RAPID COMMUNICATIONS IN MASS SPECTROMETRY
Rapid Commun. Mass Spectrom. 2003; **17**: 330–336
Published online in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/rcm.919

Stereochemical analysis of 3,4-methylenedioxymethamphetamine and its main metabolites by gas chromatography/mass spectrometry

Nieves Pizarro[1], Amadeu Llebaria[2], Silvia Cano[1], Jesús Joglar[2], Magí Farré[1,3], Jordi Segura[1,4] and Rafael de la Torre[1,4]*

[1]Pharmacology Research Unit, Institut Municipal d'Investigació Mèdica (IMIM), Dr. Aiguader 80, 08003 Barcelona, Spain
[2]RUBAM, Department of Biological Organic Chemistry, Institut d'Investigacions Químiques i Ambientals de Barcelona (IIQAB-CSIC), Jordi Girona 18-26, 08034 Barcelona, Spain
[3]Universitat Autònoma de Barcelona, Dr. Aiguader 80, 08003, Barcelona, Spain
[4]Universitat Pompeu Fabra, Dr. Aiguader 80, 08003 Barcelona, Spain

Received 25 September 2002; Revised 10 December 2002; Accepted 10 December 2002

3,4-Methylenedioxymethamphetamine (MDMA, ecstasy) is consumed as the racemate but some metabolic steps are enantioselective. In addition, chiral properties are preserved during MDMA biotransformation. A quantitative analytical methodology using gas chromatography/mass spectrometry (GC/MS) to determine enantioselective disposition in the body of MDMA and its main metabolites including 3,4-methylenedioxyamphetamine (MDA), 4-hydroxy-3-methoxymethamphetamine (HMMA), and 4-hydroxy-3-methoxyamphetamine (HMA) was developed. Plasma and urine samples were collected from a male volunteer. The analysis of MDMA, MDA, and 4-hydroxy-3-methoxy metabolites by GC/MS required a two-step derivatization procedure. The first step consisted of derivatization of the amine with enantiomerically pure Mosher's reagent ((*R*)-MTPCl). Triethylamine was used as a base to neutralize hydrochloric acid formed during the reaction allowing quantitative derivatization, which resulted in a substantial improvement in the sensitivity of the method compared with other previously described techniques. Further treatment with ammonium hydroxide was required since both amine and hydroxyl groups underwent derivatization in the reaction. Ammonium hydroxide breaks bonds formed with hydroxyl groups without affecting amine derivatives. The second derivatization step using hexamethyldisilazane was needed for metabolites containing phenol residues. This derivatization method permitted the stereochemically specific study of MDMA and its main monohydroxylated metabolites by GC/MS. A detailed study of the chemical reactions involved in the derivatization steps was indispensable to develop a straightforward, sensitive, and reproducible method for the analysis of the parent drug compound and its metabolites.

3,4-Methylenedioxymethamphetamine (MDMA, ecstasy) is a methamphetamine derivative extensively used among youth. Although amphetamine and mescaline possess obvious structural similarities, MDMA has specific pharmacological properties that have led to categorization of this compound into a new drug group of substances known as entactogens.[1] MDMA in humans is responsible for acute intoxication cases and ultimately death.[2–5] The main concern, however, relates to medium- and long-term serotonergic neurotoxicity.[6,7]

The main metabolic reactions of MDMA in humans include *O*-demethylenation followed by *O*-methylation (mainly in position 3 of the benzene ring), and conjugation with glucuronic acid and sulfate. *N*-Demethylation is a minor metabolic pathway. Major metabolites found in biological fluids are 3,4-dihydroxymetamphetamine (HHMA) and 4-hydroxy-3-methoxymethamphetamine (HMMA). 3,4-Methylenedioxyamphetamine (MDA) and 4-hydroxy-3-methoxyamphetamine (HMA) are minor metabolites.[8–14]

Oxidative *O*-demethylenation of MDMA to HHMA is an enantioselective pathway, favored for the (*S*)-(+)-MDMA stereoisomer, mainly catalyzed by the isoenzyme of cytochrome P450, CYP2D6.[8,9,15–20] The higher affinity of (*S*)-(+)-MDMA for CYP2D6 results in a *R*/*S* ratio >1 for MDMA (consumed as the racemate) in biological fluids.[9,19,21–23] *R*/*S* ratios reported for MDA are lower than 1. Some authors explain these ratios as a result of diminished bioavailability of (*S*)-(+)-MDMA.[17,19] In a recent study conducted by our group,[23] including monitoring a major MDMA metabolite (HMMA), it was observed that the HMMA *R*/*S* ratio was constant and close to 1, although it might have been expected

**Correspondence to*: R. de la Torre, PharmD, Pharmacology Research Unit, Institut Municipal d'Investigació Mèdica (IMIM), Doctor Aiguader 80, 08003 Barcelona, Spain.
E-mail: rtorre@imim.es

that HMMA enantiomeric ratios would be clearly in the opposite direction to those found for MDMA. This observation may be explained by catalysis of the MDMA biotransformation to HHMA by P450 isoenzymes other than CYP2D6 (e.g., CYP1A2, CYP2B6 and CYP3A4) which are not enantioselective for MDMA.[13] Consequently, the *R*/*S* ratio for HMMA is balanced whereas the MDMA *R*/*S* ratio is >1 but lower than expected.[23] If CYP2D6 was the only cytochrome P450 involved in the *O*-demethylenation of MDMA, higher MDMA *R*/*S* ratios than those determined experimentally would be observed.

Most pharmacokinetic data available for the MDMA enantioselective disposition have been focused on MDMA and MDA. Nevertheless, it has been postulated that the neurotoxicity of MDMA is mainly related to metabolites other than these compounds.[24] The (*S*)-enantiomers of MDA and MDMA are more potent than the (*R*)-enantiomers as inducers of the release of both serotonin and dopamine, and as inhibitors of serotonin reuptake.[25,26] On the other hand, both enantiomers have shown similar activity in inhibiting monoamino oxidase type A.[27] According to recent observations for the two enantiomers of the MDMA-like drug 3,4-methylenedioxyethylamphetamine (MDE),[28] the so-called entactogenic effects are likely to be caused by the (*S*)-enantiomer, whereas (*R*)-MDE appears to be responsible for neurotoxic effects. Therefore, the availability of analytical methods for stereoselective detection of MDMA and its main metabolites in biological fluids, especially in plasma, is highly relevant for pharmacological and toxicological characterization of the drug effects.

Drug analysis by gas chromatography/mass spectrometry (GC/MS) cannot distinguish between enantiomers unless their chemical transformation into diastereoisomers is performed. This reaction is usually carried out with an enantioselectively pure reagent. When metabolites bear other functional groups not involved in their chirality, a second derivatization reaction is required in order to make the compound amenable to GC analysis. The aim of this study was to develop a GC/MS method that would allow assessment of the enantioselective disposition of MDMA in humans. The presence of different chemical groups susceptible to derivatization before chromatography prompted us to conduct a detailed study of reactions that resulted in significant improvements of the analytical method compared to previous reports.[19] This GC/MS method permitted the determination of MDMA, HMMA, MDA, and HMA in plasma and urine human samples.

EXPERIMENTAL

Materials

MDMA, MDA, HMMA, HMA and MDMA-D_5 (1-[3,4-(methylenedioxy)phenyl)]-2-[1,2-dideutero-3,3,3-trideutero-methylaminopropane]) were purchased from Lipomed (Arlesheim, Switzerland); MDA-D_5 (1-[3,4-(methylenedioxy) phenyl)]-2-[1,2,3,3,3-pentadeuteroaminopropane]) was obtained from Cerilliant (Austin, TX, USA); and pholedrine (4-hydroxymethamphetamine) was kindly supplied by the Deutsche Sporthochschule, Biochemistry Department (Cologne, Germany). Enantiomerically enriched standards (*S*)-MDMA and (*S*)-HMMA were previously synthesized by our group.[29]

(*R*)-(−)-α-Methoxy-α-trifluoromethylphenylacetyl chloride (Mosher's reagent, (*R*)-MTPCl; 98% ee/GLC) and 1,1,1,3,3,3-hexamethyldisilazane (HMDS; 99.9%) were purchased from Aldrich (Steinheim, Germany). *N*-Methyl-bis(trifluoroacetamide) (MBTFA; GC grade) was purchased from Macherey-Nagel (Düren, Germany). Triethylamine (Et_3N; 99%) and β-glucuronidase from *Helix pomatia* (HP-2) were supplied by Sigma (St. Louis, MO, USA). Ethyl acetate (HPLC grade), phosphorus pentoxide (extra pure) and ammonium hydroxide (NH_3; 25%, analysis grade) were obtained from Merck (Darmstadt, Germany), and hexane (HPLC grade) was from Sharlau Chemie (Barcelona, Spain).

Plasma and urine samples

Plasma and urine samples analyzed by the present analytical methodology were collected from a volunteer who participated in a clinical trial.[30] The dose administered was 100 mg of (*R*,*S*)-MDMA·HCl.

Instrumentation

A gas chromatograph (6890 N; Agilent Technologies, Wilmington, DA, USA) equipped with a mass selective detector (5973 Network, AT) and an autosampler injector (7683 series, AT) was used. Enantiomeric separation of analytes was performed on a cross-linked 5% phenylmethylsiloxane column, 12 m × 0.22 mm × 0.33 μm film tickness (Ultra 2, AT). The temperature program was as follows: from 150°C (starting temperature maintained during 1 min) to 290°C at 20°C/min. Final temperature was maintained for 7 min. 2 μL of sample were injected in the split mode (1:10).

The mass spectrometer was operated in electron impact ionization (70 eV) mode, using selected ion monitoring (SIM). Three *m*/*z* values were selected for the identification of each analyte (ion ratios were checked for each analysis and were considered acceptable when there was <20% deviation), although only one *m*/*z* value was used for their quantification (see Table 1). Dwell time per SIM chanel was 30 ms in all

Table 1. GC/MS parameters for the enantiomeric analysis of MDMA and its metabolites. The *m/z* values in bold font are those used for quantification. For compounds for which no enantiomeric standards were available, enantiomers are labeled 1 and 2 corresponding to their elution order

rt (min)	Derivatized enantiomer	Ions
6.47	(1)-MDA-D_5-*N*-MTP	136, **167**, 264
	(1)-MDA-*N*-MTP	135, **162**, 260
6.60	(2)-MDA-*N*-MTP	135, **162**, 260
	(2)-MDA-D_5-*N*-MTP	136, **167**, 264
6.82	(1)-PHOLEDRINE-*N*-MTP-*O*-TMS	**206**, 179, 274
	(1)-HMA-N-MTP-*O*-TMS	209, **236**, 469
6.91	(2)-PHOLEDRINE-*N*-MTP-*O*-TMS	**206**, 179, 274
6.94	(1)-MDMA-D_5-N-MTP	136, 164, **278**
	(*R*)-MDMA-*N*-MTP	135, **162**, 274
7.00	(2)-MDMA-D_5-*N*-MTP	136, 164, **278**
	(*S*)-MDMA-*N*-MTP	135, **162**, 274
7.02	(2)-HMA-*N*-MTP-*O*-TMS	209, **236**, 469
7.22	(*R*)-HMMA-*N*-MTP-*O*-TMS	**236**, 274, 483
7.35	(*S*)-HMMA-*N*-MTP-*O*-TMS	**236**, 274, 483

cases, and effective resolution of the mass spectrometer was 0.5 u (specified by high-resolution parameters from the tune file of the mass spectrometer).

Working standards

Solutions of racemic MDMA, MDA, HMMA, and HMA (1 mg/mL) were prepared by dissolving 10 mg of each substance in 10 mL methanol. Working solutions of 0.1, 1, and 10 μg/mL of each compound were prepared by further diluting the corresponding starting solutions. MDMA and MDA deuterated analogues (D_5) were used as internal standards (IS) for their analysis. Pholedrine (4-hydroxymethamphetamine) was used as IS in HMMA and HMA analyses. A mixture (20 μL) containing 10 μg/mL of both MDMA-D_5 and pholedrine, and 1 μg/mL of MDA-D_5, were added to each sample. Enantiomerically enriched standard solutions of 100 μg/mL of (*S*)-MDMA and (*S*)-HMMA were used for enantiomeric identification.[29]

Calibration and sample preparation quality control

Calibration curves were prepared by adding appropriate volumes of working solutions to test tubes each containing 1 mL of drug-free plasma. Final concentrations were 25, 100, 200, 300, and 400 μg/L of racemic MDMA and HMMA, and 2.5, 10, 20, 30 and 40 μg/L of racemic MDA and HMA. Control plasma samples containing appropriate analytes at different concentrations were prepared in drug-free plasma and kept frozen at −20°C in 1-mL aliquots. Control concentrations were as follows: 30, 150 and 350 μg/L of racemic MDMA; 30 and 350 μg/L of racemic HMMA; 3, 15 and 35 μg/L of racemic MDA; and 3 and 35 μg/L of racemic HMA. Control samples were prepared with working standard solutions different from those used for the preparation of calibration curves.

Hydrolysis and extraction procedures

Both hydrolysis and extraction procedures were performed as described previously.[23,31] In summary, samples were subjected to an enzymatic hydrolysis using β-glucuronidase from *Helix pomatia* (HP-2). Samples were extracted using a solid-phase extraction procedure using Bond Elut Certify columns (BEC®). Compounds of interest were eluted using 2 mL of ethyl acetate with 2% NH_3. Eluates were evaporated to dryness under a nitrogen steam in a water-bath at 23°C and 10 psi (in order to avoid analyte losses by evaporation).

Chiral derivatization procedure

Two derivatization steps were required. Initially extracts were reconstituted in 2 mL of a mixture of ethyl acetate/hexane (1:1) which contained Et_3N (0.015%). Then, 20 μL of a 190 mM solution of (*R*)-MTPCl in hexane were added, and the tubes were capped and incubated for 20 min at 80°C. After reaching room temperature, 10 μL of NH_3 were added and a new incubation was performed for 20 min at 80°C. Tubes were then centrifuged at 3500 rpm for 5 min, and the organic phase was separated from salt precipitates and transferred to a new tube. Samples were then taken to dryness under a N_2 stream at 40°C, and kept in a vacuum oven in the presence of phosphorus pentoxide for 1 h at 50°C. Finally, a second derivatization step was performed by adding 50 μL of HMDS and incubating for 1 h at 80°C. Extracts were injected directly into the chromatographic system.

Achiral analysis

Compounds in plasma and urine samples were also analyzed following an achiral analysis as previously published.[23] Briefly, achiral quantification consisted of the same sample preparation procedure used for chiral analysis except for the derivatization step, which was performed by incubating extracts with MBTFA at 70°C for 45 min. Trifluorosilyl derivatives of the corresponding compounds (a mixture of enantiomeric pairs) were analyzed by GC/MS.

Validation procedure

The analytical chiral methodology was validated following a 3-day protocol. Calibration curve linearity was tested over the range 12.5–200 μg/L for MDMA and HMMA enantiomers, and over the range 1.25–20 μg/L for MDA and HMA enantiomers. Peak area ratios between compounds and the IS were used for calculations. A weighted (1/concentration) least-squares regression analysis was used (SPSS, version 9.0.1 for Windows). Three replicates of each level of the quality control samples were analyzed. Three and ten standard deviations (SD) of the calculated concentrations for the low control level were used to estimate the limits of detection and quantification, respectively. Intermediate precision was calculated as the relative SD of concentrations calculated for quality control samples. Inter-assay accuracy is expressed as the relative error with respect to the calculated concentrations.

RESULTS AND DISCUSSION

A unified approach for the detection and quantification in plasma of the enantiomers of MDMA, MDA, HMMA, and HMA, to be used in clinical studies of the enantiomeric disposition of MDMA in humans, was developed. It was the purpose of the present study to develop an analytical methodology for the chiral analysis of MDMA and metabolites in biological fluids. Only validation results in plasma are presented here as sensitivity requirements are more demanding for this biological fluid. However, similar validation results were obtained for urine.

By analyzing enantiomerically enriched (*S*)-MDMA and (*S*)-HMMA standards, it was observed that each (*S*)-enantiomer eluted after its corresponding (*R*)-enantiomer. Because of the lack of the appropriate enantiomeric standards, MDA and HMA enantiomers could not be assigned to the corresponding chromatographic peaks. Thus, for these two metabolites, the members of each pair of enantiomers were identified and designed using their elution order, as the (1)- and (2)-enantiomers.

The enantioselective determination of MDMA and some analogues, such as MDEA and its metabolites, has been achieved by applying different analytical approaches. GC/MS and capillary electrophoresis (CE) are the most common methods used for that purpose,[19,21,23] although HPLC has also been used with some success. Both GC/MS and CE have some advantages and disadvantages. On the one hand, CE

does not require a very demanding sample preparation because the derivatization step is not required since working buffers can provide chiral conditions. In contrast, since enantiomers have identical behavior under chromatographic conditions, conventional GC/MS requires specific enantiomer derivatization to diastereoisomers, that are chromatographically distinguishable. On the other hand, GC/MS provides a sensitivity that is difficult to reach by CE methods, although some strategies to decrease limits of detection have been developed for electrophoresis.[32,33] With regard to HPLC, enantiomers of amphetamine-like compounds are usually separated with the use of specific chiral columns, resulting in a substantial increase in cost.

Recently, a method for the analysis of MDEA and its main metabolites by chiral HPLC has been developed.[34] However, two chromatographic phases (β-cyclodextrin in the mobile phase for MDEA and MDA and a chiral protein phase, chiral-CBH, for the remaining compounds) and two types of detector (fluorescence and electrochemical detectors) were required for analysis. A useful method to analyze urine samples by CE has been recently developed.[23] MDMA, MDA and HMMA enantiomers could be analyzed but MDA required further concentration of samples. Also, the electrophoretic conditions for enantiomeric analysis of MDMA and HMMA were slightly different, and two runs were required for quantification of all analytes. When considering analysis of plasma samples, a higher turnaround compared with urine samples, and a further increase in sensitivity, are required. As current CE developments did not meet these criteria, we turned our attention to GC/MS methodology, and therefore the derivatization of enantiomeric analytes to its diastereomers was required.

There are several chiral derivatizing reagents, of high purity, which have been used for the stereochemical study of amphetamine-like compounds: (*R*)-(−)-α-methoxy-α-trifluoromethylphenylacetyl chloride (Mosher's reagent, (*R*)-MTPCl),[19] *N*-trifluoroacetyl-L-prolyl chloride (LTPCl),[35,36] 1*R*,2*S*,5*R*-(−)-menthylchloroformate (MCF),[37] and *N*-heptafluorobutyryl-L-prolyl chloride (HFBPCl).[9,22] Some studies indicate that LTPCl and HFBPCl can undergo an unpredictable racemization.[19] In addition, HFBPCl has to be synthesized as it is not commercially available. MCF has successfully been used for amphetamine and methamphetamine derivatization. Mosher's reagent is stereochemically stable and readily available commercially, and has been successfully used in a study of MDMA stereochemical metabolism.[19] These reasons led us to select it as the derivatization reagent for the present study.

For our purposes, a second derivatization step was required for metabolites bearing hydroxyl groups. In related studies, both *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) and *N*-methyl-bis(trifluoroacetamide) (MBTFA) have previously been used.[9,22] More reproducible results and a higher detector response were observed with silyl derivatives.[22]

The derivatization procedures previously described in the literature for MDMA and related compounds are simple and faster than the method reported here. However, taking into account some chemical considerations, important improvements over previously described methods were obtained.[19] Firstly, with our method, higher chiral reaction yields compared with other reported procedures are obtained. Secondly, when hydroxylated metabolites are also target compounds, previously published methodologies were of limited usefulness. However, by following the present method, these metabolites were suitably derivatized and quantified.

Validation results

Calibration curves were linear in the concentration range tested for each enantiomer, with correlation coefficients (R^2) higher than 0.99 in all cases. Detection and quantification limits were appropriate for every enantiomer (lower than the corresponding first concentration calibration level). Precision and accuracy were always lower than 20%, values that satisfied standard acceptance criteria. Validation data for each enantiomer are summarized in Table 2.

Sample analysis

MDMA, MDA, HMMA, and HMA enantiomers were analyzed using the present chiral methodology in both plasma and urine samples from a healthy volunteer. Figures 1 and 2 show the selected ion chromatograms for chiral analysis of plasma and urine samples, respectively. Results obtained by analyzing samples following both achiral and chiral methodologies were compared and were found to be consistent for both plasma and urine samples (see Table 3).

Primary and secondary amines derivatization

Amines were derivatized with the enantiomerically pure reagent (*R*)-MTPCl (Mosher's reagent) using an improved procedure (see Experimental section). Accordingly, enantiomeric pairs, which are not separated with a non-chiral procedure, were converted into diastereomers that could be easily separated and analyzed in a conventional capillary column by GC/MS. Two important considerations in the amine derivatization step should be considered. These involve the role of two of the reagents, triethylamine and NH_3.

Table 2. Validation data for chiral analysis of MDMA and its metabolites

	R^2	Detection limit (μg/L)	Quantification limit (μg/L)	Precision (RSD, %)	Accuracy (error %)
(*R*)-MDMA	0.998	2.4	7.3	4.2	6.1
(*S*)-MDMA	0.996	3.1	9.3	4.5	5.4
(1)-MDA	0.998	0.3	1.0	11.8	13.8
(2)-MDA	0.996	0.2	0.8	14.6	17.7
(*R*)-HMMA	0.996	1.3	4.1	14.8	14.0
(*S*)-HMMA	0.994	0.9	2.8	14.0	15.6
(1)-HMA	0.993	0.2	0.5	13.1	18.2
(2)-HMA	0.992	0.2	0.6	14.4	14.9

RCM

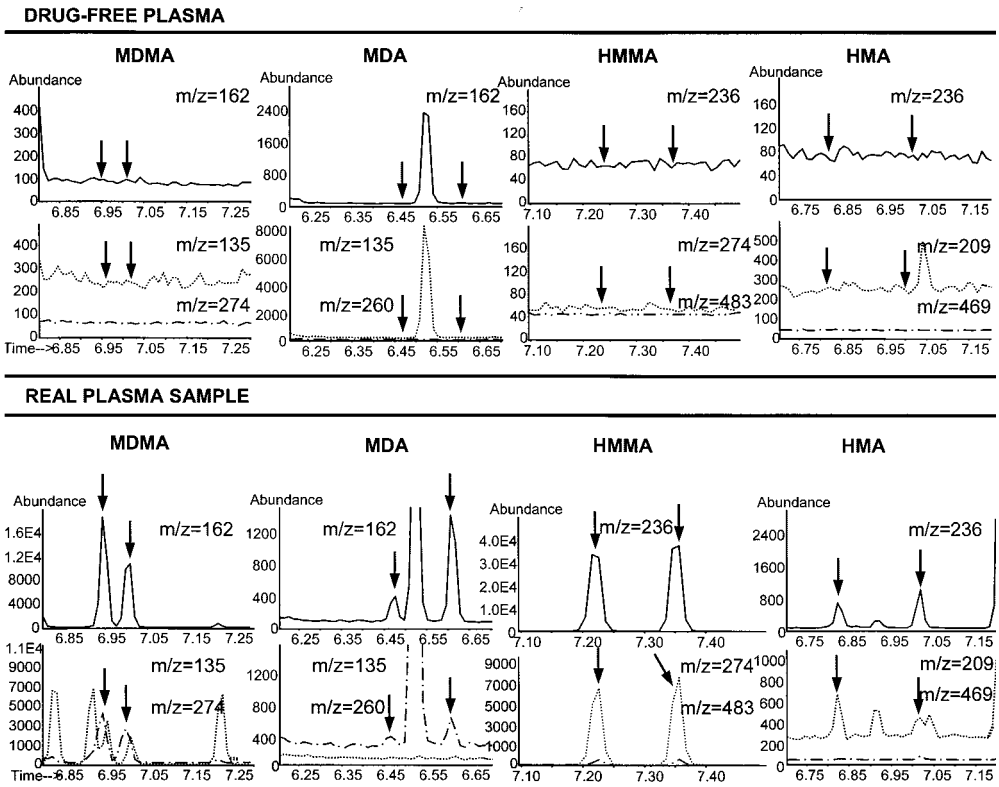

Figure 1. SIM chromatograms for plasma samples using the chiral GC/MS methodology. In each case the top chromatogram is that used for quantification, and arrows indicate the retention times of the target analytes. The top set of chromatograms corresponds to drug-free plasma (control) and the bottom set to a plasma sample from the dosed volunteer subject.

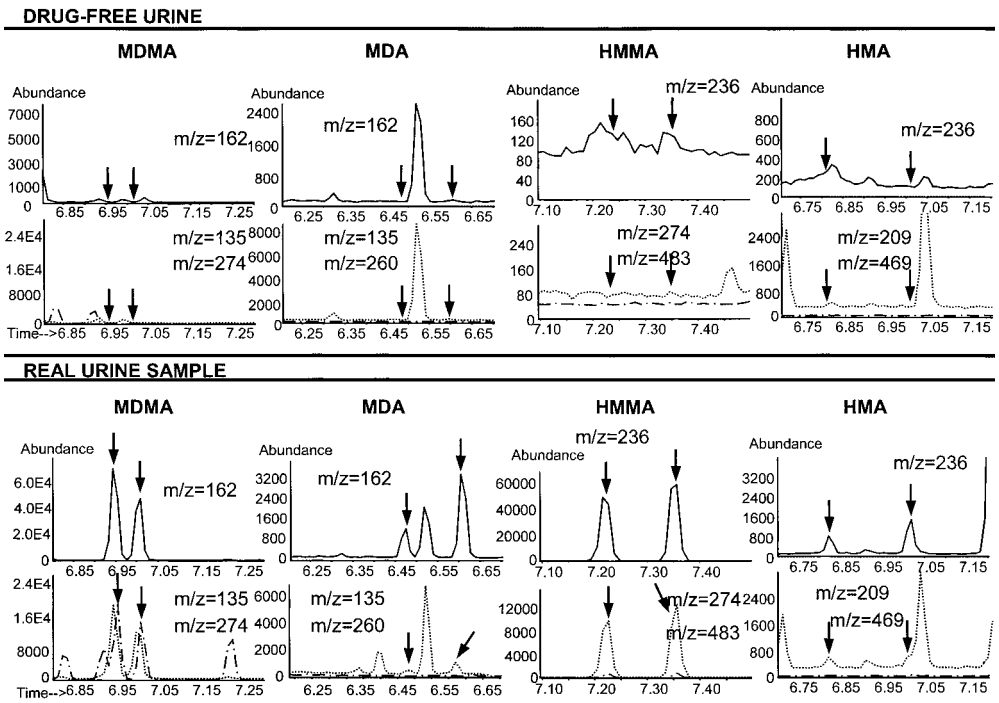

Figure 2. SIM chromatograms for urine samples using the chiral GC/MS methodology. In each case the top chromatogram is that used for quantification, and arrows indicate the retention times of the target analytes. The top set of chromatograms corresponds to drug-free plasma (control) and the bottom set to a urine sample from the dosed volunteer subject.

Table 3. Results obtained using both chiral and achiral methodologies for plasma and urine samples from a healthy volunteer dosed with 100 mg of racemic MDMA

	Plasma sample*			Urine sample*		
Compound	Chiral Analysis	Achiral analysis	Ratio *R/S*	Chiral analysis	Achiral analysis	Ratio *R/S*
(R,S)-MDMA		**98.7**			**449.8**	
(R)-MDMA	53.1	**(92.1)****	**1.36**	265.3	**(466.9)****	**1.32**
(S)-MDMA	39.0			201.6		
(R,S)-MDA		**3.1**			**7.8**	
(1)-MDA	0.6	**(2.1)****	**0.43**	2.2	**(7.9)****	**0.39**
(2)-MDA	1.4			5.7		
(R,S)-HMMA		**173.0**			**326.7**	
(R)-HMMA	83.6	**(169.7)****	**0.97**	154.5	**(324.2)****	**0.91**
(S)-HMMA	86.1			169.7		
(R,S)-HMA		**0**			**0**	
(1)-HMA	0			0	**(2.7)****	
(2)-HMA	0			2.7		

*Concentrations in μg/L.
**Sum of both enantiomer determinations.

With respect to triethylamine, as a result of the reaction of every primary and secondary amine with the Mosher reagent, an amide bond is formed and HCl is produced. HCl is neutralized by the amines present forming the corresponding hydrochlorides, since protonated amines are unable to react with (*R*)-MTPCl. Thus, an additional base is required to neutralize the HCl formed and to allow complete reaction of the analytes. Pyridine and Et_3N were tested at different amounts, incubation times and temperatures. Both bases significantly improved the analytes' response (increasing with derivatization temperature), but better results were obtained with Et_3N (data not shown). When Et_3N (0.015%) was added to the derivatization mixture and incubation was performed at 80°C for 20 min, the response was twice that obtained in the absence of base for MDMA (see Figs. 3(a) and 3(b)). With regard to HMMA in the presence of Et_3N, the amide bond was formed in a quantitative way from the amine group. Nevertheless, in addition, the formation of an ester bond with the phenol group by Mosher's reagent was also observed (see Figs. 3(a) and 3(b)).

Thus, in the presence of Et_3N, a considerable number of target compounds reacted their amine and phenol groups with (*R*)-MTPCl, producing an amide and an ester (see Fig. 3(b)), respectively. Gentle amine derivatization conditions avoided formation of esters, but lower responses were obtained. NH_3 was able to reconvert all esters to their original hydroxyl groups without affecting the amides (Fig. 3(c)). When adding NH_3, ammonium chloride precipitates interfere with the next derivatization step. Solids were efficiently removed by centrifugation for 5 min at 3500 rpm, and the remaining supernatant was transferred to another tube and taken to dryness under a nitrogen stream at 40°C and 20 psi.

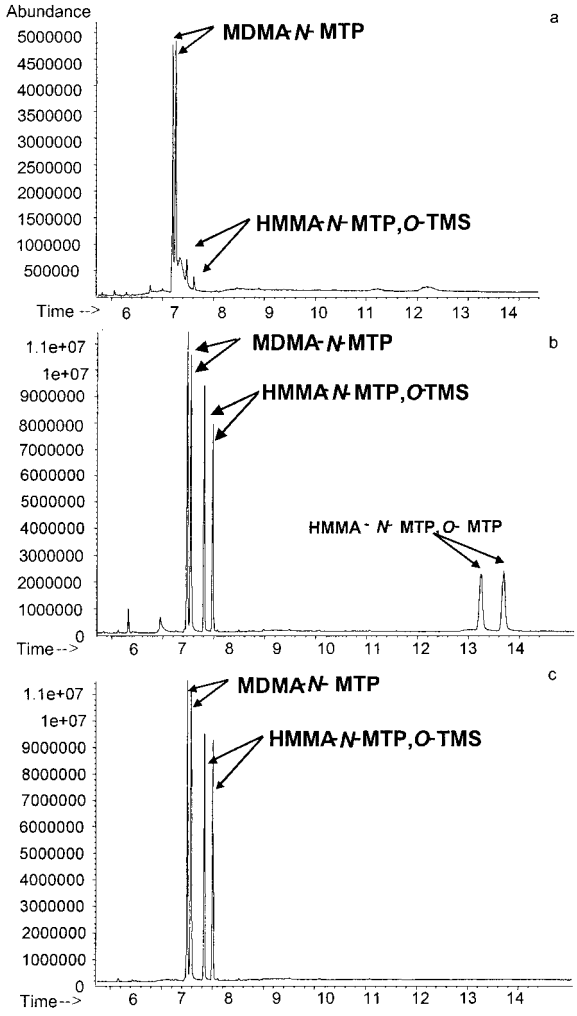

Figure 3. Chiral GC/MS analyses of MDMA and HMMA using different derivatizing conditions. (a) Mosher's reagent with no added base; (b) Mosher's reagent with triethylamine added, using optimized conditions (see text); and (c) Mosher's reagent with triethylamine, followed by a second incubation with ammonia to reconvert *O*-MTP esters to the hydroxyl groups (see text). HMDS was used for alcohol derivatization to *O*-TMS derivatives.

Phenol derivatization

The common silylation agents, such as *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA), produced the desired *O*-TMS derivatives but also to some extent the *N*-TMS derivatives for primary amines which, after amide formation via Mosher's reagent, still had a proton available for reaction. Hexamethyldisilazane (HMDS), a mild silylation reagent, allowed alcohol derivatization without affecting

primary amines, and the most suitable reaction conditions were found to involve heating for 60 min at 80°C (see Fig. 3(c)).

Sample quantification

One plasma sample from a volunteer dosed with 100 mg of (*R*,S)-MDMA·HCl was analyzed by both chiral and achiral methods. The sample selected for illustration of the results corresponds to 90 min after drug ingestion, close to peak MDMA plasma concentration values. Good agreement between the two methodologies is observed, particularly for major compounds. HMA concentrations were below the limit of quantification in both chiral and achiral analytical approaches. A urine sample from the same volunteer, corresponding to the 0–2-h collection period after MDMA ingestion, was analyzed under chiral and achiral conditions.

Plasma and urine chiral analysis confirmed the stereoselective disposition of MDMA,[23] that is, MDMA *R*/*S* enantiomeric ratios were >1 (1.36 and 1.32 for plasma and urine samples, respectively), and HMMA enantiomeric ratios were close to 1 (0.97 and 0.91 for plasma and urine samples, respectively).

CONCLUSIONS

The derivatization procedure described here allows the stereochemical study of MDMA and its main monohydroxylated metabolites using GC/MS. A detailed study of all chemical reactions involved in derivatization steps allowed us to develop a straightforward, sensitive, and reproducible analytical method for these compounds.

Acknowledgements

This study was funded by research grants FIS 98/0181, FIS 01/1336, and CIRIT 2001SGR00407. Rafael de la Torre is currently recipient of a NATO senior fellowship at the School of Pharmacy, Center of Molecular and Cellular Toxicology, University of Texas, Austin. We thank Marta Pulido for editing the manuscript and editorial assistance.

REFERENCES

1. Nichols DE. *J. Psychoactive Drugs* 1986; **18**: 305.
2. Holden R, Jackson MA. *Lancet* 1996; **347**: 1052.
3. Coore JR. *J. R. Soc. Med.* 1996; **89**: 51.
4. Mueller PD, Korey WS. *Ann. Emerg. Med.* 1998; **32**: 377.
5. Henry JA, Jeffreys KJ, Dawling S. *Lancet* 1992; **340**: 384.
6. Green AR, Goodwin GM. *Br. Med. J.* 1996; **312**: 1493.
7. McCann UD, Eligulashvili V, Ricaurte GA. *Neuropsycobiology* 2000; **42**: 11.
8. Lim HK, Foltz RL. *Chem. Res. Toxicol.* 1989; **2**: 142.
9. Lim HK, Su Z, Foltz RL. *Biol. Mass Spectrom.* 1993; **22**: 403.
10. Helmlin H-J, Bracher K, Bourquin D, Vonlanthen D, Brenneisen R. *J. Anal. Toxicol.* 1996; **20**: 432.
11. de la Torre R, Farre M, Roset PN, Hernández López C, Mas M, Ortuño J, Menoyo E, Pizarro N, Segura J, Camí J. *Ann. N. Y. Acad. Sci.* 2000; **914**: 225.
12. de la Torre R, Farré M, Ortuño J, Mas M, Brenneisen R, Roset PN, Segura J, Camí J. *Br. J. Clin. Pharmacol.* 2000; **49**: 104.
13. Kreth K-P, Kovar K-A, Schwab M, Zanger UM. *Biochem. Pharmacol.* 2000; **59**: 1563.
14. Kraemer T, Maurer HH. *Ther. Drug Monit.* 2002; **24**: 277.
15. Kumagai Y, Lin LY, Hiratsuka A, Narimatsu S, Suzuki T, Yamada H, Oguri K, Yoshimura H, Cho AK. *Mol. Pharmacol.* 1994; **45**: 359.
16. Tucker GT, Lennard MS, Ellis SW, Woods HF, Cho AK, Lin LY, Hiratsuka A, Schmitz DA, Chu TYY. *Biochem. Pharmacol.* 1994; **47**: 1151.
17. Hensley D, Cody JT. *J. Anal. Toxicol.* 1999; **23**: 518.
18. Cho AK, Hiramatsu M, Distefano EW, Chang AS, Jenden DJ. *Drug Metab. Dispos.* 1990; **18**: 686.
19. Fallon JK, Kicman AT, Henry JA, Milligan PJ, Cowan DA, Hutt AJ. *Clin. Chem.* 1999; **45**: 1058.
20. Fitzgerald RL, Blanke RV, Poklis A. *Chirality* 1990; **2**: 241.
21. Lanz M, Brenneisen R, Thormann W. *Electrophoresis* 1997; **18**: 1035.
22. de Boer D, Tan LP, Gorter P, van de Wal RMA, Kettenes-van den Bosch JJ, de Bruijn EA, Maes RAA. *J. Mass Spectrom.* 1997; **32**: 1236.
23. Pizarro N, Ortuño J, Farré M, Hernández-López C, Pujadas M, Llebaria A, Joglar J, Roset PN, Mas M, Segura J, Camí J, de la Torre R. *J. Anal. Toxicol.* 2002; **26**: 157.
24. Esteban B, O'Shea E, Camarero J, Sanchez V, Green AR, Colado MI. *Psychopharmacology* 2001; **154**: 251.
25. Azmitia EC, Murphy RB, Whitaker-Azmitia PM. *Brain Res.* 1990; **510**: 97.
26. McKenna DJ, Guan XM, Shulgin AT. *Pharmacol. Biochem. Behav.* 1991; **38**: 505.
27. Leonardi ETK, Azmitia EC. *Neuropsychopharmacology* 1994; **10**: 231.
28. Spitzer M, Franke B, Walter H, Buechler J, Wunderlich AP, Schwab M, Kovar K-A, Hermle L, Grön G. *Neuropharmacology* 2001; **41**: 263.
29. Pizarro N, de la Torre R, Farré M, Segura J, Llebaria A, Joglar J. *Bioorg. Med. Chem.* 2002; **10**: 1085.
30. Hernández-López C, Farré M, Roset PN, Menoyo E, Pizarro N, Ortuño J, Torrens M, Camí J, de la Torre R. *J. Pharmacol. Exp. Ther.* 2002; **300**: 236.
31. Ortuño J, Pizarro N, Farré M, Mas M, Segura J, Camí J, Brenneisen R, de la Torre R. *J. Chromatogr* 1999; **723**: 221.
32. Verleysen K, Sandra P. *Electrophoresis* 1998; **19**: 2798.
33. Vespalec R, Bocek P. *Electrophoresis* 1999; **20**: 2579.
34. Brunnenberg M, Kovar K-A. *J. Chromatogr.* 2001; **751**: 9.
35. Fitzgerald RL, Blanke RV, Glennon RA, Yousif MY, Rosecrans JA, Poklis A. *J Chromatogr.* 1989; **490**: 59.
36. Moore KA, Mozayani A, Fierro MF, Poklis A. *Forensic Sci. Int.* 1996; **83**: 111.
37. Hughes RO, Bronner WE, Smith ML. *J. Anal. Toxicol.* 1991; **15**: 256.