL in plasma and 3.5 ng/mL in urine using a Selected Ion Monitoring detection mode. Selectivity, accuracy, precision, and recovery met the required criteria for the method validation. This GC–IT/MS method provides high sensitivity and adequate performance characteristics for the simultaneous quantification of MDMA, MDA, HMA and HMMA in the studied matrices.

1. Introduction

The recreational use of 3,4-methylenedioxymethamphetamine (MDMA, ecstasy), represents one of the most significant trends in drug abuse over the past decades. Although considered as a "safe drug" by many, ecstasy-related deaths and emergency department visits for overdose and unexpected reactions have peaked in recent years [1]. The underlying toxicity mechanisms are diffuse and stem from the capacity of the drug to profoundly disturb the release, transport and receptor responses of the biogenic neurotransmitters such as catecholamines (dopamine and noradrenaline) and serotonin [2]. Also contributing to its toxicological effects is the metabolic bioactivation of MDMA and the metabolic bioactivation of the released neurotransmitters [3]. Long-term toxicity is also worrisome, since chronic abuse has been linked to neurodegeneration of the serotonergic neurons [4]. Measurement of MDMA metabolites is important for the pharmacokinetic profiling of the drug and for the determination of terminal elimination half-lives ($t_{1/2\beta}$) that vary substantially in humans after oral MDMA administration. Metabolites frequently have longer termination half-lives thus increasing the window of drug detection. In a pharmacokinetic study conducted with eight volunteers administered with 100 mg MDMA dose the following mean $t_{1/2\beta}$ values were reported: 9.0 ± 2.3 h for MDMA; 24.9 ± 14.5 h for 3,4-methylenedioxyamphetamine (MDA); 11.2 ± 2.9 h for 3,4-dihydroxymethamphetamine (HHMA) and 37.4 ± 17.9 h for 3,4-dihydroxyamphetamine (HHA) [3]. Also, for toxicokinetic research, MDMA metabolism has received much attention since it has been increasingly acknowledged the role of the metabolic bioactivation of the drug in most of its acute and long-term toxic effects including its neurotoxicity, hepatotoxicity, renal toxicity and cardiotoxicity [5–9]. MDMA metabolism and excretion in humans and animal models is well docu-

Abbreviations: GC–IT/MS, gas chromatography–ion trap mass spectrometry; GC–MS/MS, gas chromatography–tandem mass spectrometry; GC-NPD, gas chromatography-nitrogen/phosphorus detector; HMA, 4-hydroxy-3-metoxyamphetamine; HMMA, 4-hydroxy-3-methoxymethamphetamine; HPLC-UV, high performance liquid chromatography-UV detection; IS, internal standard (4-hydroxy-3-methoxy-benzylamine); LC–MS, liquid chromatography–mass spectrometry; MDA, methylenedioxyamphetamine; MDMA, 3,4-methylenedioxymethamphetamine; NMR, nuclear magnetic resonance; SIM, selected ion mode; ($t_{1/2\beta}$), terminal elimination half-lives.

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mented [10–13]. Over 80% of the ingested MDMA dose is eliminated after hepatic metabolism and around 20% of the dose is excreted unchanged in human urine [14]. N-demethylation of MDMA produces MDA which accounts for less than 10% of the ingested dose [15]. Both MDMA and MDA are O-demethylenated to HHMA and HHA, respectively. These intermediate catechol metabolites, HHMA and HHA, are subsequently O-methylated to 4-hydroxy-3-methoxymethamphetamine (HMMA) and 4-hydroxy-3-methoxyamphetamine (HMA). HMMA is reported to be the major urinary metabolite [10,12], with urinary concentrations approximately equal to those of unchanged MDMA, MDA and HHA are considered minor metabolites in humans [12]. HHMA, HHA, HMMA, and HMA are excreted as glucoronide and sulphate conjugates [10]. The same metabolic pathways are operant in different animal models. The rat has been the most extensively used animal model for the toxicological investigation of MDMA. As with humans, the cytochrome P-450 catalysed O-demethylenation of MDMA followed by the O-methylation of the resulting catechols catalysed by COMT are the main metabolic pathways in this species [13].

Several chromatographic methods including HPLC-UV [16,17], HPLC-fluorescence detection [18–20], GC–MS [21–27], GC-NPD [28] and LC–MS [29–33] methods, have been described in the literature for the determination of MDMA and its metabolites in blood and urine. Among these, GC–MS is the instrumental technique most commonly used for this purpose. Derivatization is usually required to improve chromatography, sensitivity, and reproducibility of these primary and secondary amines [34]. The stereochemical analysis of MDMA and main metabolites has also been reported [35–38].

Every method presents different strengths and limitations, For example, LC–MS methods enable the direct analysis of the glucuronide and sulphate conjugates of the metabolites without need of previous hydrolysis of the samples as long as reference standards of the conjugates are available for quantification [31]. However, these methods have higher LOQ values compared to those obtained with the GC–MS [30,33]. On the other hand, GC–MS methods seem to provide higher sensitivity for the simultaneous quantification of the target analytes [21,22]. Thus, the aim of the present work was to develop and validate an optimized GC–IT/MS methodology that could provide a robust and highly sensitive method to determine simultaneously MDMA and some of its most important metabolites in plasma and urine that could overcome the use of less practical methodologies and of multiple deuterated internal standards.

2. Materials and methods

2.1. Chemicals and reagents

All chemicals and reagents were of the highest grade commercially available. Trifluoroacetic anhydride (TFAA), 4-hydroxy-3-methoxybenzylamine hydrochloride (IS), and Type HP-2 β-glucuronidase from *Helix pomatia* were obtained from Sigma Aldrich (Saint Louis, Missouri), (±)-3,4-Methylenedioxymethamphetamine (MDMA) hydrochloride was obtained by extraction and purified from high-purity MDMA tablets, which were provided by the Portuguese Criminal Police Department. The obtained salt was pure and fully characterized by NMR and MS methodologies. The metabolite 3,4-methylenedioxyamphetamine (MDA) hydrochloride was kindly supplied by the United Nation Drug Control Program (Vienna, Austria). The 4-hydroxy-3-methoxyamphetamine (HMA) and 4-hydroxy-3-methoxymethamphetamine (HMMA) metabolites were synthesized by the Department of Chemistry, Faculty of Science and Technology, University of Nova of Lisboa. The preparation of 3-OMe-α-MeDA (**1**) and 3-OMe-N-Me-α-MeDA (**2**) followed a previously reported procedure for the synthesis of the α-MeDA and N-Me-α-MeDA [5] starting from the corresponding 3-methoxy-4-hydroxybenzaldehyde and nitroethane (Fig. 1). The nitroethene (**3**) was reduced with $LiAlH_4$ to the corresponding primary amine (**1**) which, for stability purposes, reacts subsequently with dry HCl ethereal solution to form the corresponding hydrochloride salt, The preparation of the secondary amine involved an alternative reduction to the ketone (**4**) followed by reductive amination with methylamine and palladium hydrogenation to **2** (Fig. 1). The obtained salts were pure and fully characterized by nuclear magnetic resonance and mass spectrometry methodologies.

2.2. Biological specimens

Drug free (blank) urine and plasma were collected from adult female Wistar rats (Charles-River Laboratories, Barcelona, Spain), weighing 250–300 g. After acclimatization, rats were kept in metabolic cages for 24 h and urine samples were collected over this period. Food and water were available *ad libitum*. To collect plasma, rats were anaesthetized and after surgical incision, blood was collected from caudal vena cava, Whole blood samples were then centrifuged at 1600 × *g* for 15 min for plasma separation, Blank urine and plasma samples were kept at −80 °C before analysis.

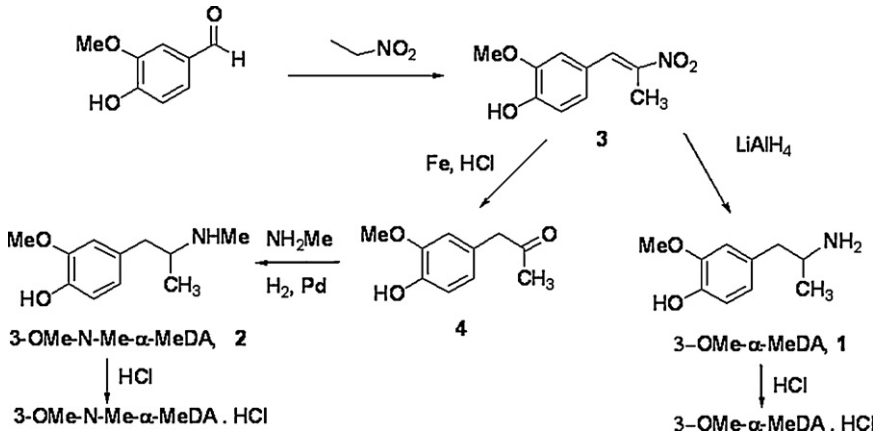

Fig. 1. Representative scheme for the preparation of MDMA metabolites, 3-OMe-α-MeDA (HMA; **1**) and 3-OMe-N-Me-α-MeDA (HMMA; **2**).

For proof of applicability, urine and plasma samples of six adult female Wistar rats belonging to a group of animals of an ongoing experiment were collected 1 h and 24 h after the i.p. administration of 20 mg/kg MDMA, respectively. Sample collection and storage were the same as described above for the blank specimens.

Housing and experimental treatment of the animals were conducted under the European Community guidelines for the use of experimental animals (European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes, 1986, and Protocol of Amendment to the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes, 1998).

2.3. Calibrators and quality control samples

Standards and internal standard stock solutions were prepared at 1 mg/mL in methanol. All intermediate solutions were also prepared in methanol. Working calibrators for both plasma and urine at 2, 10, 20, 25, 50, 100, 200, 500, 800, 1000, 1250, 2000, 5000, and 10,000 ng/mL were prepared by fortifying 1 mL of blank urine or 0.5 mL of blank plasma with the four compounds. Ten μL of 250 μg/mL 4-hydroxi-3-methoxybenzylamine hydrochloride (IS) were added to each urine and plasma specimen before hydrolysis to obtain a final concentration of 2500 ng/mL in urine and 5000 ng/mL in plasma.

2.4. Sample preparation

Hydrolysis of glucuronide and sulphate conjugates. Urine, aliquots of 1 mL of urine samples were transferred to a 25 mL glass tube with 1 mL of 0.2 M sodium acetate buffer (pH 5.2) and 50 μL of Type HP-2 β-glucuronidase from *H. pomatia*, All samples were incubated at 37 °C for 24 h.

Plasma. Aliquots of 500 μL of plasma samples were transferred to a 25 mL glass tube with 500 μL of 0.2 M sodium acetate buffer (pH 5.2) and 25 μL of Type HP-2 β-Glucuronidase from *H. pomatia*, All samples were incubated at 37 °C for 24 h.

Extraction of compounds of interest for GC–IT/MS analysis. After hydrolysis, the samples were extracted using 1 cm^3 (30 mg) OASIS MCX SPE columns obtained from Waters (Milford, Massachusetts), The totality of the hydrolysed sample was applied onto the column, Immediately after, 2 mL of 0.1 M HCL followed by 2 mL of methanol were applied onto the column and the eluates were discarded. Finally, the compounds of interest were eluted into a glass tube using 2 mL of a 5% NH_4OH/methanol solution. The obtained solution was evaporated to dryness under nitrogen flow. To eliminate residual water all tubes were dried under reduced pressure over P_2O_5 and KOH and left open in the exsicator overnight.

Derivatization procedure. To the dry residue, 50 μL of ethyl acetate and 50 μL of TFAA were added, Incubation was preformed at 70 °C for 30 min. After cooling to room temperature the solution was dried under nitrogen flow. The obtained residue was dissolved in 100 μL of ethyl acetate and used without further preparation for GC–IT/MS analysis. A summary of the procedure is shown in Fig. 2.

2.5. Analytical instrument settings

Gas chromatography–mass spectrometry analysis. GC–MS analysis was performed with a Varian CP-3800 gas chromatograph (USA) equipped with a VARIAN Saturn 4000 Ion Trap mass selective detector (USA) and a Saturn GC/MS workstation software version 6.8, The GC was equipped with a VF-5ms (30 m × 0.25 mm × 0.25 μm) capillary column from VARIAN, The carrier gas was Helium C-60 (Gasin, Portugal), at a constant flow of 1 mL/min; 1 μL of derivatized urinary or plasma extract was injected (split ratio was 1:30). The injector port was heated to 220 °C. The initial column temperature of 100 °C was held for 1 min, followed by a temperature ramp of 15 °C/min to 300 °C, with a 10 min post run hold. Total separation run time was 9 min. The transfer line, manifold and ion trap temperatures were 280, 50 and 180 °C, respectively. Ionization was maintained off during the first 4 min, to avoid solvent overloading. The emission current was 50 μA, and the electron multiplier was set in relative mode for the auto-tune procedure. All mass spectra were acquired in electron impact (EI) mode. The mass range was *m*/*z* 50–600, with a scan rate of 6 scan/s. The maximum ionization time was 25,000 μs, with an ionization storage level of 35 *m*/*z*. The data acquisition was performed in Full Scan and/or in Selected Ion Monitoring (SIM) mode. In SIM mode *m*/*z* ions are selected before

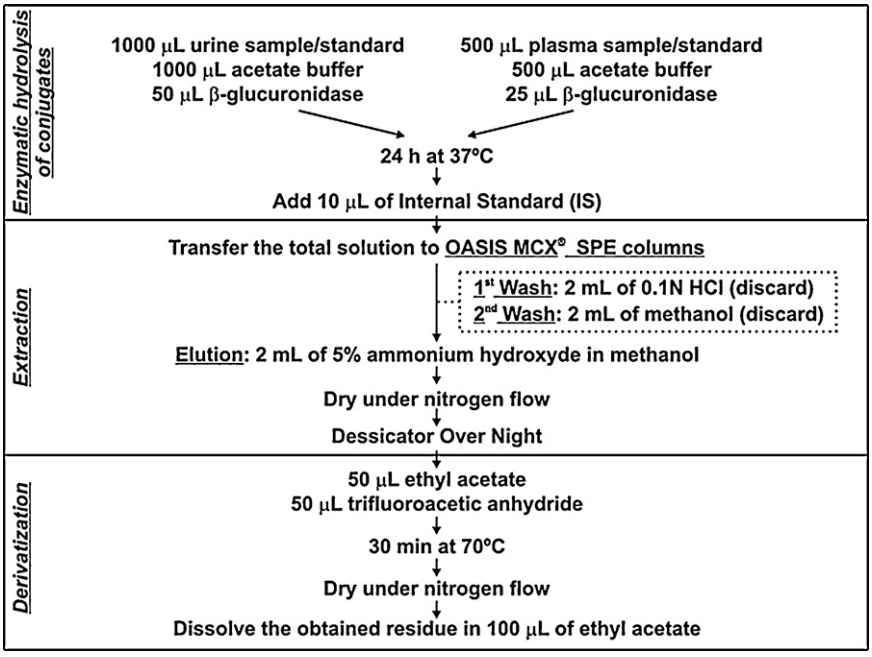

Fig. 2. Summary of sample preparation procedure.

molecule fragmentation, hence only *m*/*z* selected are analysed. The selected ions were: internal standard *m*/*z* 232 and *m*/*z* 345; HMA *m*/*z* 140 and *m*/*z* 260; HMMA, *m*/*z* 154 and *m*/*z* 260; MDMA and MDA *m*/*z* 135 and *m*/*z* 162.

2.6. Assay validation and acceptance criteria

2.6.1. Method linearity

Working calibrators for both plasma and urine at 2, 10, 20, 25, 50, 100, 200, 500, 800, 1000, 1250, 2000, 5000, and 10,000 ng/mL for the four compounds were injected. The method linearity was determined by evaluation of the regression curve (ratio of analyte peak area and IS peak area versus analyte concentration) and indicated by the squared correlation coefficient (R^2). Because the metabolite quantification in urine is of special interest for clinical and/or forensic applications and these metabolites can be found at very low concentrations if sufficient time elapses, linearity for the MDA, HMA, and HMMA metabolites at a lower range of concentrations (0–500 ng/mL) was further tested. Three independent calibration curves were obtained and the mean slopes were calculated.

2.6.2. Limits of detection and quantification

To determine the sensitivity of the method, the 10 ng/mL working calibrators (for MDMA, MDA, HMA and HMMA) of each matrix were progressively diluted with blank plasma or urine to determine the detection (LOD) and quantification limits (LOQ). A signal-to-noise ratio of 3 was considered acceptable for estimating the detection limit [39]. The quantification limit (LOQ) for each calibrator was estimated based on a signal-to-noise ratio of 10. The determined LOD and LOQ values corresponded to the lowest concentration obtained by the successive dilutions of standards that originated a sharp and symmetrical chromatographic peak, In both cases, if peaks were excessively broad, showed tailing or shoulders, or did not resolve to within 10% baseline and if the relative % of the calibration ions was not maintained, they were not considered, and the higher concentration just before was taken [40]. The lowest concentration found to fit these criteria was injected five times onto the GC–IT/MS instrument.

2.6.3. Sample storage/stability

Stability of MDMA, MDA, HMA, and HMMA at three levels of concentration in derivatized plasma extracts (20, 50 and 800 ng/mL) and in derivatized urine extracts (20, 250 and 1250 ng/mL) was evaluated. These samples were kept at 4 °C during a week.

2.6.4. Precision and accuracy

Precision was assessed by calculating the mean, standard deviation and coefficient of variation (CV%) of the observed values. The intra-day precision of the extraction method was performed by extracting five times the same urine sample (500 ng/mL) and five times the same plasma sample (200 ng/mL). The intra-day precision of the apparatus was determined by analysing five times, on the same day, one concentration of each calibration curve (500 ng/mL urine sample, and 200 ng/mL plasma sample). For urine and plasma, the inter-day precision of the method was determined by analysing, at three different days, three different concentrations spanning the linear dynamic range of the assay for MDMA and metabolites in both matrices (50, 100 and 500 ng/mL for urine, and 50, 100 and 200 ng/mL for plasma).

The accuracy of the method (A%) was investigated by the standard additions of low, medium and high control samples (50, 100 and 200 ng/mL for plasma, and 50, 100 and 500 ng/mL for urine), and calculating the percent deviation between the calculated value and the nominal value [accuracy (%) = (mean calculated concentration/nominal concentration) × 100].

2.6.5. Extraction recovery (%)

Recovery for each analyte was assessed at three different concentrations within the linear range of the assay by adding the internal standard to one set of low, medium and high concentration control samples (20, 50 and 800 ng/mL for both plasma and urine), before solid phase extraction and to a second set after extraction, but before evaporation to dryness. Recovery (percentage) was calculated by comparing the peak area ratios of analyte to IS for extracted and non-extracted samples.

2.6.6. Evaluation of interferences and specificity

To evaluate interference and method specificity, several blank (no analyte or IS added) urine and plasma samples were evaluated for co-eluting chromatographic peaks that might interfere with detection of analytes of interest or IS. Internal standard was also tested alone without the interference of the other compounds.

3. Results and discussion

3.1. Sample preparation

For the GC/ITMS analysis of MDMA and its main metabolites, prior cleavage of the conjugates is necessary since the majority of the O-methylated metabolites are excreted in urine as conjugates. O-methylation of MDMA was recently confirmed both in rats and in humans, and it was shown that more than 50% of HMMA was excreted as conjugates in both species (over 72% in humans and over 54% in rats) [41]. Sulfatation is quantitatively more significant than the glucuronidation for HMMA in humans. The opposite was found in the rat where over 99% of conjugated HMMA was excreted as the glucuronide [41]. These results are very important for the selection of the appropriate hydrolysis of the conjugates. Whereas the acidic hydrolysis is adequate for both types of conjugates [25], the enzymatic hydrolysis should be conducted with an enzyme that has both sulfatase and β-glucuronidase activities, as is the case of the Type HP-2 β-glucuronidase from *H. pomatia* used in the present study.

In spite of the multiples steps involved the sample preparation (enzymatic hydrolysis of urine and plasma for conjugate cleavage, SPE extraction, evaporation of solvent, and derivatization) prior to the GC–IT/MS injection, the good results obtained with the reproducibility studies assured the final accurate results as can be seen below. The use of a selective SPE phase (OASIS MCX columns) contributed to the good reproducibility obtained.

3.2. Gas chromatographic separation

The GC conditions for the determination of MDMA, MDA, HMA, and HMMA, resulted in well resolved peaks eluting in less than 9 min. The acquisition of all mass spectra of the complete chromatogram (Full Scan mode) guaranteed the identification of all peaks in each chromatogram. On the other hand, the use of specific ions for the integration allowed more precise peak integration, which is especially important for small peaks. A split ratio of 1:30 was chosen for the analysis. However, if lower limits of quantification are further required the split ratio can be decreased, without compromising the column overloading and peak resolution.

As shown in Fig. 3 the retention times for HMA, MDA, HMMA and MDMA, were respectively 7.5, 7.8, 8.2 and 8.7 min. The IS retention time was 7.0 min. The total time of analysis was 24 min. The chromatogram in Fig. 3 corresponds to a Full Scan reconstructed chromatogram with the following selected ions: *m*/*z* 135, *m*/*z* 162 (for both MDA and MDMA), *m*/*z* 154 (for HMMA), *m*/*z* 260 and 140 (for HMA), and *m*/*z* 232 (for IS). The acquisition of the chromatogram in Full Scan mode allows the access to some other chromatographic peaks with interest that could be identified latter.

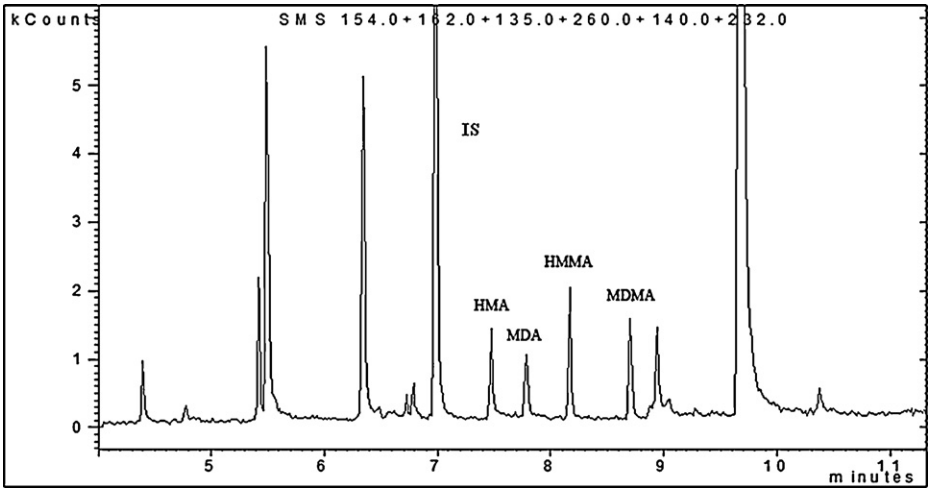

Fig. 3. Reconstructed Full Scan chromatogram with the following ions: m/z = 135, m/z = 162 (both for MDA and MDMA) m/z = 154 (HMMA), m/z = 260 and 140 (HMA), and m/z = 232 (IS), HMA (7.5 min), MDA (7.8 min), HMMA (8.2 min) and MDMA (8.7 min), IS (7.0 min).

Table 1
Calibration curves for HMA, MDA, HMMA and MDMA in plasma and urine for high a range of concentrations (0–10,000 ng/mL).

	Linearity study		
	Equation	Range (ng/mL)	R^2
Plasma			
HMA	$y = 0.005x + 0.0022$	0–10,000	0.9944
MDA	$y = 0.0004x + 0.0074$	0–10,000	0.9983
HMMA	$y = 0.0007x + 0.0194$	0–10,000	0.9963
MDMA	$y = 0.0004x - 0.0139$	0–10,000	0.9995
Urine			
HMA	$y = 0.0008x - 0.0177$	0–10,000	0.9985
MDA	$y = 0.0046x - 0.0972$	0–10,000	0.9989
HMMA	$y = 0.0021x - 0.0778$	0–10,000	0.9933
MDMA	$y = 0.0045x + 0.263$	0–10,000	0.9996

However, the method sensitivity can be increased by analysing the extracts in SIM mode (with selected ions in the ion trap source).

3.3. Quantification procedure and choice of suitable IS

Several chromatographic methods have been developed to determine MDMA and metabolites in plasma and urine [17–20,23,24,29,30,32,33], including stereochemical analysis [35–38]. Of these, some address the combined determination of all four target analytes [16,21,22,25–28,31], and these require the synthesis of the ortho-methylated metabolites.

Among these methods, GC–MS techniques are the most frequently adopted since they seem to provide higher sensitivity for the simultaneous quantification of the target analytes [21,22]. An advantage of the LC–MS methods is the possibility of analysing directly the glucuronide and sulphate conjugates of the metabolites without need of previous hydrolysis of the samples as long as reference standards of the conjugates are available for quantification [31]. However, these methods have higher LOQ values compared to those obtained with the GC [30,33]. Only the LC/MS method described by Shima et al. has comparable LOQ values for free HMMA [31]. Even greater LOQ (above 90 ng/mL) were obtained in human urine samples analysed by GC-NPD [28].

Quantification was carried out by comparison of peak area ratio (analyte versus IS), with calibration curves obtained with spiked calibrators for both plasma and urine matrices. In most of the methods previously reported several IS have been used for the simultaneous determination of MDMA and its metabolites. These included the deuterated analogues of MDMA and MDA and pholedrine for the catechol-O-methylated metabolites [21,25–27]. In the present study we tested one single IS that proved to be suitable for the quantification of all the target analytes. The 4-hydroxy-3-methoxybenzylamine compound selected as IS has a chemical behavior similar to the analytes both for the extraction and derivatization procedures, and its chromatographic retention time is adequate. Therefore, the utilization of this IS for GC–IT/MS is an advantage compared to those previously reported, since deuterated internal standards are much more expansive.

3.4. Method validation

3.4.1. Selectivity

Several blank urine and plasma extracts were analysed to evaluate chromatographic interference. No interference peaks were

Table 2
Calibration curves for HMA, MDA, and HMMA in plasma and urine for a 0–500 ng/mL concentration range.

	Linearity study		
	Equation	Range (ng/mL)	R^2
Plasma			
HMA	$y = 0.0002(0.00005)x + 0.0154(0.00242)$	0–500	0.9813
MDA	$y = 0.0007(0.00005)x - 0.0281(0.00449)$	0–500	0.9843
HMMA	$y = 0.0008(0.00005)x - 0.0261(0.0003179)$	0–500	0.9807
Urine			
HMA	$y = 0.001(0.000306) + 0.0015(0.000458)$	0–500	0.9996
MDA	$y = 0.002567(0.0014) + 0.0677(0.0065)$	0–500	0.9996
HMMA	$y = 0.0019(0.000321) + 0.026(0.0251)$	0–500	0.9999

n = 3 (SD).

Table 3
LOD and LOQ values in ng/mL for HMA, MDA, HMMA and MDMA in plasma and urine.

	Full Scan (n = 10)		SIM (n = 3)	
	LOD	LOQ	LOD	LOQ
Plasma				
HMA	3.5	10 (7.1)	2.0	5 (4.8)
MDA	3.5	10 (4.5)	2.0	5 (8.0)
HMMA	3.5	10 (8.4)	2.0	5 (9.2)
MDMA	3.5	10 (10.3)	2.0	5 (6.8)
Urine				
HMA	5.0	15 (12.5)	3.5	10 (13.2)
MDA	5.0	15 (9.8)	3.5	10 (4.2)
HMMA	5.0	15 (4.1)	3.5	10 (4.1)
MDMA	5.0	15 (7.0)	3.5	10 (12.0)

( ): CV%.

detected, neither in the retention time of the analytes nor in the IS retention time.

3.4.2. Linearity

Regression analysis of calibration data achieved satisfactory linearity over a wide concentration range (0–10,000 ng/mL). Square correlation coefficients (R^2) were always higher than 0.99, indicating a linear relationship from five point calibration curves for MDMA, MDA, HMA, and HMMA in plasma and urine. Linearity studies were also performed for a narrower concentration range (0–500 ng/mL), only for the metabolites. The obtained slopes and square correlation coefficients are presented in Tables 1 and 2.

3.4.3. Sensitivity

LOD values were established by extraction of urine and plasma samples containing decreasing concentrations of MDMA, MDA, HMA, and HMMA, The mean LOD for each compound was determined for both Full Scan and SIM detection modes. The LOD values in Full Scan mode were 5 ng/mL for urine samples, and 3.5 ng/mL for plasma samples, for all the target compounds. Using the SIM-ITMS detection mode it was possible to decrease the LOD to 2 ng/mL in plasma, and 3.5 ng/mL in urine for all analytes. The use of GC–MS in SIM mode also improved the LOQ values (Table 3).

3.4.4. Precision

This GC–IT/MS method also showed satisfactory (CV < 15%) intra- and inter-assay precision.

3.4.4.1. Intra-day precision of the method and apparatus. The intra-day precision of the extraction method was estimated based upon five independent manipulations of the same urine (500 ng/mL) and plasma (200 ng/mL) samples. For urine, the coefficient of variation (CV%) varied between 4.8 and 11%, for plasma the CV% varied between 3.1% and 9.9% (Table 4).

Table 4
Intra assay precision for the determination (ng/mL) of HMA, MDA, HMMA and MDMA.

	Precision (%)			
	HMA	MDA	HMMA	MDMA
Plasma				
GC–MS method	3.96	4.12	5.00	4.87
Extraction method	3.10	9.92	4.96	9.88
Urine				
GC–MS method	6.81	8.76	9.65	3.57
Extraction method	4.77	12.37	9.11	11.03

Precision (%)—SD/average (n = 5).

Table 5
Inter-assay precision and accuracy for the determination (ng/mL) of HMA, MDA, HMMA, and MDMA in plasma.

Plasma	Expected concentration	Observed concentration	Precision (%)	Accuracy (%)
HMA	50	57.3 (8.3)	14.50	113.2
	100	120.3 (12.1)	10.10	106.3
	200	215.5 (5.03)	2.33	107.8
MDA	50	53.3 (5.18)	9.72	106.6
	100	92.1 (14.7)	15.90	92.1
	200	193.8 (12.4)	6.37	96.9
HMMA	50	52.8 (2.28)	4.32	105.6
	100	95.1 (4.10)	4.32	95.1
	200	180.6 (2.23)	1.23	90.3
MDMA	50	47.1 (2.98)	6.33	94.2
	100	115.5 (15.9)	13.79	115.5
	200	216.3 (12.2)	5.61	108.2

n = 3, samples injected in duplicate.

The intra-day precision of the apparatus was determined after five injections of the same extract of urine (500 ng/mL) and plasma (200 ng/mL), The obtained CV% results varied between 3.6 and 9.7% for urine and between 4 and 5% for plasma (Table 4).

3.4.4.2. Inter-day precision and accuracy of the method. The results for the two matrices at three representative concentrations within the linear range of the assay are presented in Tables 5 and 6. The obtained CV% was always inferior to 15%, except for the plasma MDA 100 ng/mL calibrator (CV 15.9%), The average coefficients of variation (ACV%) for all the tested concentrations were lower than 10% (6.6% for plasma and 7.4% for urine).

The accuracy, calculated as the percentage of target concentration, was 92.6–119.8% for urine, 90.3–115.5% for plasma, The proposed acceptance limits for this parameter was 100 ± 20% [39], and the obtained accuracy results were within these limits (Tables 5 and 6).

3.4.5. Extraction recovery

At three different concentrations, spanning the linear dynamic range of the assay (20, 50 and 800 ng/mL for both plasma and urine), mean overall extraction efficiencies for urine were >89% (75–99%) and >85% (72–99%) for plasma (Table 7).

3.4.6. Processed sample stability

The stability of the derivatized extracts at three levels of concentration for the two matrices was evaluated during a week. Samples were kept at 4 °C and the same extract was injected just after

Table 6
Inter-assay precision and accuracy for the determination (ng/mL) of HMA, MDA, HMMA, and MDMA in urine.

Urine	Expected concentration	Observed concentration	Precision (%)	Accuracy (%)
HMA	50	59.9 (3.8)	6.30	119.8
	100	94.3 (3.7)	3.90	94.3
	500	484.1 (12.4)	2.60	96.8
MDA	50	52.7 (3.3)	6.34	105.4
	100	93.2 (4.1)	6.63	93.2
	500	466.9 (29.6)	6.34	93.4
HMMA	50	52.2 (12.2)	10.20	104.4
	100	92.6 (12.3)	13.30	92.6
	500	492.1 (15.1)	3.10	98.4
MDMA	50	48.4 (3.0)	6.20	96.8
	100	116.7 (12.2)	12.10	116.7
	500	556.2 (58.3)	10.50	111.2

n = 3, samples injected in duplicate.

Table 7
Recovery results for the determination of HMA. MDA, HMMA and MDMA in urine and plasma.

	Recovery (%)			
Concentration, ng/mL	HMA	MDA	HMMA	MDMA
Plasma				
20	99	86	90	97
50	86	79	76	98
800	83	72	74	74
Urine				
20	88	87	84	91
50	99	94	88	92
800	95	84	75	85

being prepared, and 3 and 5 days after preparation. Table 8 shows the CV (%) for each day of analysis. Intra-day coefficients of variation are low, even at the 5th day. However, at that time, several unknown peaks interfered in the base line of the chromatogram, The urine extract was deteriorated (visual aspect), some coloured compounds were formed, which can pollute the insert liner (injector) and consequently damage the chromatographic column. It is therefore recommendable that the extracts are analysed in a relatively short period after preparation.

3.5. *Proof of applicability*

Urine and plasma of six adult female Wistar rats were collected after the i.p. administration of 20 mg/kg MDMA. The plasma concentrations determined 1 h after MDMA administration is shown in Table 9. A representative chromatogram of a urine sample collected 24 h after MDMA administration is also presented in Fig. 4. The plasma concentrations ranged between 1510 and 3420 ng/mL for MDMA; 218 and 615 ng/mL for MDA, 22 and 33 ng/mL for HMA and 257 and 928 ng/mL for HMMA. In a previously published study with male Dark Agouti rats to which the same 20 mg/kg i.p. MDMA dose was administered, the authors report very similar values and also noted some variability in the plasma profile of MDMA and metabolites in their 2 h study (e.g. Cmax for MDMA: 1767–4559 ng/mL; Cmax for MDA: 688–1417 ng/mL) [27]. Our results agree well to this previous study where the same animal species, MDMA dose and route of drug administration were used and with comparable sampling times.

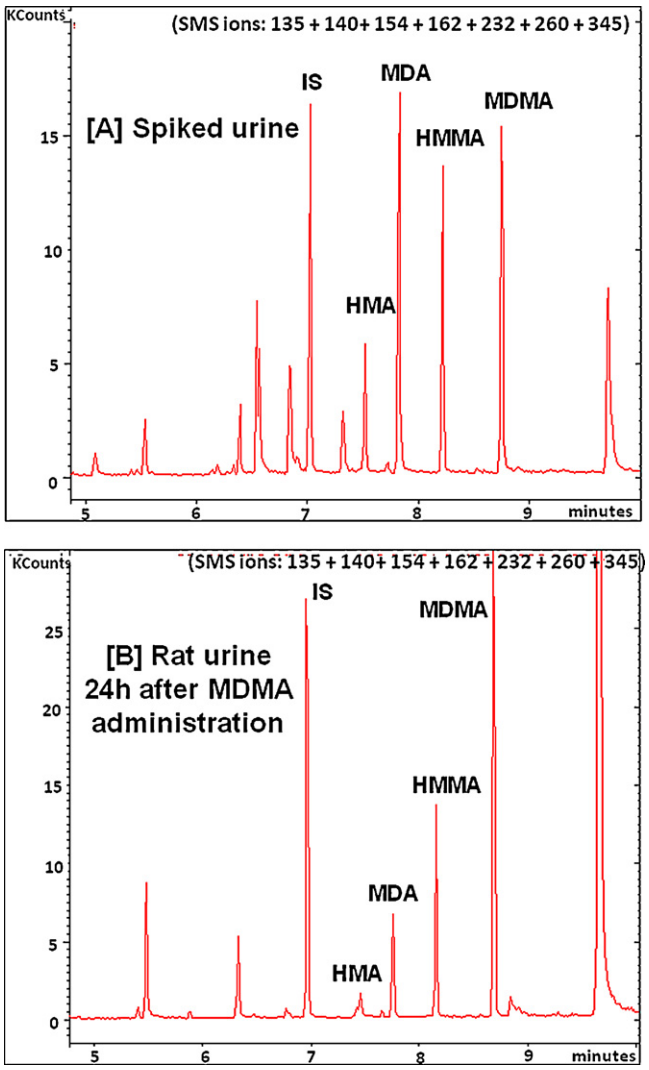

Fig. 4. Reconstructed Full Scan chromatograms with the following ions: $m/z = 135$, $m/z = 162$ (both for MDA and MDMA) $m/z = 154$ (HMMA), $m/z = 260$ and 140 (HMA), and $m/z = 232$ (IS), HMA (7.5 min), MDA (7.8 min), HMMA (8.2 min) and MDMA (8.7 min), IS (7.0 min), chromatogram obtained with rat urine spiked with MDMA and metabolites (A), chromatogram obtained with rat urine 24 h after 20 mg/kg MDMA i.p. administration (B).

Table 8
Results from the stability studies of the derivatized extracts.

	Plasma					Urine			
	HMA	MDA	HMMA	MDMA		HMA	MDA	HMMA	MDMA
Day 1					Day 1				
CV% (20 ng/mL)	11.33	11.61	8.33	8.55	CV% (20 ng/mL)	5.14	6.85	5.20	1.15
CV% (50 ng/mL)	9.27	7.17	1.78	9.00	CV% (250 ng/mL)	4.69	5.52	6.43	5.51
CV% (800 ng/mL)	9.63	3.17	7.23	5.30	CV% (1250 ng/mL)	6.96	10.70	2.40	9.61
Day 3					Day 3				
CV% (20 ng/mL)	7.13	4.52	8.37	10.28	CV% (20 ng/mL)	9.86	7.67	4.09	3.10
CV% (50 ng/mL)	7.37	11.61	1.83	6.92	CV% (250 ng/mL)	1.94	2.16	2.53	2.17
CV% (800 ng/mL)	3.58	2.46	4.17	2.67	CV% (1250 ng/mL)	5.62	4.20	3.77	2.34
Day 5					Day 5				
CV% (20 ng/mL)	6.87	7.08	6.23	8.86	CV% (20 ng/mL)	10.66	13.62	6.48	12.65
CV% (50 ng/mL)	3.90	11.83	3.82	10.67	CV% (250 ng/mL)	8.22	4.86	6.10	4.80
CV% (800 ng/mL)	4.81	3.43	6.43	2.53	CV% (1250 ng/mL)	9.70	10.00	6.18	6.00
Interday					Interday				
ACV% (20 ng/mL)	9.74	28.96	4.18	21.86	ACV% (20 ng/mL)	4.38	1.68	13.00	8.18
ACV% (50 ng/mL)	21.20	13.28	14.84	16.20	ACV% (250 ng/mL)	7.75	2.65	20.89	26.84
ACV% (800 ng/mL)	6.57	10.83	19.03	20.22	ACV% (1250 ng/mL)	3.11	3.30	24.93	28.93

Table 9
Concentration of MDMA and metabolites (ng/mL) in plasma of female Wistar rats 1 h after 20 mg/kg MDMA i.p. administration.

Plasma samples				
T (1 h)	MDMA	MDA	HMA	HMMA
1	3282.98	276.83	26.6	920.92
2	3170.71	275.9	29.39	703.07
3	2477.67	615.24	32.4	498.9
4	1510.75	217.81	22.06	257.51
5	3419.99	284.34	33.41	928.55
6	1847.51	268.08	25.8	371.46
Mean	2618.3	323.0	28.3	613.4
SD	803.8	145.1	4.3	282.9

$n = 6$.

4. Conclusions

A robust, highly sensitive and relatively inexpensive GC–IT/MS assay for the simultaneous quantification of MDMA, MDA, HMA, and HMMA in plasma and urine is presented. The developed and validated GC–IT/MS method presently described proved to be an optimized alternative to those previously published in the literature. By using a single and commercially available internal standard the method proved to be robust, to have high sensitivity and a wide linearity range. This is especially important for the clinical and/or forensic application of the method, since the concentrations of MDMA and of its metabolites are highly variable and depend upon sampling time, the ingested formulations and doses, and also on the individual metabolism. Most of the MDMA administered doses are excreted within the first 24 h after exposure [35]. Therefore, although identification of parent drug of abuse is needed for unequivocal determination of MDMA abuse (e.g. MDA is abused per se and is a result methamphetamine metabolism), the detection of metabolites will longer termination half-lives can become extremely helpful if long periods of time have elapsed. For either in vivo or in vitro toxicokinetic studies it is also common to find high concentrations of the parent drug but much lower concentrations of the metabolites. For example, after oral administration of 100 mg MDMA to eight volunteers, mean plasma concentration attained were 222.5 ± 26.1 ng/mL for MDMA; 13.1 ± 4.5 ng/mL for MDA; 236.7 ± 87.1 ng/mL for HMMA and 7.5 ± 4.0 ng/mL for HMA [3]. These concentrations fall well within the sensitivity and linearity range of this method. Therefore, the developed analytical methodology has direct application not only to research purposes but, also importantly, to the control of drug abuse in humans.

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