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In vivo Formation of Aromatic Hydroxylated Metabolites of 3,4-(Methylenedioxy)methamphetamine in the Rat: Identification by Ion Trap Tandem Mass Spectrometric (MS/MS and MS/MS/MS) Techniques†

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Aromatic hydroxylation has been established as a pathway for the *in vivo* metabolism of 3,4-(methylenedioxy)methamphetamine (MDMA) in the rat. Hydroxylation occurred at positions 2, 5 and 6 of the 3,4-methylenedioxyphenyl ring, but is favored at the 6 position. All three regioisomers of both hydroxy-MDMA and hydroxy-3,4-(methylenedioxy)amphetamine (hydroxy-MDA) were detected in the rat liver when 20 mg kg^{-1} of MDMA was administered. However, 6-hydroxy-MDMA and 6-hydroxy-MDA were the only hydroxylated metabolites detected in the rat brain and plasma and no hydroxylated metabolites were detected in the urine. The hydroxylated metabolites were identified by co-injection of synthetic reference compounds and comparison of the mass spectra of the trifluoroacetyl derivatives of the metabolites with the synthesized reference compounds. The regioisomers of both hydroxy-MDMA and hydroxy-MDA could not be distinguished by either single-stage or two-stage mass analysis. However, employment of a third stage of mass analysis produced distinctly different mass spectra for each of the three regioisomers.

INTRODUCTION

several animal species, including rat,[1] mouse,[2] guinea g[3] and monkeys,[4] 3,4-(methylenedioxy)methamphetmine (MDMA, Fig. 1) causes damage to the serotonerc neurons. At neurochemical and neuroanatomical vels MDMA causes loss of tryptophan hydroxylase tivity,[1] depression of brain concentrations of serotnin and its metabolite, 5-hydroxyindoleacetic acid,[1] duction in serotonin uptake sites,[5] and damage to rotonergic nerve terminals.[6] The toxic effects are parlly mediated by endogenously released dopamine[7] d are quantitatively different for the *d*- and *l*-antiomers.[5] There are species differences in MDMA-duced neurotoxicity; monkeys and mice are most d least sensitive, respectively, to the neurotoxic effects MDMA.[2,4] Despite the extensive characterization of e neurotoxic effects of MDMA, the mechanism of urotoxicity remains unexplained.

A metabolite of MDMA, instead of the parent compund, is thought to be responsible for the neuroticity since direct intracerebral injection of MDMA led to produce neuronal degeneration.[8] Therefore, the identification of MDMA metabolites is essential to the elucidation of the mechanism of neurotoxicity of MDMA. We previously reported the identification of eight *in vivo* metabolites of MDMA in the rat;[9] all but one of these metabolites have also been shown to be formed in man.[10] The identified metabolites are formed by metabolic pathways which include *N*-demethylation, *O*-dealkylation, deamination and conjugation. It has been postulated that dopamine undergoes aromatic hydroxylation at position 6 to form 6-hydroxydopamine, a potent neurotoxin.[11] Similarly, compounds structurally similar to 6-hydroxydopamine may be formed by aromatic hydroxylation of MDMA and/or certain of its metabolites, especially those from *N*-demethylation and/or *O*-dealkylation pathways. This report describes the detection of additional metabolites of MDMA formed in the rat by aromatic hydroxylation, and the application of tandem mass analysis techniques (MS/MS and MS/MS/MS) performed on an ion trap mass spectrometer to identify the regioisomers of the hydroxylated metabolites.

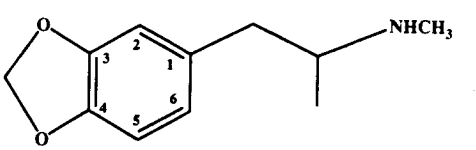

Figure 1. Structure of 3,4-(methylenedioxy)methamphetamine (MDMA).

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EXPERIMENTAL

Materials

The following chemicals were purchased from Aldrich Chemical Co. (Milwaukee, Wisconsin): 2,4,5-trimethoxybenzaldehyde (98%), 2,3,4-trimethoxybenzaldehyde (99%), 3,4,5-trimethoxybenzaldehyde (98%), boron tribromide (99+%), cesium fluoride (99%), nitroethane (96%), anhydrous ammonium acetate, glacial acetic acid (99.8%), powder iron, 325 mesh (97%), anhydrous ferric chloride (98%), hydrochloric acid (37%), methylamine hydrochloride (98%), sodium chloride (99%), sodium cyanoborohydride (95%), Celite 521, lithium aluminum hydride (95%), sodium hydroxide (98%), sodium acetate trihydrate (99+%), anhydrous sodium sulfate (99+%), *N*-methylbis(trifluoroacetamide) (MBTFA, 98%) and potassium sodium tartrate tetrahydrate (99%). β-Glucuronidase (*Helix pomatia*, type H-1) was obtained from Sigma Chemical Co. (St Louis, Missouri). Trifluoroacetic anhydride (99%) was obtained from Pierce Chemical Co. (Rockford, Illinois). All solvents were glass distilled, high-performance liquid chromatography (HPLC) grade, and were obtained from Burdick and Jackson (Muskegon, Michigan).

Mass spectrometry

Tandem mass spectrometric analyses were performed on a Finnigan MAT ion trap mass spectrometer (ITMS) coupled to a Hewlett-Packard model 5890 gas chromatograph. All data were acquired using an IBM/AT microcomputer loaded with version 2.1 of the ITMS scan editor software. The buffer gas pressure in the ion trap cell was initially set to an ion gauge reading of 1.5×10^{-5} torr at a manifold temperature of 120 °C by adjusting the flow of the carrier gas. Additional helium was then introduced into the ion trap manifold through a metering valve to give an ion gauge reading of 2.0×10^{-5} torr. The ITMS was then calibrated using perfluorotributylamine. Protonated molecules were formed by chemical ionization (CI) with methanol as the CI reagent gas introduced into the ion trap manifold through a metering valve to give a total manifold pressure of 4.5×10^{-5} torr. The pressure of the methanol was at 2.5×10^{-5} torr. The scan function depicting the sequence of events in a typical tandem mass analysis experiment is shown in Fig. 2. Both MS/MS and MS/MS/MS experiments began by ionization of the CI reagent gas for 1 ms at an r.f. level of 20 amu followed by selective mass storage of protonated methanol ions using a DC pulse of −32 V. The r.f. level was raised to 25 amu to allow CI of the analyte for 200 ms. Selective mass storage of the parent ion was accomplished by ramping the r.f. to eject all ions below the parent ion and then applying a DC voltage with an amplitude set at −0.998 V multiplied by the *m/z* of the parent ion (Table 1). Selective mass storage of the daughter ion at *m/z* 275 was incorporated in the MS/MS/MS scan function. The r.f. was then relaxed to 60 amu to efficiently store the daughter or granddaughter ions formed from collison-induced dissociation (CID) of

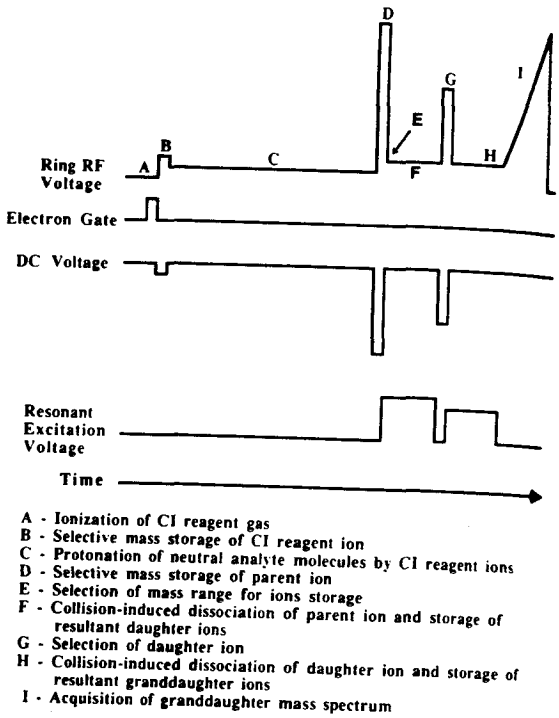

Figure 2. A scan function for CI, selective mass storage and sequential mass analysis (CI-r.f./DC-MS/MS/MS). Additional stages of mass analysis can be conducted by repeating steps G and H. The scan function schematic is not drawn to scale.

Table 1. Typical operating parameters for MS/MS and MS/MS/MS analysis

Parameters	MS/MS		MS/MS/MS	
	m/z 402	*m/z* 388	*m/z* 402	*m/z* 388
DC voltage for isolation of parent ion (V)	−401	−387	−401	−387
CID parameters for parent ion:				
Resonant excitation frequency (Hz)	52 899	54 823	52 899	54 823
Resonant excitation voltage (mV)	300	280	300	280
Resonant excitation time (ms)	62	30	62	30
DC voltage for isolation of daughter ion (*m/z* 275)			−274	−274
CID parameters for daughter ion (*m/z* 275):				
Resonant excitation frequency (Hz)			77 654	77 654
Resonant excitation voltage (mV)			290	262
Resonant excitation time (ms)			45	26

the precursor ion. CID of the parent or daughter ion was achieved by first setting the frequency of the resonant excitation voltage to the secular frequency of the selected ion. This was followed by setting the amplitude and duration of the excitation voltage such that the intensity of the ion was attenuated by more than 60%. Finally, the r.f. was ramped to eject ions over a mass range of 60–450 Da. The CID efficiency was calculated by dividing the sum of the daughter or granddaughter ion abundances by the abundance of the parent ion prior to CID.[12] The CID efficiencies for the two-stage mass analyses for 2-OH-MDMA, 5-OH-MDMA, 6-OH-MDMA and 6-OH-MDA were 76, 81, 63 and 73%, respectively. In general, slightly lower CID efficiencies were obtained when additional stages of mass analysis were performed. For example, the efficiencies for CID of the *m/z* 275 daughter ion from 2-OH-MDMA, 6-OH-MDMA and 6-OH-MDA were 43, 67, 41 and 49%, respectively.

Gas chromatography (GC)

Samples were introduced directly into the ion trap cell via a gas chromatograph fitted with a 15 m × 0.25 mm i.d. 0.25 μm film thickness, DB-5 capillary column (J&W Scientific, Rancho Cordova, California). The linear velocity was set at 40 cm s^{-1} at an oven temperature of 100 °C. The injector and transfer line temperatures were 260 and 250 °C, respectively. After splitless injection, the column temperature was held at 100 °C for 0.5 min and then programmed to 300 °C at 10 °C min^{-1}. *N*-Trifluoroacetyl-3,4-(methylenedioxy)methamphetamine (500 pg on-column) was injected to evaluate the performance of the GC/ITMS system prior to analysis of the samples.

Chemical synthesis of metabolites

All the reference compounds were characterized by GC/MS after being synthesized according to published procedures for preparation of similar compounds. All retention time and mass spectral data are for trifluoroacetyl (TFA) derivatives. The *m/z* values and relative intensities (in parentheses) are given for the ions in the spectra of each TFA derivative.

2-Hydroxy-4,5-(methylenedioxy)methamphetamine (6-OH-MDMA). Demethylation of 2,4,5-trimethoxybenzaldehyde by boron tribromide according to a procedure reported by McOmie *et al.*[13] gave 2,4,5-trihydroxybenzaldehyde: GC/CI-MS, *m/z* 443 (MH^+, base peak). Methylenation of 2,4,5-trihydroxybenzaldehyde using cesium fluoride and dichloromethane according to published methylenation procedures for catechol-containing compounds[14] yielded 2-hydroxy-4,5-(methylenedioxy)benzaldehyde: GC/CI-MS, *m/z* 263 (MH^+, base peak). Condensation of 2-hydroxy-4,5-(methylenedioxy)benzaldehyde with nitroethane[15] gave 2-hydroxy-4,5-(methylenedioxy)-β-nitrostyrene: GC/CI-MS, *m/z* 320 (MH^+, base peak). Refluxing of 2-hydroxy-4,5-(methylenedioxy)-β-nitrostyrene in the presence of iron and concentrated hydrochloric acid according to the reported procedure of Morgan and Beckett[16] gave (2-hydroxy-4,5-(methylenedioxy)phenyl)acetone: GC/CI-MS, *m/z* 291 (MH^+, base peak). Reductive amination of (2-hydroxy-4,5-(methylenedioxy)phenyl)acetone with methylamine and sodium cyanoborohydride[9] yielded 6-OH-MDMA: retention time, 7.16 min; GC/CI-MS, *m/z* 402 (MH^+, base peak); GC/EI-MS, 402 (MH^+, 5), 275 (14), 274 (68), 247 (4), 178 (7), 155 (5), 154 (100), 147 (9), 121 (7), 110 (54), 91 (7), 79 (5), 69 (34,) 63 (7), 57 (6), 56 (15), 53 (12), 51 (5), 42 (55); MS/MS from CID of *m/z* 402, 275 (100), 403 (22).

2-Hydroxy-3,4-(methylenedioxy)methamphetamine (2-OH-MDMA). This compound was synthesized from 2,3,4-trimethoxybenzaldehyde in a manner similar to that described for 6-OH-MDMA. Two compounds with identical electron impact (EI), CI and daughter mass spectra but different retention times and different granddaughter mass spectra were formed: 2-OH-MDMA and 4-hydroxy-5,6-(methylenedioxy) methamphetamine. 2-OH-MDMA: retention time, 6.93 min; GC/CI-MS, *m/z* 402 (MH^+, base peak); GC/EI-MS, 402 (MH^+, 3), 275 (18), 274 (55), 247 (10), 155 (9), 154 (100), 111 (7), 110 (34), 100 (23), 98 (12), 97 (12), 83 (11), 71 (12), 70 (11), 69 (32), 57 (23), 56 (14), 55 (16), 43 (28), 42 (12), 41 (29); MS/MS from CID of *m/z* 402, 275 (100), 403 (21); MS/MS/MS from CID of *m/z* 275, 275 (17), 257 (17), 255 (17), 247 (24), 245 (29), 231 (7), 227 (6), 225 (6), 219 (15), 161 (100), 143 (14), 133 (59), 115 (17), 105 (20).

4-Hydroxy-5,6-(methylenedioxy)methamphetamine. Retention time, 7.54 min; GC/CI-MS, *m/z* 402 (MH^+, base peak); GC/EI-MS, 402 (MH^+, 4), 401 (M^+, 10), 275 (14), 274 (48), 155 (12), 154 (100), 110 (18), 69 (6); MS/MS from CID of *m/z* 402, 275 (100), 403 (18); MS/MS/MS from CID of *m/z* 275, 275 (5), 247 (100), 161 (2), 133 (2).

3-Hydroxy-4,5-(methylenedioxy)methamphetamine (5-OH-MDMA). Synthesis of this compound from 3,4,5-trimethoxybenzaldehyde was carried out as described for 6-OH-MDMA. 5-OH-MDMA: retention time, 7.73 min; GC/CI-MS, *m/z* 402 (MH^+, 100); GC/EI-MS, 402 (MH^+, 6) 275, (12), 274 (85), 247 (18), 212 (14), 211 (7), 155 (7), 154 (100), 110 (34), 79 (8), 78 (6), 42 (15); MS/MS from CID of *m/z* 402, 275 (100), 403 (18).

2-Hydroxy-4,5-(methylenedioxy)amphetamine (6-OH-MDA). This compound was synthesized by modification of the procedure used for 6-OH-MDMA. 2-Hydroxy-4,5-(methylenedioxy)-β-nitrostyrene was reduced with lithium aluminum hydride[9] to give 6-OH-MDA. The retention time for 6-OH-MDA was 9.47 min using the above GC program but with the rate set at 5 °C min^{-1}. GC/CI-MS, *m/z* 388 (MH^+, 100); GC/EI-MS, 388 (MH^+, 8), 387 (M^+, 17), 275 (9), 274 (100), 248 (8), 247 (40), 199 (8), 183 (6), 178 (11), 177 (8), 151 (10), 150 (6), 147 (11), 140 (25), 121 (6), 92 (5), 69 (8), 50 (5); MS/MS from CID of *m/z* 388, 275 (100), 389 (18).

In vivo metabolism

Male Sprague-Dawley rats (200–250 g, $n = 6$) were fasted overnight prior to either subcutaneous or oral

administration of either 10 or 20 mg kg^{-1} MDMA (calculated as free base). Control rats ($n = 3$) received only the vehicle. Food and water were available to the animals for the duration of the study. All the rats were sacrificed by decapitation 6 h after drug administration. Brain and liver were removed from each rat, rinsed in chilled physiological saline solution, patted dry with Kimwipes and wrapped in aluminium foil prior to storage. Blood was also collected, pooled and centrifuged at 10000 × *g* to separate the plasma. In another experiment, male Sprague–Dawley rats (200–250 g, $n = 3$) which had only received the vehicle were placed individually in metabolic cages and 24 h control urine was collected. These rats were then injected subcutaneously with 10 mg kg^{-1} MDMA and 24 h urine was collected as described. All specimens were stored at −20 °C until analysis the following day.

Enzymatic hydrolysis

Pooled rat brains and individual rat liver were homogenized in 0.4 M perchloric acid (three times the weight of the tissue) containing 0.05% sodium metabisulfite, and then centrifuged at 10000 × *g* for 20 min. Supernatant equivalent to 2 g of wet tissue was used for analysis. To precipitate proteins, 4 ml of cold 10% trichloroacetic acid was added to 4 ml of plasma prior to centrifugation at 1025 × *g* for 20 min. The whole supernatant was then used for analysis. The pH of the supernatants (brain, liver and plasma) or the urine (4 ml) was adjusted to about 5 with 10 M KOH, followed by addition of 4 ml of 1 M sodium acetate buffer (pH 4.8) containing 16000 units of β-glucuronidase. In addition, 40 mg of sodium metabisulfite was added to the urine. The samples were then incubated for 16 h at 37 °C.

Extraction and derivatization

The biological specimen, in a 30 ml test tube with a Teflon-lined screw-cap, was basified to pH 8.5 with 10 M KOH, saturated with sodium chloride and extracted with 2 × 8 ml of dichloromethane–isopropanol (3:1 v/v) by gentle rocking for 15 min. The liquid phases were separated by centrifugation at 1025 × *g* for 20 min. The pooled organic extract was washed with 4 ml of 0.1 M ammonium hydroxide solution and centrifuged as above. The organic extract was then dried over anhydrous sodium sulfate and again centrifuged as above. The liquid phase was transferred to a 15 ml test tube containing 400 µl of 1% methanolic HCl and then hand vortexed for 10 s. Half of the organic extract was transferred to a 10 ml test tube and evaporated to dryness under a gentle stream of air at 60 °C. This was repeated until all the organic extract was evaporated to dryness.

Ethyl acetate (100 µl) and trifluoroacetic anhydride (200 µl) were added to the residue, and the tube was tightly capped, hand vortexed for 10 s, and then heated for 20 min at 80 °C. Just before analysis, the excess organic solvent and trifluoroacetic anhydride were removed under a gentle stream of nitrogen at 50 °C. The residue was reconstituted in 50 µl of MBTFA, heated at 80 °C for 5 min and then cooled to room temperature prior to analysis of a 1 µl aliquot by GC/MS.

RESULTS AND DISCUSSION

The ITMS has proven to be a powerful tool for the identification of structurally unknown metabolites because it can provide high full-scan sensitivity with either electron or chemical ionization.[9,10] In addition the ITMS is capable of performing multiple-stage mass analysis with high efficiency.[17,18] These capabilities facilitate detection of low concentrations of metabolites in relatively crude biological extracts, without lengthy purification procedures, or in the presence of co-eluting compounds which cannot be readily separated by a high-resolution capillary column. The particular strategy that we have employed is to first obtain full-scan CI mass spectra under conditions that generate abundant protonated molecules. These spectra indicate the unknown's molecular weight, and also often contain structurally significant fragment ions. Further structural information is then obtained by CID of the protonated molecule. If necessary, the CID-generated daughter ions can be trapped and caused to undergo further CID to granddaughter ions, thereby providing additional structural information. The postulated structure of the metabolite is then confirmed by either comparison of the daughter or granddaughter mass spectrum with that of derivatized but unpurified synthetic product, or by co-injection of the synthetic reference compound and analysis by selected reaction monitoring. The justification for the use of unpurified synthetic reference material is to facilitate the rapid identification of a postulated metabolite without having to perform a time-consuming purification of the standard. Once the metabolite has been identified, time can be appropriately invested in the purification of the standard for pharmacodynamic or toxicodynamic studies.

The initial analyses by GC/CI-MS resulted in the identification of only the major regioisomers owing to the inability to deconvolute the minor regioisomers from the co-eluting compounds in the dirty biological extracts. However, all the regioisomers of hydroxy-MDMA and hydroxy-MDA were successfully identified using GC/CI-MS/MS analysis despite the similarity of the daughter mass spectra obtained for the three regioisomers of hydroxy-MDMA and of hydroxy-MDA. Even though GC/CI-MS/MS/MS analysis was not necessary for the identification of these regioisomers, the ability of an additional stage of mass analysis beyond MS/MS to produce three very different granddaughter mass spectra corresponding to the three regioisomers of hydroxy-MDMA and of hydroxy-MDA served to further corroborate the previous identifications by MS/MS.

Detection of hydroxylated metabolites of MDMA

Hydroxylation of MDMA at the 6 position of the aromatic ring would give a compound structurally similar to 6-hydroxydopamine, which is a potent dopaminergic neurotoxin. Therefore, we sought to detect and characterize ring-hydroxylated metabolites of MDMA which may be responsible for the neurotoxic effects of MDMA.

The molecular weight of the *N,O*-bis-TFA derivative of a monohydroxylated MDMA would be 401. Therefore, CI of such a compound would be expected to give an abundant protonated molecule at *m/z* 402. GC/CI-MS analysis of hydrolyzed and derivatized liver extract from a dosed rat showed a peak in the *m/z* 402 ion current profile which was not present in the corresponding ion current profile from a rat that had not received MDMA. CID of the *m/z* 402 ion generated an abundant daughter ion at *m/z* 275, which is consistent with the loss of $CH_3N{=}C(OH)CF_3$ from the protonated molecule of the TFA derivative of a hydroxy-MDMA via a McLafferty rearrangement. This fragmentation pathway is commonly observed in the CI mass spectra of the TFA derivatives of MDMA and its nitrogen-containing metabolites.[9] The absence of a fragment ion corresponding to the loss of trifluoroacetic acid in the CI mass spectrum indicated that the metabolite was hydroxylated on the aromatic ring rather than on the side chain from β-hydroxylation. Elimination of trifluoroacetic acid produces a prominent fragment ion in the CI mass spectra of compounds such as the TFA derivative of pseudoephedrine, which has a trifluoroacetate attached to the benzylic carbon.[19]

MDMA is known to undergo *N*-demethylation to form 3,4-(methylenedioxy)amphetamine (MDA) in the rat.[9] It seemed likely that the metabolically produced MDA would also undergo *in vivo* ring hydroxylation. Consequently, we searched for and found a CI mass spectrum containing an abundant ion at *m/z* 388, which would correspond to the mass of the protonated molecule of the TFA derivative of the anticipated hydroxylated MDA. CID of the *m/z* 388 ion again gave an abundant daughter ion at *m/z* 275 which corresponds to the loss of $HN{=}C(OH)CF_3$ from the protonated molecule by the same fragmentation pathway observed for the hydroxylated MDMA compound. The formation of the same daughter ion as that from the hydroxylated MDMA compound provided further evidence that this compound was the *N*-demethylated analog of ring-hydroxylated MDMA.

These results suggested the formation of ring-hydroxylated metabolites *in vivo*, but did not indicate the positions on the aromatic ring where hydroxylation had occurred.

Identification of the regioisomers of hydroxy-MDMA and hydroxy-MDA

The technique of selected reaction monitoring provided a very selective and sensitive means of detecting hydroxylated MDMA and hydroxylated MDA in complex biological matrices. The ion current at *m/z* 275 corresponding to the daughter ion formed by CID of the protonated molecule of MDMA (*m/z* 402) was monitored during the analysis of hydrolyzed and derivatized liver extract from a rat dosed with MDMA. The resulting ion current profile (Fig. 3(a)) showed three distinct peaks, presumably corresponding to hydroxylation at each of the three available positions on MDMA's aromatic ring. In order to confirm this presumption and to determine the location of hydroxylation for each of the compounds, it was necessary to synthesize 2-, 5-, and 6-hydroxy-MDMA. TFA derivatives of each of the synthetic isomers were then separately co-injected with derivatized rat liver extract. The resulting ion current profiles are shown in Fig. 3 (profiles B, C and D). The identity of each of the three metabolite peaks is indicated by the enhanced peak intensity due to co-elution with the corresponding synthetic isomer. As a result, the peaks at retention times of 6.93, 7.16 and 7.73 min were conclusively identified as 2-, 6- and 5-hydroxy-MDMA, respectively.

The ion current profile resulting from co-injection of 2-OH-MDMA shows an additional chromatographic peak which corresponds to 4-OH-5,6-(methylenedioxy) methamphetamine, a co-product of the synthesis of the 2-OH-MDMA (see Experimental section).

In a similar manner, the rat liver extract was found to contain three hydroxy-MDA isomers (Fig. 4), the largest of which was identified as the TFA derivative of 6-OH-MDA (retention time = 9.47 min) by co-injection of the TFA derivative of synthetic 6-OH-MDA. The other two peaks in the ion current profile at retention times 8.97 and 10.67 min were tentatively assigned the structures of 2- and 5-OH-MDA, respectively, on the basis of comparison of their elution profiles with those of the three regioisomers of hydroxy-MDMA.

Based on the areas of the 6-OH-MDMA and 6-OH-MDA peaks in Figs 3 and 4, hydroxylation at the 6 position is favored over hydroxylation at the 2 and 5 positions. This is in agreement with the published report on the preferential *para*-hydroxylation of *O*-substituted phenolic amine compounds such as methoxyphenamine[20] since the 3,4-methylenedioxy moiety has been reported to behave metabolically like the methoxy functionality.[21] The role of these aromatic ring-hydroxylated metabolites of MDMA in MDMA-induced neurotoxicity is under investigation.

The identification of the three regioisomers of hydroxy-MDMA and hydroxy-MDA by either two- or three-stage mass analysis requires downloading of two separate scan functions on two different runs. Therefore, considerable time was required for the analysis of samples. In an effort to reduce the time required for multiple-stage mass analysis, a scan function was written which permitted the isolation of a narrow mass range in the ion trap cell, followed by consecutive CID of two parent ions at *m/z* 402 and 388 prior to scanning the ions out of the trap during data acquisition. Figure 5 shows the application of such a scan function to the analysis of the derivatized rat liver extract; all the regioisomers of hydroxy-MDMA and hydroxy-MDA were detected in a single run. Consequently, the analysis time was reduced by approximately 50%. We are continuing to use this type of scan function for rapid screening of samples for the presence of hydroxylated metabolites.

Tandem mass spectrometry of ring-hydroxylated metabolites of MDMA

Tandem mass spectrometry has proven to be extremely useful for the differentiation of isomeric compounds which cannot be readily distinguished by their EI or CI mass spectra. Recent examples include the differentia-

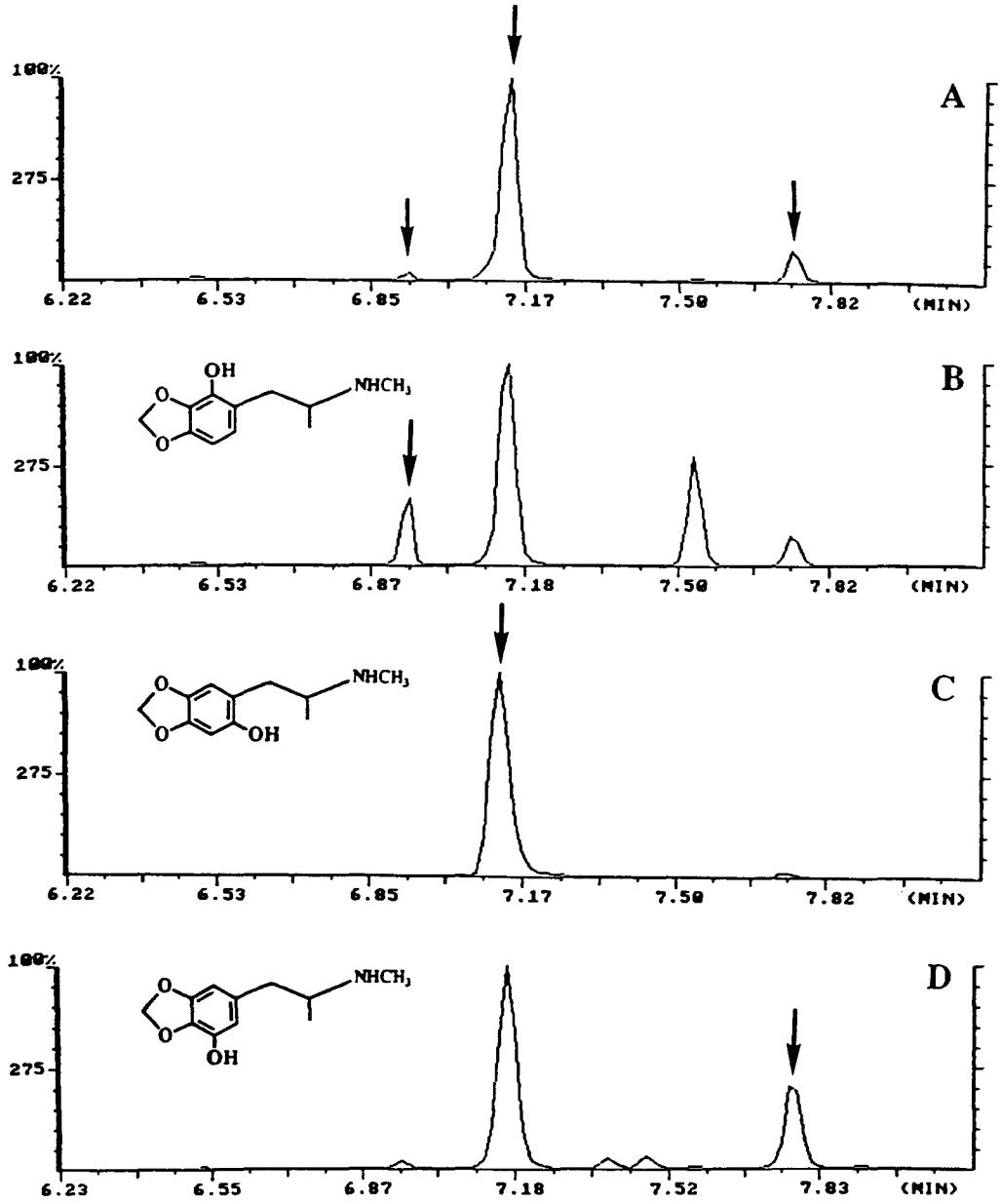

Figure 3. Reconstructed daughter ion profiles (m/z 402 → 275) from analysis of hydrolyzed and derivatized liver extract from a rat dosed with 20 mg kg^{-1} MDMA. The gas chromatographic conditions used are as described in the text. The arrows indicate the peaks corresponding to the three regioisomers of hydroxy-MDMA (a). Co-injection with derivatized synthetic reference compounds permitted identification of 2-OH-MDMA (b), 6-OH-MDMA (c) and 5-OH-MDMA (d).

tion of isomeric pyranocoumarins[22] and hydroxyindoles[23] by CID of the molecular ions formed in an ITMS. Unfortunately, CID of the protonated molecules of the TFA derivatives of the regioisomers of hydroxy-MDMA all gave identical spectra, each consisting of a single abundant daughter ion peak at m/z 275 resulting from loss of $CH_3N{=}C(OH)CF_3$ from the respective protonated molecules. Similarly, the daughter mass spectra observed for the regioisomers of OH-MDA were all identical. However, further CID of the m/z 275 daughter ions derived from MS/MS analysis of the protonated molecules of the three regioisomers of hydroxy-MDMA gave very different granddaughter mass spectra (Fig. 6). The granddaughter mass spectrum of the TFA derivative of 6-OH-MDA was found to be essentially identical to the granddaughter mass spectrum from the TFA derivative of 6-OH-MDMA. This was expected since the granddaughter mass spectra are produced by CID of structurally identical daughter ions at m/z 275 from each regioisomer of hydroxy-MDMA and hydroxy-MDA. Therefore, comparison of the granddaughter mass spectra of the regioisomers of both hydroxy-MDMA and hydroxy-MDA from MS/MS/MS analysis of hydrolyzed and trifluoroacetylated dosed rat liver extract with authentic standards was used to corroborate the previous structural assignments derived by co-injection of synthetic reference compounds.

The most abundant granddaughter ions occur at different masses for each of the regioisomers. For 2-OH-

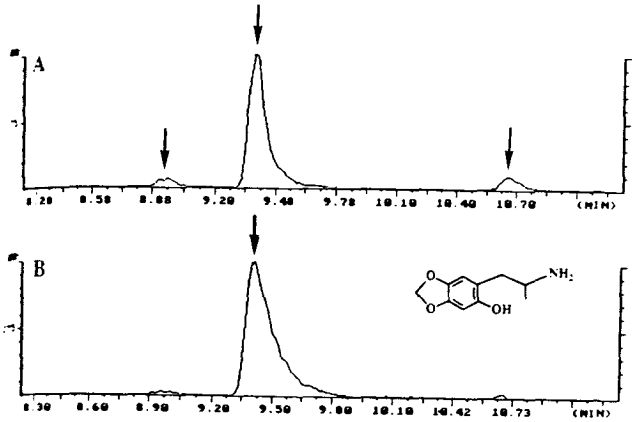

Figure 4. Reconstructed daughter ion profiles (m/z 388 → 275) from analysis of the same samples as in Fig. 3. The gas chromatographic conditions were the same except for the temperature program rate, which was 5 °C min^{-1}. The arrows indicate the peaks corresponding to the three regioisomers of hydroxy-MDA (a). Co-injection of a derivatized synthetic reference compound permitted identification of 6-hydroxy-MDA (b).

MDMA (Fig. 6(a)), the formation of granddaughter ions at m/z 161 is due to the loss of trifluoroacetic acid from the daughter ion at m/z 275. The granddaughter ions at m/z 143 ($-H_2O$), 133 ($-CH_2{=}CH_2$), 115 (−HCOOH) and 105 ($-CH_2{=}CH_2$ and CO) were shown to be formed by further fragmentation of the m/z 161 ions by isolation and further dissociation (MS/MS/MS/MS) of the m/z 161 ions. Also, the granddaughter mass spectrum of 2-OH-MDMA shows ions at m/z 257, 255, 247, 245 and 219, similar to a series of ions in the granddaughter mass spectrum of 6-hydroxy-MDA.

In contrast to 2-OH-MDMA, the m/z 275 daughter ion from 6-OH-MDMA is dissociated by several pathways, leading to the formation of a cluster of abundant ions (Fig. 6(b)). The most favorable fragmentation pathways is the ring-opening of the 3,4-methylenedioxy moiety with subsequent elimination of formaldehyde to form the base peak at m/z 245. Other prominent daughter ions can be rationalized as due to the losses of H_2O (m/z 257), HF (m/z 255), $CH_2{=}CH_2$ (m/z 247), CO_2 (m/z 231) and HCOOH (m/z 229) from the m/z 275 ion.

The granddaughter mass spectrum of 5-OH-MDMA (Fig. 6(c)) is relatively simple; it is dominated by an abundant ion at m/z 247 which is due to the loss of ethylene from the m/z 275 daughter ion. The granddaughter mass spectrum of 5-OH-MDMA also contains ions similar to those in the granddaughter mass spectrum of 2-OH-MDMA but in lower abundances (m/z 161, 143, 133, 115 and 105).

Comparison of mass spectrometric, MS/MS and MS/MS/MS analysis

The reconstructed ion current profiles corresponding to hydroxy-MDA from the analysis of hydrolyzed and derivatized dosed-rat liver extract serve to illustrate the differences between the three modes of mass analysis (Fig. 7). The signal-to-noise of the minor peak corresponding to 6-OH-MDA in the ion current profile from GC/CI-MS analysis (Fig. 7(*a*)) was significantly enhanced when a similar sample was analyzed by two-stage mass analysis (Fig. 7(b)). Employment of a third stage of mass analysis (MS/MS/MS) resulted in a substantial decrease in sensitivity, as indicated by the absence of the two minor regioisomers (2- and 5-OH-MDA) in the granddaughter ion current profile (Fig. 7(c)). The decreased sensitivity in this example is primarily due to the distribution of the ion current amongst many granddaughter ions formed from CID of the daughter ion at m/z 275. Only in the multi-stage modes of mass analysis were we able to obtain mass spectra free of ions due to co-eluting contaminants.

Distribution of metabolites

Only metabolites corresponding to the hydroxylation at position 6 of MDMA and its *N*-demethylated metabo-

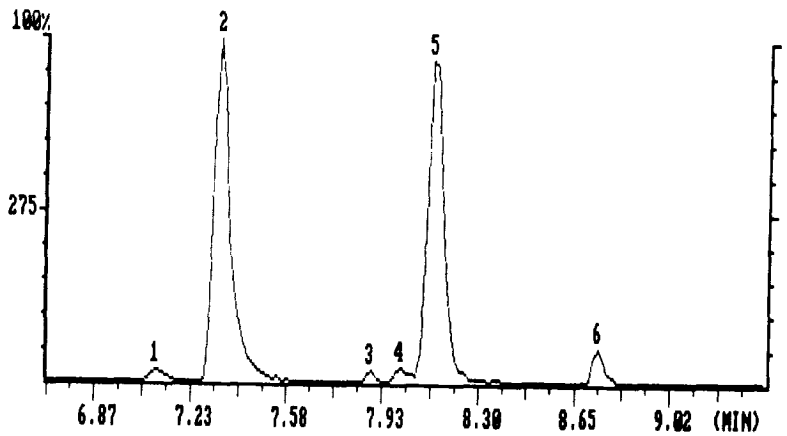

Figure 5. A reconstructed daughter ion profile showing the three regioisomers of both hydroxy-MDMA and hydroxy-MDA from analysis of the same sample as in Fig. 3. The modified gas chromatographic conditions necessary for the separation of all the compounds were: 100 °C, 2 min, 8 °C min^{-1}, 300 °C. The peak identities are: (1) 2-OH-MDA, (2) 6-OH-MDA, (3) 2-OH-MDMA, (4) 5-OH-MDA, (5) 6-OH-MDMA and (6) 5-OH-MDMA. The analysis was performed using a CI-r.f./DC-MS/MS scan function with selective mass storage of a narrow mass range (m/z 385 to 405) and consecutive CID of two parent ions (m/z 388 and 402).

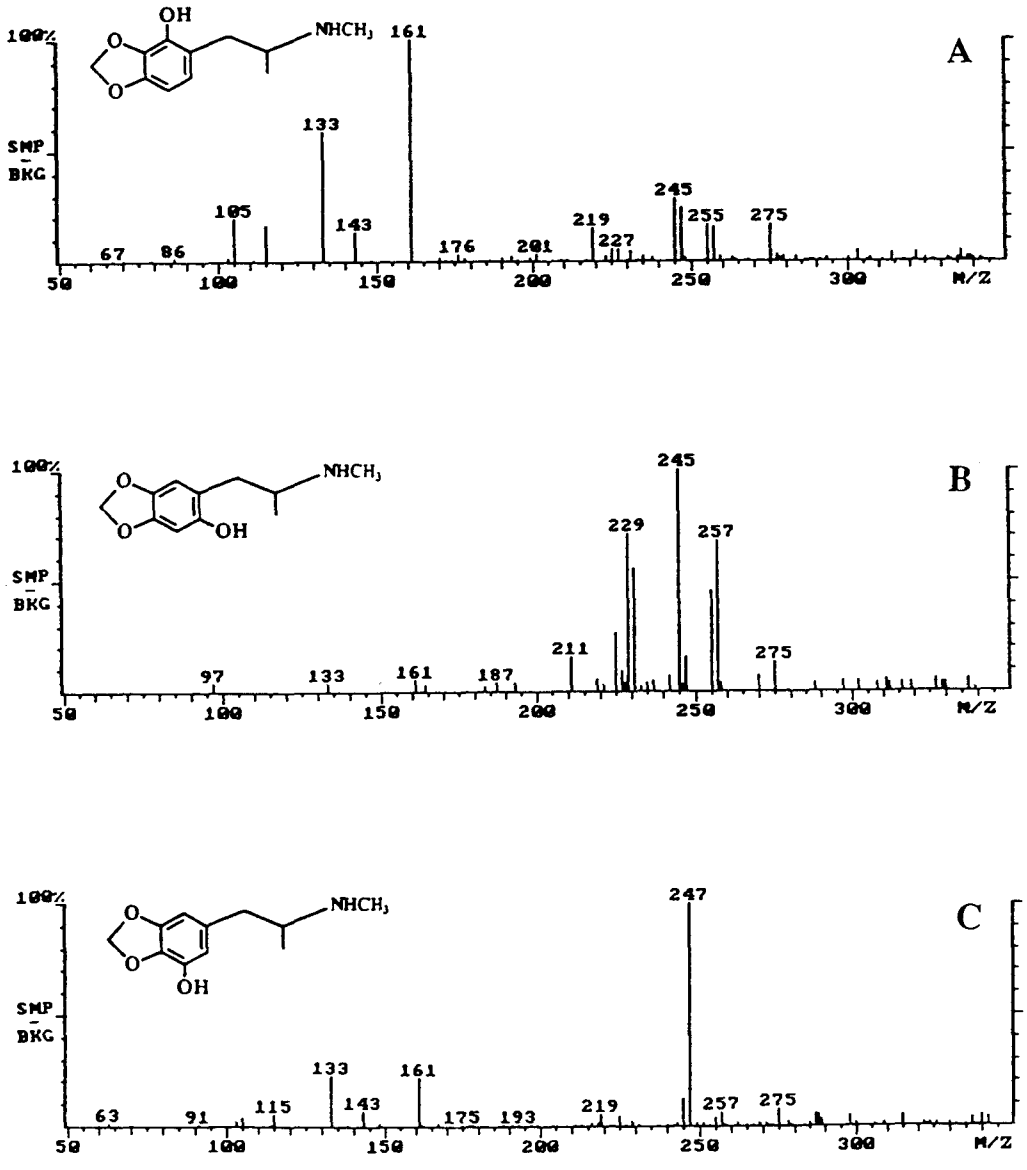

Figure 6. Differentiation of the three regioisomers of hydroxy-MDMA by tandem mass spectrometry as shown by the three distinct grand-daughter mass spectra of the TFA derivatives of 2-OH-MDMA (a), 6-OH-MDMA (b) and 5-OH-MDMA (c).

lite (MDA) were detected in the liver following administration of 10 mg kg^{-1} MDMA, by either subcutaneous or oral routes. However, all the regioisomers of hydroxy-MDMA and hydroxy-MDA were found in the liver when the dose was increased to 20 mg kg^{-1} MDMA. In contrast, only 6-OH-MDMA and 6-OH-MDA were detected in the brain and plasma even when the dose of MDMA was increased from 10 to 20 mg kg^{-1}. Also, these hydroxylated metabolites of MDMA were not detected in the urine at either dose of MDMA.

The potential significance of the identification of these hydroxylated MDMA and MDA isomers in rat brain following administration of MDMA was recently heightened by our finding that 6-OH-MDMA undergoes *in vitro* O-dealkylation to 2,4,5-trihydroxymethamphetamine,[24] a compound that has been shown to be highly neurotoxic to rats.[25]

CONCLUSIONS

Three regioisomers of hydroxy-MDMA and hydroxy-MDA were identified as *in vivo* metabolites of MDMA by ion trap multiple-stage mass analysis techniques. Hydroxylation at the 6 position is favored, but it also occurs at the 2 and 5 positions. All three regioisomers of both hydroxy-MDMA and hydroxy-MDA were detected in the rat liver following subcutaneous administration of 20 mg kg^{-1} of MDMA. However, only the 6-hydroxy isomers of both compounds were detected in the brain and plasma and none of the hydroxylated metabolites were detected in the urine. With both hydroxy-MDMA and hydroxy-MDA, the regioisomers gave identical EI and CI mass spectra, as well as identical daughter ion spectra following CID of their proto-

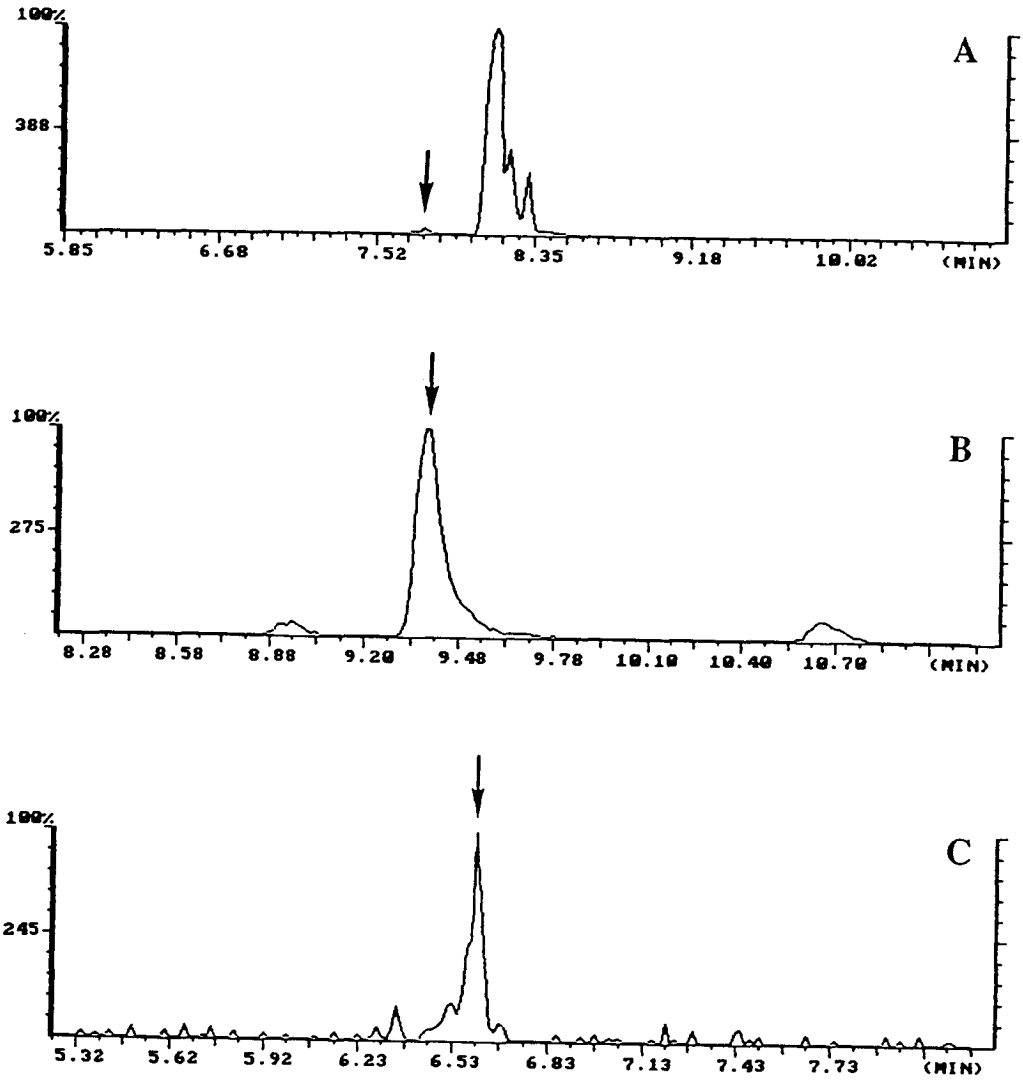

Figure 7. Reconstructed ion profiles from analysis of similarly prepared samples from MDMA-treated rats by three modes of mass analysis: a) GC/CI-MS; the gas chromatographic conditions were the same as in Fig. 4 except for the column, which was a 15 m × 0.2 mm i.d., SB-Phenyl-5 capillary column with a film thickness of 0.25 μm (Lee Scientific, Salt Lake City, Utah); (b) GC/CI-r.f./DC-MS/MS; gas chromatographic conditions as in Fig. 4, and GC/CI-r.f./DC-MS/MS/MS; gas chromatographic conditions as in (a) except for the rate, which was at 10 °C min^{-1}. In each profile, the arrow indicates the retention of 6-hydroxy-MDA.

nated molecules. However, three different granddaughter mass spectra were formed by tandem mass spectrometry when an additional stage of mass analysis was used (MS/MS/MS).

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