Determination of MDMA and its Metabolites in Blood and Urine by Gas Chromatography–Mass Spectrometry and Analysis of Enantiomers by Capillary Electrophoresis

Nieves Pizarro[1], Jordi Ortuño[1], Magí Farré[1,2], Cándido Hernández-López[1], Mitona Pujadas[1], Amadeu Llebaria[3], Jesús Joglar[3], Pere N. Roset[1], Marta Mas[1], Jordi Segura[1,4], Jordi Camí[1,4], and **Rafael de la Torre[1,2,4,*]**

[1]Pharmacology Research Unit, Institut Municipal d'Investigació Mèdica (IMIM); [2]Universitat Autònoma and [4]Universitat Pompeu Fabra; and [3]Department of Biological Organic Chemistry, Institut de Investigacions Químiques i Ambientals, E-08003 Barcelona, Spain

Abstract

A gas chromatography–mass spectrometry (GC–MS) method was used for the simultaneous quantitation of 3,4-methylenedioxymethamphetamine (MDMA) and the 3,4-methylenedioxyamphetamine (MDA), 4-hydroxy-3-methoxymethamphetamine (HMMA), and 4-hydroxy-3-methoxyamphetamine (HMA) metabolites in plasma and urine samples after the administration of 100 mg MDMA to healthy volunteers. Samples were hydrolyzed prior to a solid-phase extraction with Bond Elut Certify® columns. Analytes were eluted with ethyl acetate (2% ammonium hydroxide) and analyzed as their trifluoroacyl derivatives. Linear calibration curves were obtained at plasma and urine concentration ranges of 25–400 ng/mL and 250–2000 ng/mL for MDMA and HMMA, and of 2.5–40 ng/mL and 100–1000 ng/mL for MDA and HMA. Following the same urine preparation procedure but without the derivatization step, a capillary electrophoresis (CE) method for enantiomerical resolution of compounds was developed using (2-hydroxy)propyl-β-cyclodextrin at two different concentrations (10 and 50mM in 50mM H_3PO_4, pH 2.5) as chiral selector. Calibration curves for the CE method were prepared with the corresponding racemic mixture and were linear between 125 and 2000 ng/mL, 50 and 1000 ng/mL, and 125 and 1500 ng/mL for each enantiomer of MDMA, MDA, and HMMA, respectively. Stereoselective disposition of MDMA and MDA was confirmed. HMMA disposition seems to be in apparent contradiction with MDMA findings as the enantiomer ratio is close to 1 and constant over the time.

* Author to whom correspondence should be addressed. Rafael de la Torre, Pharmacology Research Unit, Institut Municipal d'Investigació Mèdica (IMIM), Doctor Aiguader 80, E-08003 Barcelona, Spain. E-mail: rtorre@imim.es.

Introduction

3,4-Methylenedioxymethamphetamine (MDMA, "ecstasy") is an amphetamine derivative widely used as a recreational drug among youth. It has been involved in an increasing number of acute intoxications, some of which have resulted in death (1–4). Deleterious long-term effects of the drug seem to be associated with progressive neurodegeneration of the serotoninergic system (5).

In relation to the metabolism of MDMA, its main metabolites include 3,4-methylenedioxyamphetamine (MDA), 4-hydroxy-3-methoxymethamphetamine (HMMA), and 4-hydroxy-3-methoxyamphetamine (HMA), with 3,4-dihydroxyamphetamine (HHA) and 3,4-dihydroxymethamphetamine (HHMA) as metabolic intermediates. The pharmacological effects of MDMA in humans have been evaluated in a limited number of studies with single-dose protocols. Verebey et al. (6) tested 50 mg of MDMA in one volunteer and quantitated MDMA and MDA in plasma and urine samples. Helmlin et al. (7) studied two patients given 1.5 mg/kg of MDMA and quantitated HMMA and HMA in urine. MDMA and vasopressin hormone were studied by Henry et al. (8) in eight volunteers who received 40 mg of MDMA. In a previous study by our group (9), pharmacokinetics and cardiovascular and neuroendocrine effects of MDMA and MDA at doses of 75 and 125 mg were assessed. We recently reported quantitation of HMMA and HMA in plasma samples of healthy volunteers treated with 100 mg MDMA (10).

MDMA is a chiral compound used in its racemic form (*R*,*S*-MDMA), the (*S*)-MDMA enantiomer being the most psychoactive (11). In animal models (12,13), MDMA showed a stereoselective disposition resulting in *R*/*S* MDMA and HMMA ratios > 1 and *R*/*S* MDA and HMA ratios < 1, except in mice that excreted similar amounts of enantiomers. Enantioselective

metabolism in humans has also been reported. Urinary results from MDMA intoxications (14,15) seem to confirm findings in animal models. Lanz et al. (16) analyzed urine samples of two subjects who received 1.5 mg/kg of racemic MDMA and found the following: most MDMA was excreted unchanged, *R/S* ratios increased progressively over time, and (*R*)-MDMA was practically the only compound detected at 72 h after drug administration. On the other hand, MDA showed an inversion in its ratio in both volunteers and HMMA results reflected large interindividual differences (no enantiomerical identification could be done for either MDA or HMMA). Hensley et al. (17) reported that *R/S* MDA ratio crossed between approximately 24–36 h after dosing, and Fallon et al. (18) simultaneously performed a very comprehensive MDMA and MDA enantiomerical study in plasma and urine samples reporting pharmacokinetic parameters and urinary recovery data.

We report a validated method for the simultaneous analysis of MDMA and its main metabolites in plasma and urine by gas chromatography–mass spectrometry (GC–MS) and a validated capillary electrophoresis (CE) method for the analysis of enantiomers of MDMA, MDA, and HMMA in urine. These methods were applied for the determination of MDMA and its main metabolites in biological fluids from clinical studies in healthy volunteers. To our knowledge, HMMA enantiomerical ratios are presented here for the first time and their possible metabolic implications are discussed.

Methods

Materials and reagents

MDMA, MDA, HMMA, HMA, and the internal standards MDMA-d_5, MDA-d_5, and codeine were pursached from Lipomed (Arlesheim, Switzerland). Enantiomerically enriched standards for (*S*)-MDMA and (*S*)-HMMA were synthesized in our laboratory (19). 3,4-Methylenedioxypropylamphetamine (MDPA) (internal standard, I.S.) was supplied by Alltech-Applied Sciences (State College, PA). Pholedrine (4-hydroxymethamphetamine) (I.S.) was generously given by Deutsche Sporthochschule (Biochemistry Department, Cologne, Germany).

N-Methyl-bis(trifluoroacetamide) (MBTFA) (GC grade) was purchased from Macherey-Nagel (Düren, Germany). Acetic acid (glacial), ethyl acetate, ortho-phosphoric acid, ammonia solution, sodium acetate trihydrate, disodium hydrogen phosphate anhydrous, sodium hydrogen phosphate monohydrate (reagent grade), and methanol (high-performance liquid chromatographic [HPLC] grade) were obtained from Merck (Darmstadt, Germany). β-Glucuronidase from *Helix pomatia* (HP-2) was supplied from Sigma (St. Louis, MO). (100,000 and 7500 units per milliliter for β-glucuronidase and sulfatase activities, respectively). α-Cyclodextrin was obtained from Merck and β-cyclodextrin, γ-cyclodextrin, (2-hydroxy)propyl-β-cyclodextrin (2-OHP-β-CD), and heptakis(2,6-di-O-methyl)-β-cyclodextrin were purchased from Hewlett-Packard (Palo Alto, CA). Ultra pure water was obtained using Milli-Q purification system (Millipore, Molsheim, France). Bond Elut Certify® solid-phase extraction columns were obtained from Varian Sample Preparation Products (Harbor City, CA). The extraction was performed on a Vac-Elut vacuum manifold (Supelco, Bellefonte, PA). Drug-free urine was a drug urine bulk supplied from Bio-Rad Laboratories, S.A. (Barcelona, Spain). Drug-free plasma was obtained from Hospital del Mar blood bank (Barcelona, Spain).

Clinical studies

Biological samples were obtained from six male healthy volunteers who were recreational users of MDMA. All participants gave the written informed consent and the study was approved by the institutional review board and authorized by the Spanish Ministry of Health (AEM no. 98/112). MDMA was obtained from the Spanish Ministry of Health, and MDMA soft gelatin pills were prepared and supplied by the Department of Pharmacy of Hospital del Mar. Participants were phenotyped with dextromethorphan for CYP2D6 enzyme activity, and all were categorized as extensive metabolizers (20). The study protocol included urine drug testing for amphetamines and synthetic drugs as well as other drugs of abuse (cocaine, cannabinoids) before participation in every experimental session. Individuals testing positive were excluded from the study. Blood samples were obtained through a catheter inserted in a peripheral vein before drug administration (baseline) and at 0, 15, 30, 45, 60, 75, and 90 min and at 2, 3, 4, 6, 8, 10, and 24 h after the administration of the drug. The heparinized blood was centrifuged at $1100 \times g$ for 10 min, and plasma was transferred to polypropylene tubes and stored at –20°C until analysis. Urine samples were collected before and after drug administration at 0–2, 2–4, 6–8, 8–10, and 10–24 h time periods and stored at –20°C until analysis.

Instrumentation

A GC (HP 6890 series GC system, Hewlett-Packard, Palo Alto, CA) equipped with a quadrupole MS (HP 5973 mass selective detector) and autosampler (7683 series injector) was used for MDMA, MDA, HMMA, and HMA analysis using MDMA-d_5 as MDMA I.S., MDA-d_5 as MDA I.S., and pholedrine as both HMMA and HMA I.S. Separation was performed using a cross-linked 5% phenylmethylsiloxane capillary column (12 m × 0.2-mm i.d., 0.3-µm film thickness) (HP, Ultra-2). Helium was used as carrier gas at a flow rate of 1.2 mL/min. The oven was maintained at 70°C over 2 min, and then four consecutive rates

Table I. MDMA and its Metabolites Selected Ions for GC–MS Identification and Quantitation

Compound	Derivative	*m/z**
MDMA	*N*-TFA	**154**, 162, 289
MDA	*N*-TFA	135, **162**, 275
HMMA	*N*-TFA, O-TFA	110, **154**, 260
HMA	*N*-TFA, O-TFA	140, 163, **260**
MDMA-d_5	*N*-TFA	**158**, 164, 294
MDA-d_5	*N*-TFA	136, **167**, 280
Pholedrine	*N*-TFA, O-TFA	110, **154**, 230

* Ions selected for quantitation in bold face.

were programmed as follows: first, from 70°C to 160°C at 30°C/min; second, from 160°C to 170°C at 5°C/min; third, from 170°C to 200°C at 15°C/min; and fourth, from 200°C to 280°C at 30°C/min with a total run time of 11.67 min. Samples were injected in the splitless mode. Insert liners packed with silanized glasswool were used. Injector and interface were set at 280°C.

The MS was operated by electron ionization (70 eV) and in the selected ion monitoring (SIM) acquisition mode. Three ions were selected for each substance and the cleanest ion trace was used for quantitation (Table I). Ions selected for quantitation were used consistently from sample to sample. Ion ratios of selected ions for the quantitation of each substance and the internal standard were always used.

The CE system (^{3D}CE, Hewlett-Packard) used for the enantiomeric study of MDMA, MDA, and HMMA was equipped with a diode-array detector. MDPA was used as I.S. for both MDMA and MDA and codeine as the HMMA I.S. Separation was performed in an untreated fused-silica capillary of 48.5-cm total length (40-cm effective length) and a standard 50-µm optical path length cell. A constant voltage of 30 kV was applied and the cartridge temperature was maintained at 15°C. The diode-array detector was set to monitor the signal at 204 nm. Two different concentrations of (2-hydroxy)propyl-β-cyclodextrin in 50mM H_3PO_4 at pH 2.5 as running buffer were selected for the chiral separation of analytes. MDMA and MDA enantiomers were separated at a concentration 10mM of cyclodextrin, whereas HMMA enantiomers were separated at 50mM. Preconditioning conditions before each experiment included 50mM H_3PO_4 for 1 min (when chiral selector concentration was 10mM) or 1.5 min (when its concentration was 50mM) and running buffer for 1 min. Injection of the sample was done by applying 50 mbar of external pressure for 2 s. Buffer solutions were freshly prepared on each experimental day.

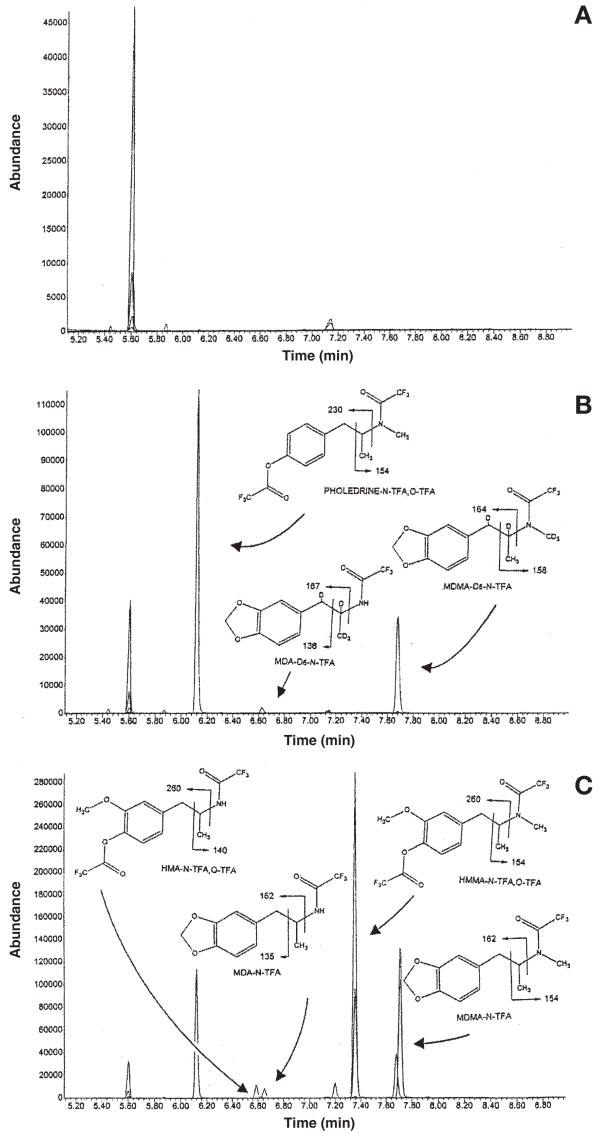

Figure 1. GC–MS ion chromatograms with proposed fragmentation pattern: urine blank (A); urine spiked with I.S., MDMA-d_5 (*m/z* 158, 500 ng/mL), MDA-d_5 (*m/z* 167, 250 ng/mL), and pholedrine (*m/z* 154, 250 ng/mL) (B); and (C) urine spiked with MDMA (*m/z* 154, 2000 ng/mL), MDA (*m/z* 162, 1000 ng/mL), HMMA (*m/z* 154, 2000 ng/mL), HMA (*m/z* 260, 1000 ng/mL), and I.S. as in B.

Synthetic procedure for enantiomerically enriched standards

Preparation of enantiomerically enriched standards has been already reported (19), Briefly, (*S*)-MDMA was obtained from enantiomerically enriched 3-(3,4-dibenzyloxyphenyl)-2-propanamine that was synthesized from commercially available 3,4-dibenzyloxybenzaldehyde after a resolution with dibenzoyl-(–)-tartaric acid. (*S*)-HMMA was obtained by an asymmetric synthesis from commercially available 4-benzyloxy-3-methoxybenzaldehyde and (*S*)-(+)-α-methylbenzylamine.

Preparation of standards

One milligram per milliliter solutions of racemic MDMA, MDA, HMMA, HMA, MDMA-d_5, MDA-d_5, and pholedrine were prepared by dissolving 10 mg of each substance in 10 mL methanol. Working solutions of 10 and 100 µg/mL of each compound were prepared by dilution of the corresponding 1 mg/mL solution.

For GC–MS analysis, a mixture of MDMA-d_5 (200 ng/mL for plasma or 500 ng/mL for urine), MDA-d_5 (20 ng/mL for plasma or 250 ng/mL for urine), and pholedrine (200 ng/mL for plasma or 250 ng/mL for urine) was prepared as I.S. working mixture from stock solutions. For the CE analysis, a mixture of MDPA (100 µg/mL) and codeine (75 µg/mL) was prepared as I.S. from stock solutions of 1 mg/mL and 100 µg/mL, respectively.

Calibration and sample preparation quality control

Calibration curves were prepared for each analytical batch. Appropriate volumes of working solutions were added to test tubes containing 1 mL of drug-free plasma or urine and vortexed vigorously. In the GC–MS method, final concentrations of MDMA and HMMA were 25, 100, 200, 300, and 400 ng/mL for the analysis of plasma samples and 250, 500, 750, 1000, and 2000 ng/mL for the analysis of urine samples. Final concentrations of MDA and HMA were 2.5, 10, 20, 30, and 40 ng/mL for the analysis of plasma samples and 100, 250, 500, 750, and 1000 ng/mL for that of urine samples. In the CE method, final concentrations were 250, 1000, 2000, 3000, and 4000 ng/mL of

racemic MDMA; 100, 500, 1000, 1500, and 2000 ng/mL of racemic MDA; and 250, 750, 1500, 2000, and 3000 ng/mL of racemic HMMA.

Control plasma samples containing appropriate analytes at three different concentrations were prepared in drug-free plasma or drug-free urine and were kept frozen at –20°C in 1-mL aliquots. In the analysis of plasma samples by GC–MS, low control concentrations were 50 ng/mL for MDMA and HMMA and 4 ng/mL for MDA and HMA; medium control concentrations were 125 ng/mL for MDMA and HMMA and 12.5 ng/mL for MDA and HMA; and high control concentrations were 300 ng/mL for MDMA, 325 ng/mL for HMMA, 32.5 ng/mL for MDA, and 30 ng/mL for HMA. In the analysis of urine samples by GC–MS, control concentrations were 300, 800, and 1500 ng/mL for MDMA and MDA and 200, 600, and 900 ng/mL for HMMA and HMA (low, medium, and high controls, respectively). In the analysis of urine samples by CE, concentracions of racemic forms were as follows: low control concentrations were 300 ng/mL for MDMA and HMMA and 200 ng/mL for MDA; medium control concentrations were 1600 ng/mL for MDMA, 800 ng/mL for MDA, and 1200 ng/mL for HMMA; and high control concentrations were 3400 ng/mL for MDMA, 1700 ng/mL for MDA, and 2500 ng/mL for HMMA.

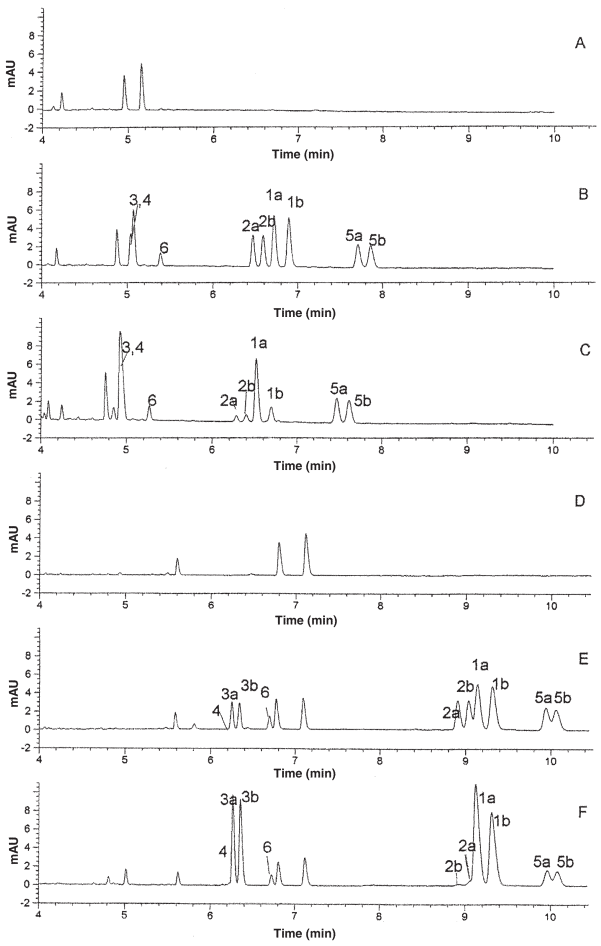

Figure 2. A–C: CE electropherograms at 2-OHP-β-CD, 10mM (A) blank of urine, 10mM; (B) urine spiked with (*R,S*)-MDMA (2000 ng/mL), (*R,S*)-MDA (1000 ng/mL), (*R,S*)-HMMA (2000 ng/mL), (*R,S*)-MDPA (1000 ng/mL), and codeine (500 ng/mL); and (C) 10–24 h diluted urine (1:10) from a volunteer who took 100 mg of (*R,S*)-MDMA ((*R,S*)-MDMA (28.3 and 7.7 μmoL of (*R*) and (*S*)-MDMA, respectively), and (*R,S*)-MDA (not quantitated)). D–F: CE electropherograms at 2-OHP-β-CD, 50mM (D) blank of urine, (E) urine spiked (same as (B)), and (F) 0–2 h undiluted urine from a volunteer who took 100 mg of (*R,S*)-MDMA [(*R,S*)-HMMA (12.3 and 13.8 μmoL)]. (1a: (*R*)-MDMA, 1b: (*S*)-MDMA; 2a, 2b: MDA enantiomers; 3a: (*R*)-HMMA, 3b: (*S*)-HMMA enantiomers; 4: elution time of (*R,S*)-HMA, 5a, 5b: MDPA enantiomers; and 6: codeine.

Hydrolysis procedure

One milliliter of plasma or 100 μL of urine mixed with 900 μL of drug-free urine were required for analysis. The pH of the sample was adjusted to 5 by adding 1 mL of 1.1M acetate buffer (pH 5.2). About 5000 Fishman units of β-glucuronidase (50 μL) were added to each sample and incubation was performed in a shaking water bath for 16 h at 37°C.

Extraction and derivatization

Samples analyzed by GC–MS were processed according to a previously reported method (21). Briefly, samples were submitted to a solid–liquid extraction procedure using Bond Elut Certify columns. Elution was done with 2 mL of ethyl acetate (2% of ammonium hydroxide). Trifluoroacyl derivatives were formed by reaction with MBTFA as derivatization agent. When analysis was performed with CE, the same extraction procedure was followed, but the derivatization step was omitted. Residues were reconstituted with 50 μL of 50mM H_3PO_4 buffer at pH 2.5.

Results and Discussion

The determination of MDMA and its main metabolites in plasma and urine by GC–MS and in urine by CE have been validated. Representative chromatograms of the GC–MS method are presented in Figure 1 and electropherograms for CE in Figure 2. Regarding methods validation, the following results were obtained.

Validation results

Calibration curves for the GC–MS methods were linear over 25–400 ng/mL (plasma) and over 250–2000 ng/mL (urine) concentration ranges for MDMA and HMMA. For MDA and HMA linearity was tested in the following concentration ranges: 2.5–40 ng/mL (plasma) and 100–1000 ng/mL (urine). Calibration curves for the CE method were prepared with the corresponding racemic mixture and were linear between 125 and 2000 ng/mL, 50 and 1000 ng/mL, and 125 and 1500 ng/mL for each enantiomer of MDMA, MDA, and HMMA, respectively.

Peak-area ratios between compounds and I.S. were used for calculations. A weighted (1/concentration) least-square regression analysis was used (SPSS for Windows, version 7.0). Mean determination coefficients (n = 4) by GC–MS method from the analysis of plasma were 0.998 ± 0.001 for MDMA, 0.996 ± 0.001 for MDA, 0.994 ± 0.002 for HMMA, and 0.996 ± 0.003 for HMA; the corresponding values from the analysis of urine were 0.996 ± 0.001, 0.991± 0.004, 0.993 ± 0.005, and 0.986 ± 0.006, respectively. Mean determination coefficients

(n = 4) by the CE method were 0.998 ± 0.001 for (*R*)-MDMA, 0.999 ± 0.001 for (*S*)-MDMA, 0.997 ± 0.001 for (1)-MDA, 0.997 ± 0.001 for (2)-MDA, 0.992 ± 0.009 for (*R*)-HMMA, and 0.992 ± 0.001 for (*S*)-HMMA. MDA enantiomers were identified following their elution order and were named (1)-MDA and (2)-MDA, respectively.

Four replicate analyses were performed with plasma or urine samples corresponding to the first level of concentrations of the calibration curves, and 3 and 10 standard deviations (SD) of the calculated concentrations at this calibration level were used for estimating the limits of detection and quantitation, respectively. Precision was calculated as the relative standard deviation (RSD) of the quality control samples concentrations. Accuracy is expressed as the relative error of the calculated concentrations. Limits of detection and quantitation as well as precision and accuracy results are listed in Table II.

Table II. Limits of Detection and Quantitation and Precision and Accuracy Results

	Detection limit (ng/mL)	Quantitation limit (ng/mL)	Within-run		Between-run	
			Precision (RSD, %)*	Accuracy (%)	Precision (RSD, %)*	Accuracy (relative error,%)
GC–MS, plasma						
MDMA	5.7	19.1	4.4	2.5	3.3	5.2
MDA	0.4	1.5	3.4	1.4	6.7	9.0
HMMA	3.0	9.9	5.0	6.16	3.85	9.7
HMA	0.1	0.4	10.1	12.0	11.1	10.4
GC–MS, urine						
MDMA	37.5	113.6			7.6	10.5
MDA	16.4	49.7			12.8	18.8
HMMA	47.0	142.5			11.3	12.0
HMA	23.1	69.9			10.5	8.3
CE method						
(*R*)-MDMA	33	99	8.4	8.7	5.1	7.4
(*S*)-MDMA	13	41	6.2	7.1	4.6	6.0
(1)-MDA	14	43	6.57	11.2	6.1	6.6
(2)-MDA	11	35	4.6	9.2	4.9	5.1
(*R*)-HMMA	16	48	15.9	17.9	11.8	10.9
(*S*)-HMMA	12	37	15.3	17.3	10.6	10.1

* RSD: relative standard deviation of the quality control samples concentrations.

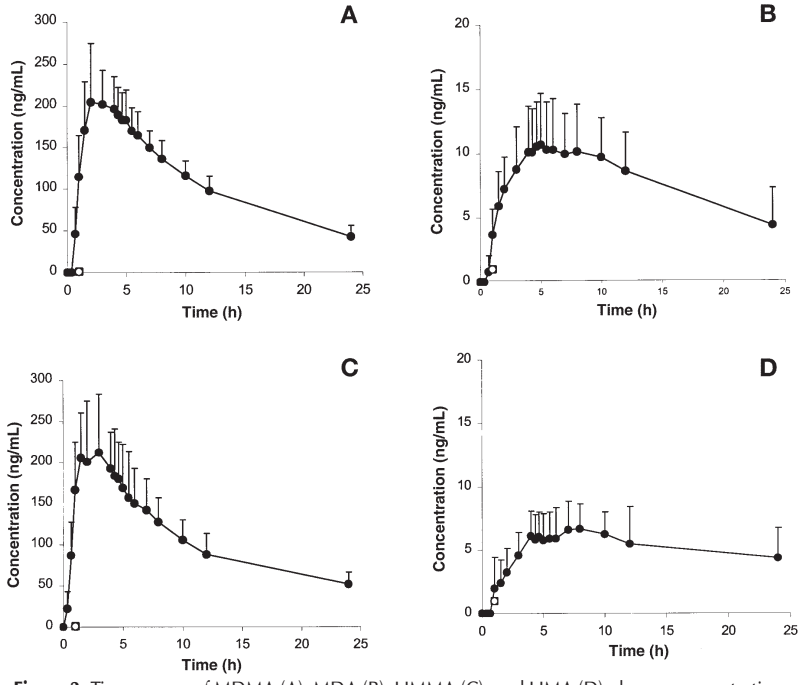

Figure 3. Time course of MDMA (A), MDA (B), HMMA (C), and HMA (D) plasma concentrations.

CE method development

Several cyclodextrins were assayed in the CE method development: α-cyclodextrin, γ-cyclodextrin, 2-OHP-β-CD, and heptakis(2,6-di-O-methyl)-β-cyclodextrin (at 5, 10, 25, and 50mM concentrations) and β-cyclodextrin (at 5, 10, and 15mM concentrations) (data not shown). A good separation of MDMA and MDA enantiomers was obtained with 2-OHP-β-CD at 10mM concentration, using racemic MDPA as I.S. (Figure 2). Each enantiomer of the two analytes was quantitated with the corresponding enantiomer of MDPA (according to the elution order). Enantiomeric separation of HMMA was performed at 50mM concentration of 2-OHP-β-CD. Codeine was used as I.S. for the two HMMA enantiomers (Figure 2). Attempts to use an alternative chiral I.S. for HMMA were unsuccessful. HMA enantiomers were not separated at the two final concentrations of 2-OHP-β-CD, and given that HMA is a minor metabolite of MDMA, other analyses were not performed.

MDMA and its main metabolites in plasma

Time-course of plasma concentrations of MDMA, MDA, HMMA, and HMA are shown in Figure 3. Experimental (C_{max}, t_{max}, and $AUC_{0-24\,h}$) and calculated pharmacokinetic parameters (using PKCALC computer program) (22) are also depicted (Table III). Other pharmacokinetic data have already been described in previous studies (10,23). MDA and HMA represented approximately 8% and 5% of MDMA concentrations, respectively (AUC comparisons), as it has already been reported in previous studies (10,23). Although HMMA concentrations were close to the detection limit when the free form was analyzed (10,23), $AUC_{0-24\,h}$ was even a 5% higher than the corresponding to MDMA after hydrolysis of HMMA. Conjugated HMMA is then the major MDMA metabolite.

MDMA and its main metabolites in urine

GC–MS results. A total of 44.7% of the dose was recovered at 24 h in urine as MDMA, MDA, HMMA, and HMA (Table IV). MDMA and MDA urine results were in accordance with those previously published (18,23). MDA represented approximately 7.4% of the dose recovered as MDMA in 24 h urine (µmoL recovered comparison) a similar figure was observed in plasma. The MDMA *N*-demethylation pathway to MDA (1.8% of dose recovered in urine) is much less relevant than for methamphetamine (24) where a 10.6 ± 3.7% of methamphetamine dose is recovered in 48 h urine as amphetamine. In the case of 3,4-methylenedioxyethylamphetamine (MDEA) (25), 2.8% of the dose was recovered as the *N*-deethylated metabolite in 32 h urine being similar the amounts of the parent compound recovered (21% dose of MDMA and 19% dose of MDEA). 4-Hydroxy-3-methoxyethylamphetamine (HMEA) represented 31.6% of dose, while 17.1% of MDMA dose was recovered as HMMA. These findings confirm that *O*-demethylenation is favored compared with *N*-demethylation when the methylenedioxy group is present in the chemical structure and that such *O*-demethylenation seems to be more extensive when the substituted secondary amine contains a larger alkyl group.

Urinary recoveries of HMMA and HMA in comparison with that of MDMA were somewhat lower and somewhat higher, respectively, than those expected according to plasma AUC comparisons (71.7% in urine versus 105% in plasma for HMMA and 7.8% vs. 5.3% for HMA, respectively) (Tables III and IV). These results are in agreement with previous observations (23) and seem to point towards a further *N*-demethylation of HMMA to HMA.

CE results. MDMA and HMMA enantiomers were the only ones quantitated in urine. MDA enantiomerical profile was determined in one volunteer with the aim of confirming results previously reported by others (16–18). The ratio (1)-MDA/(2)-MDA was lower than 1 at all time periods, increasing from 0.42 at 0–2 h to 0.86 at 10–24 h post-drug administration. These figures are compatible with an assignation of (1)-MDA as (*R*)-MDA and (2)-MDA as (*S*)-MDA. In previous studies (16,17), (*R*)-MDA concentration initially exceed those corresponding to the (*S*)-enantiomer, but over the time (*S*)-MDA concentrations rise progressively surpassing those of (*R*)-MDA at approximately 24–36 h post-drug administration. Although no enantiomer concentration crossing was observed, the enantiomerical profile found in our case agreed indirectly with an enantioselective *O*-demethylenation of MDMA. This is because the (*R*) enantiomer of MDMA less prone to be metabolized by CYP2D6 is ready available to be *N*-demethylated. As a consequence, ratios observed for MDA must be just the reverse of those corresponding to MDMA, in the absence of an unlikely enantioselective metabolism of the *N*-demethylation of MDMA. Because of being a very minor metabolite, MDA quantitation required more concentrated samples than needed for MDMA and HMMA analysis (a 1:10 sample dilution was needed for their analysis). Then to fit in the CE dynamic range, a duplicate of every sample undiluted was necessary for MDA analysis. It was observed that samples were unstable and that extracts cannot be left for long periods of time (no more than 24 h) either in the CE autosampler or at 4°C until analysis. Accordingly, it was decided to not analyze all samples for MDA and to concentrate on HMMA.

A very good correlation was found when comparing quantitative results from GC–MS (enantiomerical mixture of substances) and CE (sum of enantiomers measured separately for each substance) methods for MDMA (r = 0.99) and for HMMA (r = 0.98, but with some bias).

(*R*)-MDMA recovered was higher than (*S*)-MDMA at all time periods studied and such differences were increasing over time (Table V) showing a mean of 80.7 ± 19.0 µmoL of (*R*)-

Table III. Pharmacokinetic Parameters of MDMA and HMMA

	C_{max}* (ng/mL)	t_{max} (h)	$AUC_{0\text{-}24h}$ (ng/mL.h^{-1})	AUC_{total} (ng/mL.h^{-1})	k_a† (h^{-1})	k_e (h^{-1})	$t_{1/2a}$‡ (h)	$t_{1/2e}$ (h)
MDMA								
Mean	223.0	2.8	2554.8	3020.4	1.86	0.082	0.54	8.49
± SD	48.0	1.0	469.7	589.6	1.29	0.009	0.30	0.97
HMMA								
Mean	220.6	2.5	2684.4	3266.3	2.14	0.068	0.39	10.35
± SD	62.9	1.2	455.7	797.1	0.61	0.010	0.14	1.73

* Abbreviations: C_{max} = peak plasma concentration, t_{max} = time of peak plasma concentration, AUC_{0-24} h = area under curve from 0 to 24 h, AUC_{total} = area under curve from 0 to infinite, k_a = absorption rate constant, k_e = elimination rate constant, $t_{1/2a}$ = absorption half-life, $t_{1/2}$ = elimination half-life.
† Formation constant rate in the case of HMMA .
‡ Formation half-life in the case of HMMA.

Table IV. Urinary Recovery* of MDMA and its Metabolites

0–2 h	2–4 h	4–6 h	6–8 h	8–10 h	10–24 h	Overall 0–24 h
MDMA						
31.7 ± 11.2 (6.1%)	27.3 ± 14.7 (5.4%)	16.8 ± 8.0 (3.2%)	9.8 ± 7.1 (1.9%)	4.4 ± 3.3 (0.8%)	33.3 ± 11.0 (6.4%)	123.6 ± 24.4 (23.9%)
MDA						
0.9 ± 0.3 (0.2%)	1.5 ± 0.8 (0.3%)	1.5 ± 1.0 (0.3%)	0.7 ± 0.6 (0.1%)	0.4 ± 0.2 (0.1%)	4.1 ± 1.2 (0.8%)	9.2 ± 1.7 (1.8%)
HMMA						
13.6 ± 5.2 (2.6%)	18.6 ± 7.2 (3.6%)	13.8 ± 3.5 (2.7%)	10.0 ± 3.0 (1.9%)	6.7 ± 3.3 (1.3%)	25.9 ± 11.5 (5.0%)	88.7 ± 31.8 (17.1%)
HMA						
0.8 ± 1.2 (0.2%)	1.2 ± 1.0 (0.2%)	1.4 ± 1.2 (0.3%)	0.8 ± 0.3 (0.2%)	0.8 ± 0.3 (0.2%)	4.7 ± 2.5 (0.9%)	9.6 ± 5.5 (1.9%)

* µmoL and % dose recovered calculated by GC–MS method.

MDMA and 42.1 ± 12.5 µmoL of (*S*)-MDMA over 24 h post-drug administration (*R*/*S* ratio around 1.9). These results indicate an enantioselective disposition of (*R*) and (*S*) enantiomers. (*R*)-MDMA/(*S*)-MDMA ratio was increasing over the time from 1.36 at 0–2 h to 4.99 at 10–24 h post-drug administration (Figure 4). Similar results have been reported recently (18).

A mean of 49.8 ± 11.5 µmoL of (*R*)-HMMA and 41.4 ± 20.9 µmoL of (*S*)-HMMA were excreted over 24 h post-drug administration, with an (*R*)/(*S*) ratio of 1.2. However, the ratios of the two HMMA enantiomers were almost similar throughout the study period. In fact, (*R*)-HMMA/(*S*)-HMMA ratio was a close to 1 for all periods, although for the 10–24 h it was around 1.7 (Figure 4). Taking into account that the chiral center was not affected during all metabolic steps from MDMA to HMMA and according to MDMA stereoselective disposition, we firstly hypothesized that HMMA enantiomeric ratios would be the opposite of MDMA enantiomeric ratios. Surprisingly, not only ratios were different from that expected but also were rather constant. Reported ratios > 1 in mice and rat in 24 h urine (12) and those > 0.5 in urine samples from three unrelated MDMA intoxications (14) did not contribute to clarify the results. In a controlled trial in two volunteers carried out by Lanz et al. (16), large interindividual differences in HMMA ratios in 72 h urine samples were found. In this study, HMMA recovery was very low, which is in contrast with the majority of studies performed in humans (7).

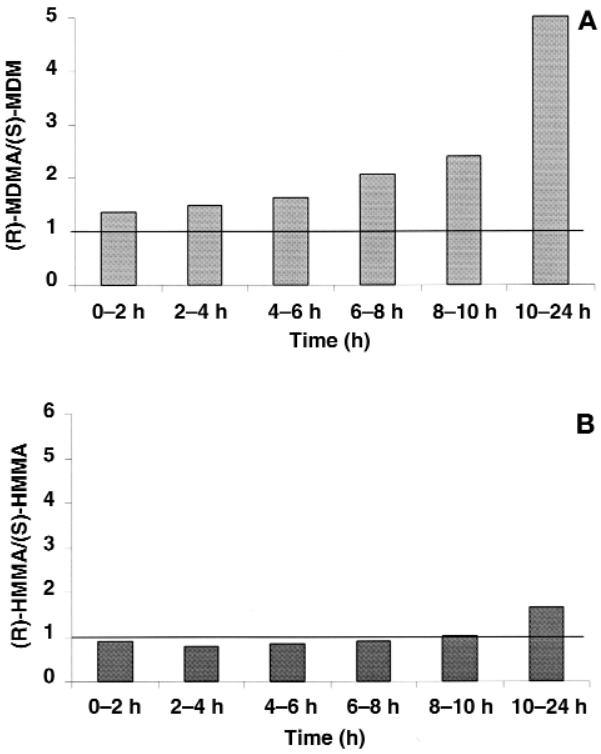

Figure 4. Enantiomeric ratio of MDMA (A) and HMMA (B) in urine over the time.

The *O*-demethylenation of MDMA to HHMA has been reported to be enantioselective. The *S*-enantiomer has a higher affinity for CYP2D6, the major isoenzyme of citochrome P450 reported to be regulating this metabolic pathway (26,27). On the basis of in vitro results, CYP2D6 should be responsible for approximately 60% of total *O*-demethylenation of MDMA, which implies that other isoenzymes are involved partially in this metabolic reaction. Recently several isoenzymes of cytochrome P450 have been identified as participating to different rates in MDMA *O*-demethylenation: CYP1A2, CYP2B6, and CYP3A4 (28). Then, theoretically, this partially enantioselective step should be translated in (*R*)-MDMA/(*S*)-MDMA ratio > 1, but lower than those reported for other amphetamine-like compounds because the involvement of enzymes without chiral requirements (29). CYP2D6 is no longer involved in further metabolic steps and hence, expected (*R*)-HHMA/(*S*)-HHMA and (*R*)-HMMA/(*S*)-HMMA ratios should be < 1, just the reverse of those observed for MDMA, as no changes in the enantiomerical ratio should be expected.

In vivo, MDMA shows a lack of linearity on its pharmacokinetics (23,30). The formation of a catalytically inactive complex in the first metabolic step, probably because an interaction of the nascent HHMA, a catechol-like compound with the enzyme has been documented by several authors (31,32), may be the basis for observations made in vivo. The progressive inactivation over time of CYP2D6 while exposed to MDMA is translated in the accumulation of MDMA in the body that accounts for a rise of plasma concentrations of about 30% (23). The relatively modest increase in MDMA plasma concentrations suggests that while the enzyme inhibition is operating other isoenzymes of cytochrome P450 (as described before) contribute to MDMA disposition. It is hypothesized that these enzymes would metabolize the (*R*)-MDMA enantiomer preferentially not because chiral requirements but because in the early stages of MDMA kinetics it

Table V. Urinary Recovery* of Enantiomers of MDMA and its Metabolites

0–2 h	2–4 h	4–6 h	6–8 h	8–10 h	10–24 h	Overall 0–24 h
(*R*)-MDMA						
18.5 ± 5.0 (3.6%)	14.9 ± 6.0 (2.9%)	10.5 ± 4.3 (2.0%)	5.5 ± 2.6 (1.0%)	4.4 ± 2.3 (0.9%)	26.9 ± 8.3 (5.2%)	80.7 ± 19.0 (15.6%)
(*S*)-MDMA						
13.9 ± 3.8 (2.7%)	10.3 ± 4.5 (2.0%)	6.5 ± 2.7 (1.2%)	2.7 ± 1.3 (0.5%)	2.0 ± 1.3 (0.4%)	6.8 ± 3.3 (1.3%)	42.1 ± 12.5 (8.1%)
(*R*)-HMMA						
6.8 ± 3.0 (1.3%)	8.3 ± 2.3 (1.6%)	7.3 ± 2.5 (1.4%)	5.0 ± 1.9 (1.0%)	4.2 ± 0.9 (0.8%)	18.3 ± 5.2 (3.5%)	49.8 ± 11.5 (9.6%)
(*S*)-HMMA						
7.6 ± 3.4 (1.5%)	10.4 ± 2.6 (2.0%)	8.6 ± 2.8 (1.7%)	5.4 ± 1.7 (1.0%)	4.2 ± 1.1 (0.8%)	11.1 ± 3.5 (2.1%)	41.4 ± 20.9 (8.0%)

* µmoL and % dose recovered calculated by CE method.

is the most abundant. Then overall the net balance of the formation of the metabolite-enzyme complex and the activity of enzymes other than CYP2D6 would be a re-equilibration of *R/S* enantiomer ratios. The *R/S* ratios observed for HMMA would support indirectly the formation of the enzyme-metabolite complex until now hypothesized based on in vitro studies.

Conclusions

This study described a GC–MS method for the simultaneous quantitation of MDMA and its main metabolites in plasma and urine samples of healthy volunteers. Subjects were given a single oral dose of 100 mg MDMA. Following the urine sample preparation for GC–MS analysis except for the derivatization step, a CE method for enantiomerical resolution of these compounds was developed. Stereoselective disposition of MDMA and MDA was confirmed ratio close to 1 and constant over the time seems reflect CYP2D6 enzyme inhibition reported in vitro.

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