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Stereoselective Disposition: Enantioselective Quantitation of 3,4-(Methylenedioxy) methamphetamine and Three of Its Metabolites by Gas Chromatography/Electron Capture Negative Ion Chemical Ionization Mass Spectrometry†

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A new chiral assay for 3,4-(methylenedioxy)methamphetamine (MDMA) and three of its metabolites in biological specimens is based on direct aqueous derivatization with *N*-heptafluorobutyryl-*S*-prolyl chloride, followed by capillary chromatographic separation of the diastereomeric derivatives and detection by a mass spectrometer operated in the electron capture negative ion chemical ionization mode. The assay is linear from 5 to 1000 ng ml^{-1} for each enantiomer and allows simultaneous quantitation of MDMA and three of its metabolites in biological specimens. Investigation of the disposition of racemic MDMA in rats and mice revealed quantitative differences in the disposition of the enantiomers of MDMA in these species; the most noteworthy result was a two-fold greater urinary excretion of the neurotoxic *S*-(+)-MDMA by mice. Only MDMA and 3,4-(methylenedioxy)amphetamine (MDA) enantiomers were detected at measurable concentrations in the frontal cortices and hippocampis from rats dosed with 10 mg kg^{-1} of racemic MDMA; in this species the enantiomeric profiles of these two compounds were similar in brain and urine.

INTRODUCTION

The illicit drug 3,4-(methylenedioxy)methamphetamine (MDMA), which contains a chiral center, is normally prepared as a racemic mixture of the *R*-(−) and *S*-(+)-enantiomers. These enantiomers display different pharmacodynamic properties: the *S*-(+)-MDMA is a more potent psychotomimetic agent than the *R*-(−)-enantiomer,[1] and apparently only *S*-(+)-MDMA is neurotoxic because in neurotoxicity studies in rats only this enantiomer caused a significant long-term depletion of serotonin and its uptake sites, and depression of tryptophan hydroxylase activity.[2,3] Also, differences in stereochemical requirements for the acute and long-term depletion of cortical serotonin suggest that the acute depletion of serotonin is unlikely to contribute to the neurotoxicity of MDMA.[2]

Whether the differences in the pharmacodynamic effects of the two enantiomers of MDMA are related to differences in their metabolism and disposition was recently investigated by administration of either racemic or individual enantiomers of MDMA to rats.[4,5] Stereoselectivity in the disposition of MDMA was indicated by differences in the mean ratios of the areas under curves of blood concentration versus time for each of the enantiomers of MDMA [*S*-(+)/*R*-(−) = 0.7] and its *N*-demethylated metabolite, MDA [*S*-(+)/*R*-(−) = 3.1].[4] The lower concentration of *S*-(+)-MDMA was attributed to greater metabolism of this enantiomer to *S*-(+)-MDA, since no appreciable differences in absorption, distribution or urinary excretion were observed between the two enantiomers of MDMA.[4]

The previously reported study of the stereochemistry of metabolism and disposition of MDMA in rats employed an assay which involved formation of diastereomers of MDMA and MDA by derivatization with *N*-trifluoroacetyl-*S*-prolyl chloride, and subsequent quantitation by gas chromatography/electron ionization mass spectrometry (GC/EIMS).[6] The linear dynamic range of that assay was 5–2000 ng per enantiomer of each analyte per milliliter of blood.

This communication reports the development of an enantioselective assay using GC/negative ion chemical ionization MS for simultaneous quantitation of individual enantiomers of MDMA and three of its primary metabolites in biological specimens. Data are shown to illustrate application of the chiral assay to investigation of the stereoselective disposition of MDMA in two animal species (rats and mice) with documented differences in susceptibilities to MDMA-induced neurotoxicity, and to a study of stereoselective disposition of MDMA in the regions of the rat brain affected by this drug.

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EXPERIMENTAL

Materials

Hydrochloride salts of racemic MDMA, MDA and 3,4-(methylenedioxy)propylamphetamine (MDPA) were purchased from Alltech Applied Science (Deerfield, Illinois). Racemic 4-hydroxy-3-methoxymethamphetamine (HMM) and 4-hydroxy-3- methoxyamphetamine (HMA) were synthesized in our laboratory according to methods reported previously.[7] All solvents obtained from Burdick and Jackson (Muskegon, Michigan) were high-performance liquid chromatography (HPLC) grade and glass distilled. The following reagents were purchased from Aldrich Chemical Co. (Milwaukee, Wisconsin): sodium hydroxide (98%), sodium acetate trihydrate (99+%), anhydrous sodium sulfate (99+%), sodium chloride (99+%), sodium bicarbonate (99%), *N*-methylbis(trifluoroacetamide) (98%), *S*-(−)-proline (99+%), heptafluorobutyric anhydride (98%), thionyl chloride (99+%), *dl*-α-methylbenzylamine (99%), *d*-α-methylbenzylamine (98%) and sodium carbonate (99+%). β-Glucuronidase (*Helix pomatia*, type H-1) was obtained from Sigma Chemical Co. (St Louis, Missouri). Ammonium hydroxide and glacial acetic acid were purchased from VWR Scientific Co. (Salt Lake City, Utah).

Instrumentation

The analysis of MDMA and three of its primary metabolites in urine and brains was performed on a Finnigan-MAT 4500 gas chromatograph/mass spectrometer system. The gas chromatograph was equipped with a 15 m × 0.25 mm i.d., 0.25 μm film thickness, DB-5 fused-silica capillary column coupled to a 15 m × 0.25 mm i.d., 0.25 μm film thickness, DB 1301 fused-silica capillary column (J & W Scientific, Rancho Cordova, California). The end of the second column was routed through the separator oven (maintained at 250 °C), and directly into the ion source of the mass spectrometer. Hydrogen, the carrier gas, was set at a linear velocity of 60 cm s^{-1} at an oven temperature of 100 °C. The injector was maintained at 260 °C and operated in the splitless mode for 0.7 min following injection. After injection, the oven temperature was held at 100 °C for 4 min and then linearly programmed to 300 °C at 10 °C min^{-1}.

The mass spectrometer was operated in the negative ion chemical ionization mode. Typical operating conditions were: electron energy 100 eV, filament emission current 15 mA, conversion dynode −3 kV, and indicated ion source temperature 100 °C. Before analysis, the mass spectrometer was tuned by opening the valve to the instrument's calibration compound inlet after bleeding in methane to an indicated ion source pressure of 0.80 torr; ion source voltages were adjusted so that the negative ions at *m/z* 452 and 633 from perfluorotributylamine displayed a maximum ion current while retaining unit mass resolution and peak symmetry. Also, the quadrupole field was dropped to a potential of ≤ +4 V with respect to the ion volume (ground potential) using both quad offset and offset program pods. In addition, the voltages applied to the extractor and quadrupole entrance lens were adjusted to give maximum ion transmission while a minimum voltage was applied to the ion focusing lens without sacrificing ion transmission efficiency. Typically the lenses were cleaned when the applied potential to the quadrupole filter exceeded +4 V.

The mass spectrometer was set to monitor the molecule anions of the *N*-heptafluorobutyryl-*S*-prolyl derivative of each compound (see Fig. 1 for structures), except for MDMA and MDPA, where the ions monitored were those due to loss of HF from their molecule anions. The ion currents were monitored at: *m/z* 466 (MDMA); *m/z* 472 (MDA); *m/z* 494 (MDPA); *m/z* 570 (HMA); and *m/z* 485 (HMM), using a sampling time of 0.05 s per mass and a total cycle time of 0.289 s. Typically, a 1 μl aliquot of derivatized racemic MDMA and related compounds (1 ng per enantiomer on-column) was injected to verify the performance of the gas chromatograph/mass spectrometer system before actual analysis of samples. All acquisitions and reductions of data were performed with the Finnigan INCOS data system.

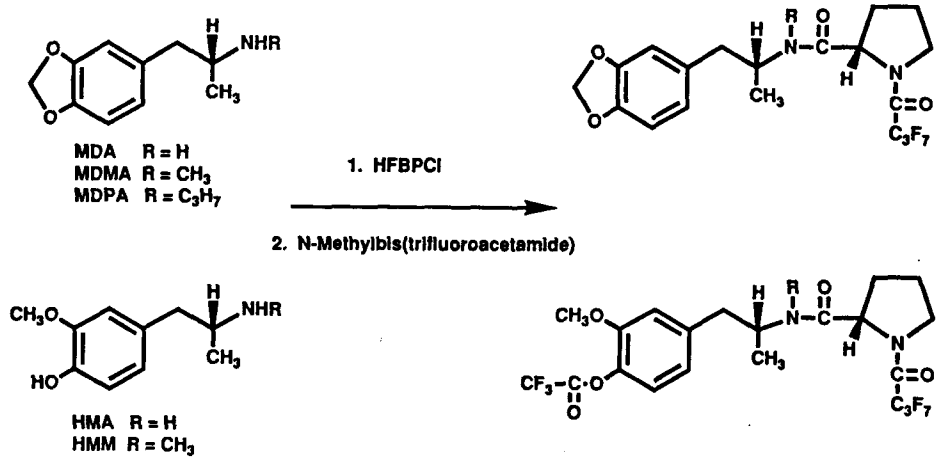

Figure 1. The structures of MDMA and related compounds before and after derivatizations.

Treatment of animals

Dosing with MDMA enantiomers. Three male Sprague–Dawley rats (200–250 g) were deprived of food overnight before being injected subcutaneously with 10 mg kg^{-1} of either *S*-(+)-MDMA or *R*-(−)-MDMA (each calculated as free base). Drugs for injection were prepared in 0.9% (w/v) NaCl solution. The rats were then housed individually in metabolic cages in a room with controlled lighting (12 h light/dark cycle) and temperature (24 °C). All the animals had access to food and water for the duration of the study. Urines were collected 24 h after drug administration. The samples were stored in polyethylene bottles at −20 °C until analysis.

Disposition study. Six male Sprague–Dawley rats (200–250 g) and five male CF-1 mice (25–30 g) were deprived of food overnight before being injected subcutaneously with 10 mg kg^{-1} racemic MDMA (expressed as free base). Urine collection and storage were as described above except that all of the mice were placed together in one metabolic cage. For measurement of concentrations of MDMA and its metabolites in brain, four male Sprague–Dawley rats (200–250 g) were injected subcutaneously with 10 mg kg^{-1} racemic MDMA (expressed as free base); 3 or 6 h after administration of the MDMA, the rats were decapitated and whole brains were removed. Frontal cortices and hippocampi were removed from the brains of half the rats; these tissues were wrapped separately in aluminum foil and stored at −20 °C until analysis. Whole brains from the remaining rats were individually wrapped with aluminum foil and stored at −20 °C.

Synthesis of chiral derivatizing agent

N-Heptafluorobutyryl-*S*-prolyl chloride (HFBPCl) was synthesized according to a previously reported procedure.[8] The final product was dissolved in a sufficient volume of chloroform to give a final concentration of 0.1 M. The HFBPCl solution was aliquoted and sealed under nitrogen prior to storage at −20 °C. The optical purity of HFBPCl, determined as previously described,[8] was greater than 98%.

Assay procedure

Rat urines. All glassware was vapor-phase silanized, rinsed with methanol, and oven-dried before use. To a 10 ml Teflon-lined, screw-capped test tube containing 1 ml of urine, 800 ng of racemic MDPA was added as the internal standard. Typically, urine samples from dosed rats were diluted 10- and 100-fold with pooled urines from control rats prior to analysis; for mice, the dilutions were 20- and 200-fold. The tube was capped and allowed to equilibrate at room temperature by gentle rocking for 15 min. The pH of the sample was adjusted to 5 with 6 M HCl, followed by addition of 0.5 ml of 1 M sodium acetate buffer (pH 4.8) containing 1000 units of β-glucuronidase. Enzymatic hydrolysis was allowed to proceed for 16 h at 37 °C. After hydrolysis, the sample was adjusted to pH 2–3 with 6 M HCl and extracted with 4 ml ethyl acetate by gentle rocking for 15 min. After centrifugation at 1377 × *g* for 5 min, the organic phase was removed by aspiration. The remaining aqueous phase was adjusted to pH 9 by addition of 10 M NaOH and 0.5 ml of a chilled 1 M sodium bicarbonate/sodium carbonate buffer (pH 9). Each tube was cooled in an ice-water bath for 25 min, 40 µl of HFBPCl was added, and the tube was immediately capped and vortexed at 1800 rpm for 30 min. After the aqueous derivatization, the urine was adjusted to pH 9, saturated with KCl and extracted with 4 ml of ethyl acetate by gentle rocking for 15 min. Phase separation was again achieved by centrifugation and the organic layer was transferred to another 10 ml test tube. The organic layer was washed with 2 ml water by rocking for 10 min. After another phase separation, the organic layer was transferred to a 10 ml disposable centrifuge tube and evaporated to dryness under a gentle stream of air at 55 °C.

To the residue in each test tube were added 25 µl each of acetonitrile and *N*-methylbis(trifluoroacetamide). The tubes were capped, hand vortexed for 5 s, and heated at 80 °C for 30 min. After cooling to room temperature, an aliquot of 1 µl of each derivatized extract was analyzed by GC/MS.

Rat brains. The frontal cortex or hippocampus was weighed into a 10 ml test tube and the weight recorded. To each tube was added 1 ml of chilled 1 M HCl and 800 ng of racemic MDPA. The tissue in each tube was homogenized using a polytron for 20 s at a setting of '6', then centrifuged at 1377 × *g* for 10 min at 5 °C. The supernatant was analyzed in the same manner as urines. For analysis of whole brain, the tissue was homogenized in chilled 1 M HCl equivalent to twice its wet weight; homogenization was as described above except that it was carried out for a longer time (1 min). Homogenate equivalent to 0.5–1 g of wet brain weight was weighed into a 12 ml polypropylene centrifuge tube and 800 ng of racemic MDPA was added. Following hand vortexing (5 s), the tubes were centrifuged at 10000 × *g* for 20 min at 5 °C; the supernatant was analyzed for individual enantiomers of MDMA and its metabolites, as described for urines.

Working standards

Separate 100 µg ml^{-1} stock solutions of racemic MDMA, MDA, HMM, HMA and MDPA were prepared by dissolving 1 mg of the free base equivalent in 10 ml of methanol. Working solutions containing all four analytes (MDMA, MDA, HMM and HMA), each at concentrations of 0.2, 0.4, 1.0, 2.0, 4.0, 10.0 and 20.0 ng $µl^{-1}$, were prepared by serial dilution of the stock solutions with water. Racemic MDPA was diluted only ten-fold with water to give a working solution with a concentration of 10 ng $µl^{-1}$. All the working solutions were freshly prepared for each set of analyses.

Calibration curves

Calibration standards containing 10, 20, 50, 100, 200, 500, 1000 and 2000 ng of each racemic analyte

(MDMA, MDA, HMM and HMA) were prepared by adding 50 μl of the respective working solutions to 1 ml of urine or brain supernatant. The samples were then processed as described in the assay procedure. Calibration curves for the *R*-(−) and *S*-(+) enantiomers of MDMA and three of its metabolites were constructed by plotting the ratios of the peak areas of the *R*-(−) and *S*-(+) enantiomers of each compound to the corresponding *R*-(−) and *S*-(+) enantiomers of MDPA *vs.* the concentrations of the enantiomers. This procedure was followed by linear regression analysis of the calibration curves. Quality-control samples containing concentrations of 10, 20, 200 and 1000 ng ml^{-1} of each racemic compound in urine or brain supernatant were also prepared and processed together with the calibration standards.

Precision studies

Intra-assay validation was conducted by analyzing on the same day four sets of quality-control samples containing racemic MDMA and three of its metabolites at concentrations of 2, 4, 10, 20, 40, 50 and 1000 ng ml^{-1}; data from this study were used for determination of the limit of quantitation of the assay. An inter-assay validation was carried out by analyzing quality-control samples containing racemic MDMA and three of its metabolites at concentrations of 20 and 1000 ng ml^{-1} on each of four days; the quality-control samples were prepared daily for this study. In each study, the concentrations were calculated from the linear regression calibration curves constructed as described above. Routinely, eight calibration standards and three quality-control standards (10, 20 and 1000 ng of each racemic compound per milliliter of urine or brain supernatant) were analyzed along with samples from rats and mice dosed with racemic MDMA.

Recovery studies

Recoveries of each enantiomeric pair of MDMA and three of its metabolites from urine were determined at concentrations of 40 and 1000 ng ml^{-1} of each racemic compound. The spiked urine samples were processed as described in the assay procedure except that an accurately measured volume of the organic phase was removed in each extraction step and the derivatized racemic MDPA (800 ng) was added to the organic extract rather than to the urine sample. The peak area ratios obtained from these samples were compared to those obtained when equal amounts of racemic MDMA and three of its metabolites were added to water and processed as described above.

RESULTS AND DISCUSSION

Development of the enantioselective GC/MS assay

Initial efforts to develop an enantioselective GC/MS assay for racemic MDMA and three of its metabolites using a commercially available chiral capillary column (Chirasil-Val) were not successful because the derivatized enantiomers could not be separated. Because of the limited selection and the high cost of chiral capillary GC columns, we chose to develop an assay based on derivatization with a chiral reagent and separation of the diastereomeric pairs with an achiral capillary column prior to mass spectrometric analysis. The ideal chiral reagent would be commercially available in high optical purity and not susceptible to racemization during derivatization. Such a reagent is (*R*)-(+)-α-methoxy-α-(trifluoromethyl)phenylacetic acid;[9] however, derivatization of racemic MDMA and three of its metabolites with this chiral reagent using published procedures[9–11] did not give a quantitative conversion of each of the compounds. In contrast, all of the diastereomeric pairs were formed quantitatively when racemic MDMA and its metabolites were subjected to aqueous derivatization with HFBPCl by modification of previous published procedures.[8,12] Under the conditions used only the amine functionalities were acylated; further derivatization of the free phenolic functionality with *N*-methylbis(trifluoroacetamide) was required in order to improve the chromatographic behavior of the phenolic amine metabolites. Commercially available *N*-trifluoroacetyl-*S*-prolyl chloride was not used for derivatization because this reagent was found to be unsuitable for aqueous derivatization of amines.[8]

An additional advantage of the *N*-heptafluorobutyryl-*S*-prolyl derivatization is that the resulting diastereomers are very efficiently ionized under electron capture negative ion chemical ionization conditions. A ten-fold lower response was obtained when the same compounds were analyzed using positive ion chemical ionization. The negative ion chemical ionization mass spectra of the diastereomeric derivatives of MDMA and its related compounds are tabulated in Table 1. The mass spectra of each pair of diastereomers are similar and are characterized by abundant molecular anions and prominent fragment ions resulting from successive losses of HF from the molecular anions. The relative abundances of the fragment ions are strongly influenced by the voltages applied to the quadrupole filter and lenses; typically, voltages which caused minimal fragmentation were selected (see section on instrumentation for details). The spectra are also strongly influenced by temperature; the relative abundance of the molecular anions decreased with increasing ionizer temperature. The optimal ionizer temperature setting (100 °C) yielded abundant molecular anions without causing significant broadening of the chromatographic peak due to condensation within the ion source. Electron capture negative ion chemical ionization mass spectra are known to be highly dependent on instrumental parameters.[13]

Chromatographic separation of the diastereomeric pairs of MDMA and its related compounds was evaluated using capillary GC columns coated with stationary phases of different polarity. Baseline separation of all the diastereomeric pairs was achieved only when a 15 m capillary column coated with 5% phenyl methylpolysiloxane was coupled to another 15 m capillary column coated with 6% cyanopropylphenyl methylpolysiloxane. The elution order of *R*-(−) and *S*-(+) enantiomers of MDMA and three of its metabolites was

Table 1. Electron capture negative ion chemical ionization mass spectra of *N*-heptafluorobutyryl-*S*-prolyl derivatives of MDMA and its related compounds

Compound	Mol. wt	Mass spectrum	
		R-(−) enantiomer	*S*-(+) enantiomer
MDMA	486	486[M⁻] (39), 467 (18), 466 (100), 446 (48), 426 (20)	486[M⁻] (33), 467 (17),466 (100), 446 (46), 426 (19)
MDA	472	473 (17), 472[M⁻] (100), 452 (36), 433 (11), 432 (58), 414 (10), 413 (10), 412 (39)	473 (16), 472[M⁻] (100), 452 (15), 433 (11), 432 (59), 414 (13), 413 (10), 412 (36)
HMM	584	585 (19), 584[M⁻] (100), 565 (18), 564 (98), 545 (21), 544 (91), 525 (15), 524 (60), 504 (23), 113 (48)	585 (18), 584[M⁻] (100), 565 (15), 564 (83), 545 (19), 544 (86), 525 (12). 524 (54), 504 (22), 113 (62)
HMA	570	571 (16), 570[M⁻] (100), 550 (64), 511 (18), 510 (73) 490 (17)	571 (17), 570[M⁻] (100), 550 (38), 511 (22), 510 (95), 490 (22)
MDPA	514	514[M⁻] (42), 495 (18), 494 (100), 475 (13), 474 (54), 454 (22)	514[M⁻] (39), 495 (18), 494 (100), 475 (12), 474 (49) 454 (20)

established from analysis of urine samples from rats dosed with optically pure *R*-(−)- or *S*-(+)-MDMA. The *R*-(−) enantiomer of each diastereomeric pair eluted earlier than the *S*-(+) enantiomer, except for HMM. This exception illustrates the unpredictability of assigning configurations to a diastereomeric pair based solely on the basis of comparison with the chromatographic behavior of related compounds.

Previously, we reported difficulty in developing an efficient, single-step extraction for MDMA and its metabolites from biological fluids because their polarities and pK_a values differ.[14] We therefore investigated an alternative approach consisting of aqueous derivatization of the amines under Schotten—Baumann conditions prior to extraction into an organic solvent. The resulting extracts were too 'dirty' to permit quantitation of the analytes at low nanogram per milliliter concentrations of each enantiomer; however, much cleaner extracts were obtained simply by extracting the hydrolyzed urine at an acidic pH before performing the aqueous derivatization. This procedure effectively removed non-basic and neutral components of the urine, so that after aqueous derivatization with HFBPCl and extraction of the derivatized amines, each diastereomer of derivatized MDMA and its metabolites could be detected at concentrations down to 2 ng ml^{-1} of each enantiomer. The detection limits of this assay are comparable to those previously reported[6] and are limited by the distribution of the ion currents amongst the numerous fragment ions present in the negative ion chemical ionization mass spectrum of each of the diastereomeric pairs (Table 1). HFBPCl has been used with similar success for the analysis of enantiomers of other drugs.[8,12]

The recoveries of each enantiomeric pair of MDMA and its related compounds by direct aqueous derivatization of urine with HFBPCl are summarized in Table 2. There were no statistically significant differences in the recoveries of *R*-(−) and *S*-(+) enantiomers of each analyte. The extraction recoveries for the internal standard (MDPA) at a concentration of 400 ng ml^{-1} of each enantiomer were comparable: 85% [coefficient of variation (CV) = 2.4%] for the *R*-(−) enantiomer and 83% (CV = 2.1%) for the *S*-(+) enantiomer.

The computer-generated regression lines of the calibration curves for *R*-(−) and *S*-(+) enantiomers of each analyte were linear from 5 to 1000 ng ml^{-1} and gave a correlation coefficient of at least 0.99 for each enantiomer of each analyte. That racemization during derivatization was minimal was indicated by the nearly identical slopes of the linear regression lines of the calibration curves for both enantiomers of each analyte.

Before the enantioselective GC/MS assay could be applied to the analysis of urine samples from the disposition study, it was necessary to rigorously establish that the assay could reproducibly measure each of the enantiomeric pairs of MDMA and the three metabolites with sufficient accuracy and precision over an extended period of time. With each batch of urine specimens, eight calibrators and three quality-control samples were analyzed. The extracts of the urine specimens were injected only if the quality-control samples were within

Table 2. Extraction recoveries of *R*-(−) and *S*-(+) enantiomers of MDMA and three of its metabolites from spiked rat urine

Amount of drug added to urine (ng ml^{-1})	% mean recovery (CV*) (n = 4)			
	MDMA	MDA	HMM	HMA
	R-(−) *enantiomer*			
20	67.4 (5.7)	69.5 (9.9)	63.8 (7.6)	89.7 (5.9)
500	67.0 (2.9)	59.7 (9.9)	76.6 (5.7)	93.8 (6.2)
	S-(+) *enantiomer*			
20	81.1 (9.4)	69.6 (7.9)	72.3 (11.2)	84.5 (11.7)
500	68.8 (3.5)	65.9 (3.5)	70.9 (4.3)	82.7 (4.5)

* CV, coefficient of variation.

Table 3. Precision and accuracy for quantitation of *R*-(−) and *S*-(+) enantiomers of MDMA and three of its metabolites in spiked rat urine

Target concentration (ng ml^{-1})	Measured concentration (ng ml^{-1}, CV*, $n = 4$) MDMA	MDA	HMM	HMA
Intra-assay	*R*-(−) *enantiomer*			
5.0	5.4 (13.2)	4.9 (4.4)	5.3 (6.7)	5.7 (8.6)
10.0	11.4 (4.7)	11.9 (6.8)	10.7 (4.2)	9.4 (12.0)
20.0	19.7 (1.9)	19.1 (3.4)	19.1 (11.0)	20.9 (4.2)
25.0	25.0 (7.7)	28.1 (7.0)	28.3 (7.1)	24.2 (4.1)
500.0	501.4 (2.5)	498.8 (3.3)	499.9 (0.2)	518.3 (1.4)
	S-(+) *enantiomer*			
5.0	5.9 (1.4)	5.0 (9.5)	4.9 (10.7)	5.3 (5.8)
10.0	10.3 (3.7)	11.1 (6.9)	10.5 (8.3)	10.5 (4.1)
20.0	20.2 (1.4)	19.4 (6.5)	21.4 (5.7)	19.8 (12.8)
25.0	27.5 (1.1)	28.1 (2.3)	27.0 (6.5)	25.2 (4.0)
500.0	497.1 (1.8)	496.5 (6.5)	511.2 (5.3)	507.9 (2.7)
Inter-assay	*R*-(−) *enantiomer*			
10.0	10.5 (6.3)	9.8 (12.9)	9.8 (6.2)	9.8 (7.9)
500.0	500.1 (1.7)	502.8 (1.1)	505.8 (0.8)	498.6 (3.9)
	S-(+) *enantiomer*			
10.0	9.9 (4.9)	9.9 (7.5)	10.4 (7.1)	9.8 (4.5)
500.0	506.6 (3.6)	486.1 (3.5)	501.0 (3.1)	492.8 (2.8)

* CV, coefficient of variation.

20% of their target concentrations. If the measured concentration of one or more of the QC samples was unacceptable, instrument maintenance was performed in an effort to improve the ion current intensities and the chromatographic performance. If the QC sample(s) continued to give an unacceptable measured concentration, new sets of calibrators, quality-control samples and specimens were extracted for analysis. Using this proto-

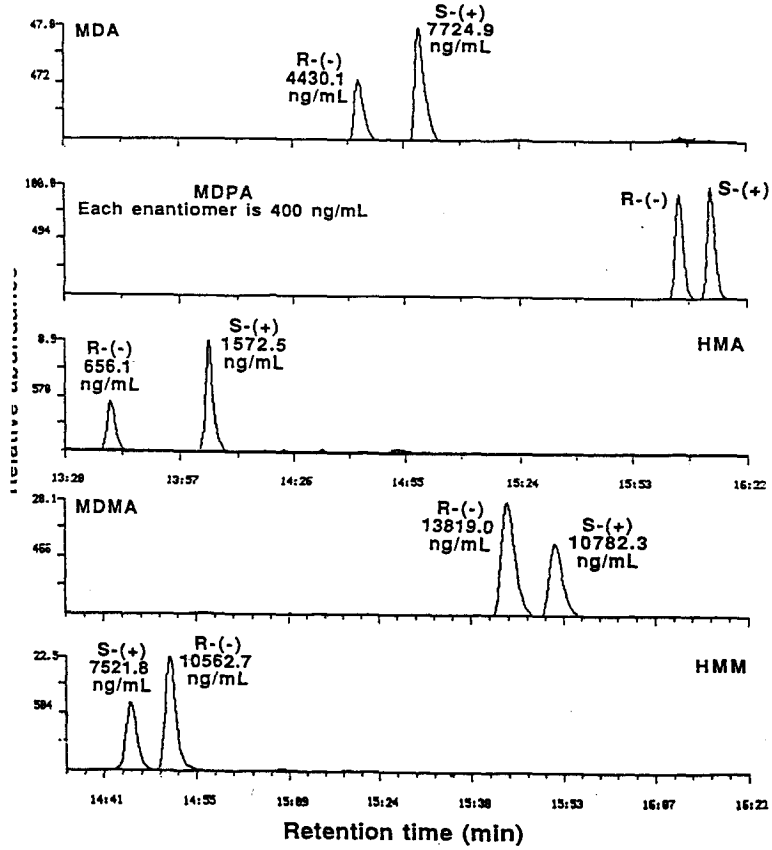

Figure 2. Selected ion current profiles corresponding to the diastereomeric pairs of MDMA and four related compounds from the gas chromatographic electron capture negative ion chemical ionization mass spectrometric analysis of a derivatized extract from a rat's hydrolyzed 24 h urine after subcutaneous injection of 10 mg kg^{-1} of racemic MDMA.

col, acceptable accuracy and precision were obtained for each enantiomeric pair of each analyte during intra- and inter-assay validation studies (see Table 3).

The data in Table 3 indicate that a simple linear regression analysis of the calibration curves is adequate for quantitation of each of the enantiomers of MDMA and three of its metabolites over the indicated concentration range. In the present study, the limit of quantitation (LOQ) was defined as the lowest concentration of each enantiomer that could be measured with an accuracy within 20% of the target concentration and with a CV $\leqslant$ 15%. The LOQs were determined by analysis of quality-control samples in quadruplicate at concentrations downward from 25 ng ml^{-1} of each enantiomer; by this definition, the LOQs of each of the *R*-(−) and *S*-(+) enantiomers were 5 ng ml^{-1} (Table 3).

Comparative stereoselective disposition

The inherent ability of optically active receptors and enzymes to interact differentially with drug enantiomers is the basis for the stereoselective pharmacodynamic and pharmacokinetic properties of a racemic drug.[15] However, this assumption has been proven experimentally only recently, when chemically synthesized enzyme enantiomers were shown to exhibit reciprocal chiral specificity in their substrate interactions.[16] Therefore, it is not surprising that the two MDMA enantiomers display different pharmacodynamic and pharmacokinetic properties.[1-4] Even though stereoselective disposition has been established for racemic MDMA,[4] earlier investigations were limited to quantitative accounts of MDMA and MDA enantiomers in rats[4] and mice[17] without investigation of the stereoselective elimination of the two compounds. Such information may be pertinent to our understanding of the relative insensitivity of mice, in comparison to rats, to MDMA-induced neurotoxicity, since the absence of neurotoxicity in mice is thought to arise from the greater urinary excretion of the parent drug by this species.[14]

Preliminary analyses of urine samples from either dosed rats or mice indicated that in some of the animals the urinary concentrations of MDMA and its metabolites were beyond the linear dynamic range of the calibration curves. Therefore, it was necessary to dilute those samples so that their concentrations fell within the linear dynamic range of the calibration curves. Each pair of analyte enantiomers was chromatographically separated from the co-extracted endogenous compounds. Figure 2 shows the ion current profiles corresponding to the enantiomeric pairs of MDMA and four related compounds from analysis of 24 h urine from a dosed rat. The ion current profiles obtained from analysis of 24 h urine pooled from dosed mice were also free of interferences. The results from investigation of the stereoselective disposition of racemic MDMA are presented in Fig. 3. The total amounts (expressed in micrograms) of each of the enantiomeric pairs [*R*-(−) and *S*-(+)] excreted in urine over 24 h by rats were: MDMA, 305.1 ± 118.0 and 222.9 ± 89.3; MDA, 90.6 ± 55.8 and 146.4 ± 68.4; HMM, 207.3 ± 113.5 and 162.2 ± 83.7; and HMA, 11.8 ± 5.6 and 35.7 ± 16.3. In

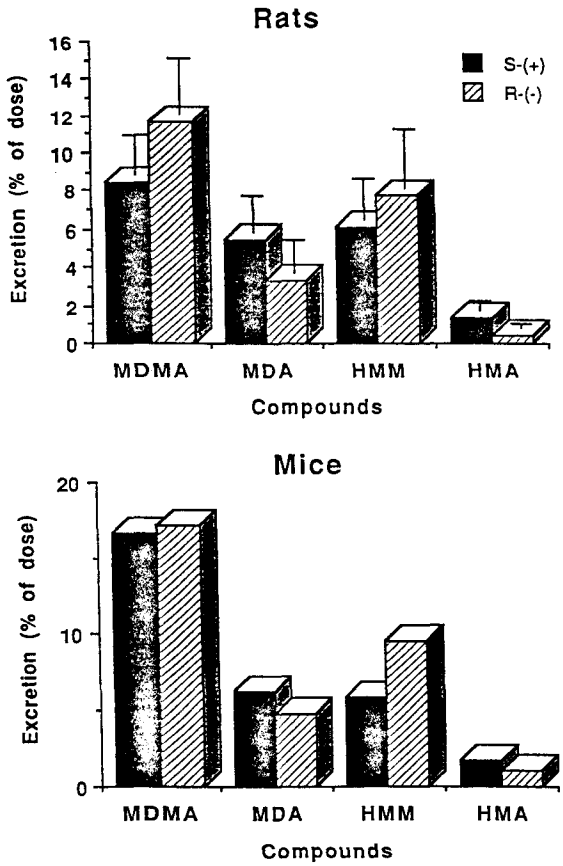

Figure 3. Elimination of *R*-(−) and *S*-(+) enantiomers of MDMA and three of its metabolites in 24 h urines from rats (each point is the mean ± SD for six rats) and mice (urine pooled from five mice) after subcutaneous injection of 10 mg kg^{-1} of racemic MDMA.

comparison, the total amounts (expressed in micrograms) of each of the enantiomeric pairs [*R*-(−) and *S*-(+)] in 24 h pooled mice urine were: MDMA, 51.9 and 50.3; MDA, 14.4 and 18.8; HMM, 28.8 and 17.6; and HMA, 3.2 and 5.2. The different amounts of each enantiomer of MDMA and the three metabolites excreted in 24 h urines from dosed rats and mice clearly indicate stereoselective disposition of MDMA in both animal species. In general, the 24 h urines from rats and mice contained more of the *R*-(−) enantiomers for the secondary amines (MDMA and HMM), while the *S*-(+) enantiomers were the predominant stereoisomers for the primary amines (MDA and HMA), except that mice excreted comparable amounts of each MDMA enantiomer. Reported enantiomeric profiles of MDMA in murine plasma are similar.[17]

Most of the stereoselectivity in disposition can be accounted for by stereoselective metabolism.[18] Stereoselectivity in the *N*-demethylation pathway is indicated because the enantiomeric profiles [*S*-(+) > *R*-(−)] of MDA and HMA are the reverse of those [*S*-(+) < *R*-(−)] for MDMA and HMM. The current data are consistent with those reported by others.[4,5,17] Stereoselectivity in the *O*-dealkylation metabolic pathway has been previously demonstrated *in vitro*.[5] Nonetheless, the *in vivo* chirality of the *O*-dealkylation pathway needs to be verified by measurement of the precursors of HMM and HMA, since stereoselectivity of this pathway *in vitro*

may not be the same as that observed *in vivo*. This anomaly has been observed with MDA.[5]

With the exception of the urinary excretion of *S*-(+)-MDMA and *R*-(−)-HMA, species differences in the urinary excretion of enantiomers are negligible. The greater urinary excretion of *R*-(−)-HMA by mice is unlikely to account for the relative insensitivity of this species to the neurotoxic effects of MDMA,[19] because racemic HMA is not neurotoxic to rats.[20] However, previously we suggested that the difference in susceptibilities of rats and mice to MDMA-induced neurotoxicity might be related to the greater urinary excretion of the parent drug by mice.[14] That conclusion is further supported by the current observation of a two-fold greater elimination of the neurotoxic *S*-(+)-MDMA enantiomer in murine urine (Fig. 3).

Stereoselective disposition in rat brain regions

Our understanding of the metabolism and disposition of either racemic MDMA or its enantiomers in the rat brain has not kept pace with the accumulation of knowledge about the neurodegenerative effects of MDMA. Hence the mechanism of MDMA-induced neurotoxicity is still elusive. Despite several studies of metabolism and disposition that have been conducted with either racemic MDMA[7,14] or its enantiomers,[4,5,17] the dispositional behaviors of MDMA enantiomers in the regions of rat brain that are susceptible to the neurotoxic effects of racemic MDMA are still unclear. The results from the application of the chiral GC/MS assay to analysis of the enantiomeric pairs of MDMA and three of its metabolites in frontal cortices, hippocampi and whole brains from dosed rats are summarized in Fig. 4. This assay successfully measured the MDMA and MDA enantiomers in rat brain; its failure to measure HMM and HMA does not imply that these phenolic amine metabolites were not present in the brain, but rather that their concentrations were below the LOQs of the chiral assay. In another study the peak brain concentrations of racemic HMM and HMA, after subcutaneous injection of 10 mg kg^{-1} of racemic MDMA, were 8.6 and 2.4 ng g^{-1}, respectively;[21] these concentrations are below the chiral assay LOQs of 5 ng g^{-1} for each enantiomer. As the development of neurotoxicity of MDMA can be prevented by co-administration of a serotonin uptake inhibitor at any time between 3 and 6 h post drug administration,[22] a neurotoxic metabolite is probably formed during that interval. This observation provided the rationale for studying the brain disposition profiles of MDMA enantiomers at those time points.

The enantiomeric profiles of MDMA and MDA in rat brain are similar to those in urine. Interestingly, comparable amounts of the *S*-(+) enantiomers of MDMA and MDA were present in the frontal cortex, hippocampus and whole brain 3 h after drug administration. However, the data also suggest that the *S*-(+)-MDMA was removed more rapidly from the brain than

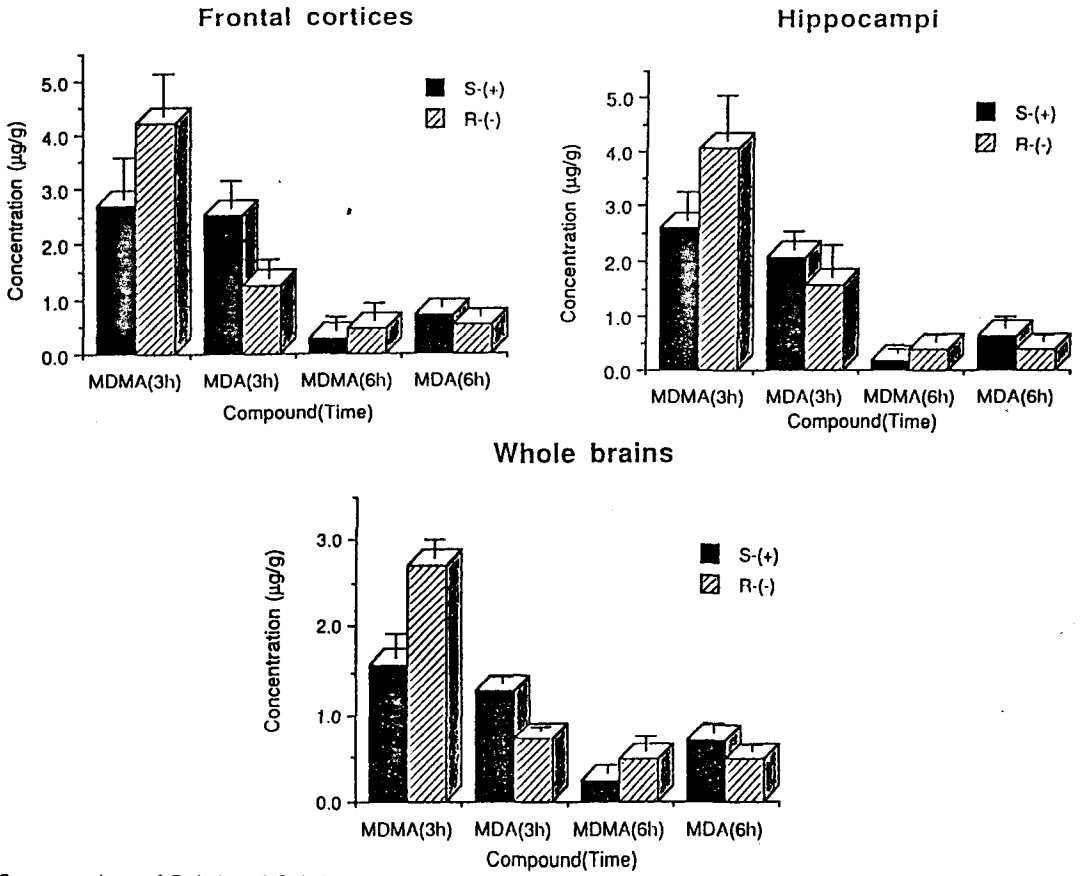

Figure 4. Concentrations of *R*-(−) and *S*-(+) enantiomers of MDMA and MDA in frontal cortices, hippocampi and whole brains from rats injected subcutaneously with 10 mg kg^{-1} of racemic MDMA. Each point is the mean ± SD for five rats.

the *S*-(+)-MDA after 3 h; this may account for the observed > two-fold difference between the concentrations of (*S*)-(+) enantiomers of MDMA and MDA at 6 h, when the differences in the concentrations of the *R*(−) enantiomers were negligible. Furthermore, a comparison of the concentrations of MDMA and MDA enantiomers in the frontal cortex and hippocampus to those of the whole brain does not support the hypothesis that selective neuronal uptake in these brain regions is responsible for the serotonergic neurotoxicity of MDMA and MDA.[23] The detection of both neurotoxic *S*-(+)-MDMA and *S*-(+)-MDA in the affected brain regions argues for the contribution of both compounds to the neurotoxicity of MDMA. Precisely how these two compounds cause neurodegeneration is unclear, but the recently identified bioactivation pathway consisting of aromatic hydroxylation at the 6-position, prior to *O*-dealkylation, may play an important role in the neurotoxicity.[24]

CONCLUSIONS

A chiral GC/MS assay has been developed for the simultaneous quantitation of the enantiomers of MDMA and three of its metabolites in biological specimens. Stereoselectivity was demonstrated in the dispositions of racemic MDMA in rats and mice using this assay. Even though no significant qualitative difference was observed between the two species in the stereoselective disposition of racemic MDMA, a quantitative difference occurred in the 24 h urinary excretion of the *S*-(+)-MDMA and *R*-(−)-HMA. The two-fold greater urinary excretion of the neurotoxic *S*-(+)-MDMA enantiomer by mice may explain the relative insensitivity of this species to the neurotoxic effects of MDMA. Furthermore, only MDMA and MDA enantiomers were detected in the rat brain by this chiral assay. The detection of the *S*-(+) enantiomers of MDMA and MDA in both the frontal cortices and hippocampis from MDMA-dosed rats suggests the potential contribution of the *S*-(+) enantiomers of these compounds to the neurotoxicity of MDMA.

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