Communications

Ion Trap Tandem Mass Spectrometric Evidence for the Metabolism of 3,4-(Methylenedioxy)methamphetamine to the Potent Neurotoxins 2,4,5-Trihydroxymethamphetamine and 2,4,5-Trihydroxyamphetamine

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Introduction

A report that intracerebroventricular injection of MDMA[1] failed to cause neurotoxicity in rats led to speculation that a metabolite, rather than the parent compound, is responsible for the neurotoxic effects of MDMA (*1*). However, the chemical structure of the neurotoxic metabolite remains elusive despite recent identifications of 14 in vivo metabolites of MDMA in the rat (*2*, *3*). Hiramatsu et al. (*4*) showed that one of these metabolites, di-HO-MA, forms a reactive quinone which they isolated as a glutathione adduct. However, because intracerebroventricular injection of this metabolite into rats failed to elicit any neurotoxic effect, we concluded that this metabolite is unlikely to contribute to the neurotoxicity of MDMA.[2] Furthermore, we and others have tested other metabolites of MDMA, including 3,4-dihydroxyamphetamine (*5*), 4-hydroxy-3-methoxymethamphetamine,[3] and 4-hydroxy-3-methoxyamphetamine[3] (*5*), for neurotoxicity; none of these compounds elicited neurotoxicity in rats. Consequently, we explored other metabolic pathways that might give rise to neurotoxic metabolites from MDMA.

6-Hydroxydopamine is valuable for neuropharmacological studies because it can selectively induce lesions in dopaminergic neurons. As a result, there has been considerable interest in the identification of 6-hydroxydopamine in neurodegenerative brain tissues from rats treated with amphetamine and related drugs. Its involvement in the neurotoxicity of these drugs is controversial due to problems in detecting it in degenerative brain tissues from treated rats (*6*, *7*). Our interest in 6-hydroxydopamine-related compounds as possible neurotoxic metabolites of MDMA is based on the recognition that the previously reported aromatic hydroxylated metabolites of MDMA are potential precursors of 6-hydroxydopamine-like compounds, since MDMA is known to undergo metabolic *O*-dealkylation (*2*). Moreover, we have recently found that intracerebroventricular injection of tri-HO-MA or tri-HO-A into rats showed these compounds to be potent neurotoxins like 6-hydroxydopamine, but with notable differences in their neurotoxicity profiles (*8*, *9*). In the present communication we report that tri-HO-MA and tri-HO-A are in vitro metabolites of 6-HO-MDMA and 6-HO-MDA, respectively. Furthermore, these potent neurotoxins are present in vivo in rats dosed chronically with 20 mg/kg MDMA.

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Materials and Methods

Materials. All solvents, reagents, and chemicals used were of the best grades available commercially. MDMA, 6-HO-MDMA, and 6-HO-MDA were obtained from Research Triangle Institute (Research Triangle Park, NC), SKF-525A was from Smith Kline and French Laboratories (Philadelphia, PA), quinine sulfate was from Lilly Research Laboratories (Indianapolis, IN), and 1-aminobenzotriazole was from Hoffmann-La Roche (Nutley, NJ). Quinidine sulfate dihydrate was purchased from Sigma Chemical Co. (St. Louis, MO).

Instrumentation. In vitro metabolism was investigated by using the GC–MS system which has been described elsewhere (*3*). Chromatographic and mass spectrometric conditions were the same unless otherwise indicated (*3*); the manifold temperature was maintained at either 130 °C (tri-HO-MA and 5,6-dihydroxy-1,2-dimethylindole) or 140 °C (tri-HO-A), and isobutane was the chemical ionization reagent gas. Collision-induced dissociation (CID) of the protonated molecule of derivatized 5,6-dihydroxy-1,2-dimethylindole at m/z 294 was performed with the following excitation parameters: resonant frequency 72 563 Hz; voltage 500 mV; and time 32 ms. For the protonated molecule of derivatized tri-HO-MA at m/z 430, the excitation parameters were as follows: resonant frequency 49 432 Hz; voltage 410 mV; and time 20 ms. CID of the protonated molecule of tri-HO-A at m/z 458 was performed with the following excitation parameters: resonant frequency 46 392 Hz; voltage 425 mV; and time 18 ms. The overall CID efficiencies for the m/z 294 and 430 ions were 76 and 91%, respectively. Following sample injection, the oven temperature was held at 100 °C for 1 min and then linearly programmed to 300 °C at 20 °C/min. The injector and transfer line temperatures were 280 and 270 °C, respectively.

The in vivo metabolism was investigated using a Finnigan-MAT 4500 GC–MS system operated in the selected ion monitoring mode. The capillary column was the same type as that used for the in vitro study except for the increased film thickness of 1 μm; the column was routed through the separator oven (maintained at 260 °C) and directly into the ion source of the mass spec-

[1] Abbreviations: MDMA, 3,4-(methylenedioxy)methamphetamine; 6-HO-MDMA, 2-hydroxy-4,5-(methylenedioxy)methamphetamine; 6-HO-MDA, 2-hydroxy-4,5-(methlenedioxy)amphetamine; tri-HO-MA, 2,4,5-trihydroxymethamphetamine; tri-HO-A, 2,4,5-trihydroxyamphetamine; di-HO-MA, 3,4-dihydroxymethamphetamine; CID, collision-induced dissociation; EI, electron ionization; CI, chemical ionization; GC–CI–MS/MS, gas chromatography–chemical ionization–tandem mass spectrometry.

[2] Johnson, M., Elayan, I. M., Hanson, G. R., Foltz, R. L., Gibb, J. W., and Lim, H. K. (1991) Effects of 3,4-dihydroxymethamphetamine and 2,4,5-trihydroxymethamphetamine, two metabolites of 3,4-(methylenedioxy)methamphetamine, on central serotonergic and dopaminergic systems. *J. Pharmacol. Exp. Ther.* (submitted for publication).

[3] M. Johnson, I. M. Elayan, G. R. Hanson, R. L. Foltz, J. W. Gibb, and H. K. Lim, unpublished results.

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trometer. The carrier gas (hydrogen) was set to a linear velocity of 80 cm/min at an oven temperature of 100 °C. The injector temperature was 270 °C and operated in the splitless mode for 0.7 min after injection. After injection, the oven temperature was held at 100 °C for 1 min and then linearly programmed to 300 °C at 10 °C/min. The mass spectrometer was operated in the positive ion chemical ionization mode at an electron energy of 100 eV and at an indicated ionizer temperature of 100 °C. The mass spectrometer was tuned using perfluorotributylamine in the presence of methane at an indicated source pressure of 0.8 Torr; ion source voltages were adjusted such that the ions at m/z 414 from perfluorotributylamine displayed a maximum ion current at unit mass resolution while retaining a symmetrical peak shape. Sample analyses were performed with a chemical ionization reagent gas consisting of a methane and ammonia mixture (1:1) with a trace of perfluorotributylamine at a combined ion source pressure reading of 0.8 Torr. The mass spectrometer was set to monitor ions at m/z 489 and 475 which correspond to the M + NH_4^+ of the *N*-(methoxycarbonyl)-*O*,*O*,*O*-tris(trimethylsilyl) derivatives of tri-HO-MA and tri-HO-A, respectively. These ions were monitored using a sampling time of 0.105 s per mass for a total cycle time of 0.218 s. All acquisitions and data processing were performed with the Finnigan-MAT INCOS data system.

Exact mass measurement was performed by peak matching on a high-resolution mass spectrometer (Varian MAT Model 731). The proton NMR spectra were recorded on a 200-MHz IBM NR-200 spectrometer; chemical shifts are reported in ppm relative to sodium 3-(trimethylsilyl)-1-propanesulfonate. Signal multiplicities are designated as follows: s, singlet; d, doublet; and m, multiplet.

Chemical Synthesis. All the reference compounds were characterized by ^1H NMR and/or mass spectrometry.

(A) 3,4-Dihydroxymethamphetamine was synthesized by O-dealkylation of MDMA with boron tribromide according to a previously reported procedure (*2*). Lyophilization afforded a pale brown solid which gave electron ionization (EI) and chemical ionization (CI) mass spectra identical to those previously reported for di-HO-MA (*2*).

(B) 2,4,5-Trihydroxymethamphetamine was synthesized from 2,4,5-trimethoxybenzaldehyde by modification of the procedure for 4-hydroxy-3-methoxymethamphetamine (*2*). The resultant 2,4,5-trimethoxymethamphetamine was purified by elution from a silica gel column (60–120 mesh) with toluene (125 mL), chloroform (125 mL), and toluene/chloroform/methanol (2:2:1). Evaporation of the solvent gave a yellow-brown oil: GC–CI/MS (trifluoroacetyl derivative), m/z 336 (MH^+, base peak). O-Demethylation of 2,4,5-trimethoxymethamphetamine was achieved by treatment with boron tribromide under nitrogen (*2*). After quenching of the reaction by methanol, the solvent was removed by rotary evaporation followed by lyophilization. The resulting brown solid was identified as tri-HO-MA by high-resolution mass spectrometry and ^1H NMR: m/z 197.1065 (M^+) (calcd for $C_{10}H_{15}NO_3$, 197.1052); ^1H NMR (^2H_2O) δ 1.20 (d, J = 6.7 Hz, 3 H, CCH_3), 2.63 (s, 3 H, NCH_3), 2.77 (m, 2 H, CH_2), 3.43 (m, 1 H, CH), 6.44, 6.66 (2 s, 2 H, aromatic).

(C) 2,4,5-Trihydroxyamphetamine was synthesized from 2,4,5-trimethoxybenzaldehyde by modification of the procedure reported for 4-hydroxy-3-methoxyamphetamine (*2*). The resultant 2,4,5-trimethoxyamphetamine was purified using the above procedure for 2,4,5-trimethoxymethamphetamine to give a brown oil: GC–CI/MS (trifluoroacetyl derivative), m/z 322 (MH^+, base peak). O-Demethylation of 2,4,5-trimethoxyamphetamine to tri-HO-A was achieved by the above procedure for tri-HO-MA to give a brown solid which was characterized by solid-probe CI/MS and ^1H NMR: m/z 184 (MH^+, base peak); ^1H NMR (^2H_2O) δ 1.30 (d, J = 6.7 Hz, 3 H, CCH_3), 2.79 (m, 2 H, CH_2), 3.66 (m, 1 H, CH), 6.52, 6.73 (2 s, 2 H, aromatic).

(D) 5,6-Dihydroxy-1,2-dimethylindole. Oxidation of tri-HO-MA by potassium ferricyanide in a sodium bicarbonate solution for 1 h and subsequent quenching of the reaction by sodium dithionite, according to a previously reported procedure for preparation of substituted indole compound by oxidation of (2,4,5-trihydroxyphenyl)ethylamine (*10*), afforded 5,6-dihydroxy-1,2-dimethylindole: GC–CI/MS (trifluoroacetyl derivative), m/z 370 (MH^+, base peak); GC–CI/MS (methoxycarbonyl derivative), m/z 294 (MH^+, base peak); GC–EI/MS [methoxycarbonyl derivative, m/z (relative intensity)], 294 (27), 293 (100), 249 (24), 234 (28), 203 (11), 190 (15), 162 (46), 159 (20), 148 (21), 147 (80), 134 (21), 132 (15), 131 (14), 119 (27), 118 (12), 91 (13), 89 (10), 78 (11), 63 (20), 59 (27), 42 (19).

In Vitro Metabolism. Supernatants (9000*g*) were prepared from liver homogenates from six untreated male Sprague-Dawley rats (200–250 g) as described previously (*2*). All incubations without inhibitors (2 mL total volume) contained 120 mM phosphate buffer (pH 7.4), 46 mM KCl, 2 mM $MgCl_2$, 0.4 mM β-nicotinamide adenine dinucleotide phosphate ($NADP^+$), 4 mM glucose 6-phosphate, 0.8 unit of glucose-6-phosphate dehydrogenase, 1 mM *N*-acetyl-L-cysteine, 0.1 mM substrate (6-HO-MDMA, 6-HO-MDA, tri-HO-A, or tri-HO-MA), and 0.4 mL of 9000*g* supernatant. For experiments with inhibitors, the incubation was carried out as above except for the addition of an inhibitor. The inhibitors examined included: 1 mM SKF-525A in 1.15% KCl, quinidine in 1% acetic acid, quinine in 1% acetic acid, and 10 mM 1-aminobenzotriazole in 20% dimethyl sulfoxide. The concentrations of quinidine and quinine were varied from 0.2 to 100 μM.

For experiments without inhibitors, the reaction was started by addition of the NADPH-generating system and the 9000*g* supernatant after a 5-min preincubation. For inhibition studies, the inhibitor was preincubated with the NADPH-generating system and the 9000*g* supernatant for 15 min before addition of the substrate. Then incubation was carried out at 37 °C for 15 min with rapid shaking. The reaction was stopped by immersing the tubes in an ice bath followed by adjustment of the pH to 9 (using 20 μL of 10 M NaOH and 1 mL of 1 M $NaHCO_3/Na_2CO_3$ buffer, pH 9) and aqueous derivatization with 100 μL of methyl chloroformate (*11*). After derivatization, the protein was precipitated with 4 mL of cold acetone. Following centrifugation at 1400*g* for 5 min, the acetone in the supernatant was evaporated under an air stream at 55 °C. Finally, the supernatant was adjusted to a pH of 9.0 and extracted twice with 4 mL of ethyl acetate. Again, the phases were separated by centrifugation at 1400*g* and the pooled organic layers were evaporated to dryness. The residue was reconstituted in 200 μL of ethyl acetate prior to analysis of a 1-μL aliquot by gas chromatography–chemical ionization–tandem mass spectrometry (GC–CI–MS/MS). For tri-HO-A, the *O*-(methoxycarbonyl) groups were exchanged with *O*-(trimethylsilyl) groups by further heating the residue in 50 μL each of acetonitrile and *N*-(trimethylsilyl)imidazole at 125 °C for 30 min. The solution was analyzed directly as above.

In Vivo Metabolism. Three male Sprague-Dawley rats (200–250 g) were dosed with 20 mg/kg MDMA (expressed as free base) using the protocol of Battaglia et al. (*12*) for neurotoxicity testing of MDMA. In another set of experiment, three female Sprague-Dawley rats (200–250 g) were similarly injected with MDMA as described above. Control rats (male or female Sprague-Dawley rats, n = 3) were injected subcutaneously with 1 mL/kg physiological saline solution. The animals were sacrificed at 13 h after last injection: whole livers were removed, rinsed in chilled 1.15% KCl solution, dried with Kimwipes, and finally individually wrapped in aluminum foil prior to storage at −20 °C until analysis the next day. About 3.5 g of the wet liver was homogenized in 1 M HCl (containing 1 mM ascorbic acid) equivalent to 2 times the weight of the liver. Homogenate equal to 2 g of wet tissue was centrifuged at 9000*g* for 15 min, and the supernatant was derivatized with methyl chloroformate as described for in vitro experiments. After derivatization, the supernatant was adjusted to pH 9 with 10 M NaOH and 0.2 mL of 1 M $NaHCO_3/Na_2CO_3$ buffer, pH 9, followed by two extractions with 5 mL of ethyl acetate with gentle rocking for 15 min. After centrifugation at 1000*g* for 5 min, the pooled organic layers were dried over anhydrous sodium sulfate, centrifuged as before, and finally evaporated to dryness under a gentle stream of air at 55 °C. Formation of the *N*-methoxy-*O*,*O*,*O*-tris(trimethylsilyl) derivative was as described for the in vitro experiments, but analysis was by GC–CI/MS under selected ion monitoring mode.

Results and Discussion

Initial attempts to isolate tri-HO-MA or tri-HO-A from biological specimens by liquid–liquid or solid-phase extractions were unsuccessful, presumably because of the

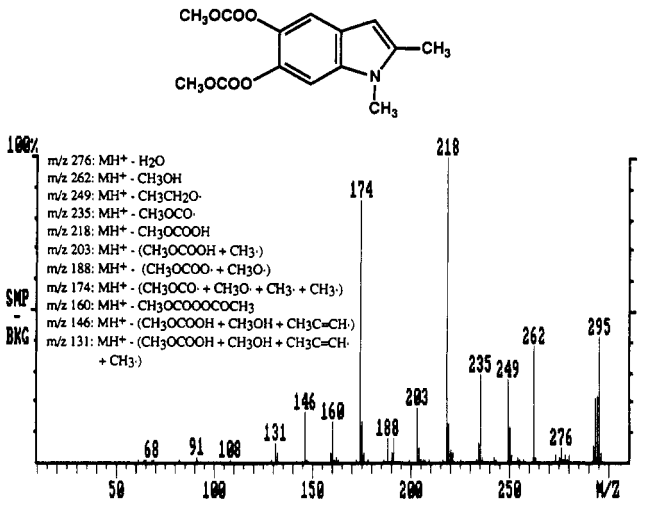

Figure 1. The daughter ion mass spectrum produced by CID of the protonated molecule of the *O,O*-bis(methoxycarbonyl) derivative of 5,6-dihydroxy-1,2-dimethylindole formed by further oxidation of tri-HO-MA in fortified 9000*g* rat liver supernatant.

compounds' susceptibility to oxidation. Therefore, we sought to isolate and identify biochemically formed oxidation products that would indicate the presence of these trihydroxy compounds. Incubation of tri-HO-MA with 9000*g* rat liver supernatant fortified with the NADPH-generating system, followed by GC–MS analysis of the derivatized extract, produced a chromatographic peak that was not present in the control. The mass spectrum of this product showed an abundant ion at m/z 294 which we tentatively identified as the protonated molecule of the *O,O*-bis(methoxycarbonyl) derivative of 5,6-dihydroxy-1,2-dimethylindole, on the basis of a report that 6-hydroxydopamine readily undergoes 1,2-intracyclization when oxidized and then subsequent rearrangement to an indole (*13*). To confirm this structural assignment by GC–CI–MS/MS, the protonated molecule (m/z 294) was subjected to collision-induced dissociation. The resulting daughter ion mass spectrum (Figure 1) agreed well with the corresponding daughter ion spectrum obtained from a synthetic sample of derivatized 5,6-dihydroxy-1,2-dimethylindole. The rationalizations of the various daughter ions are included in Figure 1. The m/z 295 ion in the daughter ion mass spectrum is due to the contribution of ^{13}C and ^{15}N isotopes of the protonated molecule; the parent + 1 ion is commonly observed in the daughter ion mass spectra obtained with an ion trap mass spectrometer due to the inherent difficulty in isolation of ions at a single m/z value (*14*).

In contrast to tri-HO-MA, only a trace amount of the corresponding indole compound (tentatively assigned the structure of 5,6-dihydroxy-2-methylindole) was observed when tri-HO-A was incubated with NADPH-fortified 9000*g* rat liver supernatant. Therefore, the poor yield of the indole compound from in vitro metabolism of tri-HO-A suggests that this compound may not be a suitable marker for the formation of tri-HO-A.

Upon further investigation we discovered that aqueous derivatization with methyl chloroformate provided a means of converting the highly reactive tri-HO-MA to a stable derivative that could be detected by GC–MS. The chemical ionization mass spectrum of the *N,O,O,O*-tetrakis(methoxycarbonyl) derivative of tri-HO-MA showed an abundant protonated molecule at m/z 430 which was further characterized by CID to give a daughter ion mass spectrum that was identical to that of derivatized synthetic tri-HO-MA; the daughter ion mass spectrum contained the following ions [m/z (relative intensity)]: 431 (24), 430 (28), 399 (11), 398 (100), 354 (6), and 322 (13). The daughter ions at m/z 398, 354, and 322 can be rationalized by the loss of CH_3OH, CH_3OCOOH, and CH_3OH plus CH_3OCO-OH, respectively, from the protonated molecule. Comparison of their respective chromatographic peak areas indicated that more than half of the tri-HO-MA had been converted to 5,6-dihydroxy-1,2-dimethylindole under the incubation conditions described.

Only a small peak corresponding to tri-HO-A was detected by GC–CI–MS/MS analysis of the in vitro incubate of tri-HO-A. The methoxycarbonyl derivative may not be suitable for the detection of trace amounts of tri-HO-A since the derivative markedly increased the boiling point of the trihydroxy compound (eluted at close to 300 °C). Detection of trace amounts of tri-HO-A near the maximum operating temperature of the column was difficult due to increased column bleed which induced space charging in the ion trap mass spectrometer with a resulting decrease in sensitivity. To improve our ability to detect these trihydroxy metabolites, we sought a more volatile derivative. This was achieved by exchanging the methoxycarbonyl groups with trimethylsilyl groups. The resulting derivatives eluted at an oven temperature about 30 °C lower than that of the corresponding methoxycarbonyl derivatives. The exchange of the trimethylsilyl groups for the methoxycarbonyl groups were performed by a procedure developed for the trace analysis of catecholamines by GC–MS (*15*). With the new sequential derivatization procedure we obtained a much larger ion current profile peak corresponding to the remaining tri-HO-A from the in vitro incubate of tri-HO-A. The CI mass spectrum contained an abundant ion at m/z 458 corresponding to the protonated molecule of the *N*-(methoxycarbonyl)-*O,O,O*-tris(trimethylsilyl) derivative of tri-HO-A. Further characterization by CID of the protonated molecule gave a daughter ion mass spectrum consisted of the following ions [m/z (relative intensity)]: 460 (22), 459 (51), 458 (8), 427 (7), 426 (78), 384 (6), and 383 (100). The presence of the ions at m/z 460 and 459 is due to the isotope peaks of the protonated molecules (m/z 458). The ions at m/z 426 and 383 can be rationalized by loss of CH_3OH and $CH_3OC(OH)$=NH, respectively, from the protonated molecule.

The conversion of tri-HO-MA to 5,6-dihydroxy-1,2-dimethylindole apparently depends on some factor in the 9000*g* rat liver supernatant, because 5,6-dihydroxy-1,2-dimethylindole was absent when the liver supernatant was omitted during incubation of tri-HO-MA. However, the conversion of tri-HO-MA to the indole is probably nonenzymatic, because the amount of 5,6-dihydroxy-1,2-dimethylindole did not decrease substantially when tri-HO-MA was incubated with boiled 9000*g* rat liver supernatant or incubated in the absence of NADPH. However, the formation of 5,6-dihydroxy-1,2-dimethylindole from tri-HO-MA was partially inhibited in the presence of cytochrome P-450 inhibitors like SKF-525A (about 54%) or 1-aminobenzotriazole (about 37%). This result suggested that oxidation of tri-HO-MA to 5,6-dihydroxy-1,2-dimethylindole may require the presence of hemoproteins. Alternatively, tri-HO-MA may be nonenzymatically oxidized to the indole by metallic cations in the S-9 fraction. In addition, we observed a decrease in the amount of 5,6-dihydroxy-1,2-dimethylindole, comparable to that seen with SKF-525A, when *N*-acetyl-L-cysteine was omitted, possibly due to the further oxidation of 5,6-dihydroxy-1,2-dimethylindole to the aminochrome which can be reduced back to its original form by *N*-acetyl-L-cysteine (*16*).

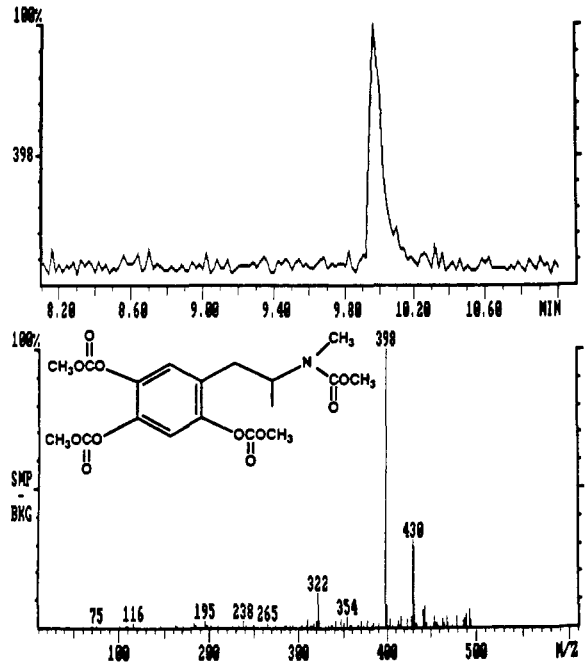

Figure 2. GC–CI–MS/MS analysis of derivatized extract from the incubation of 6-HO-MDMA with fortified 9000*g* rat liver supernatant. Top panel: Daughter ion current profile (m/z 430 → 398) showing a chromatographic peak (9.96 min) identified as the *N,O,O,O*-tetrakis(methoxycarbonyl) derivative of tri-HO-MA. Bottom panel: Daughter ion mass spectrum corresponding to the derivatized tri-HO-MA.

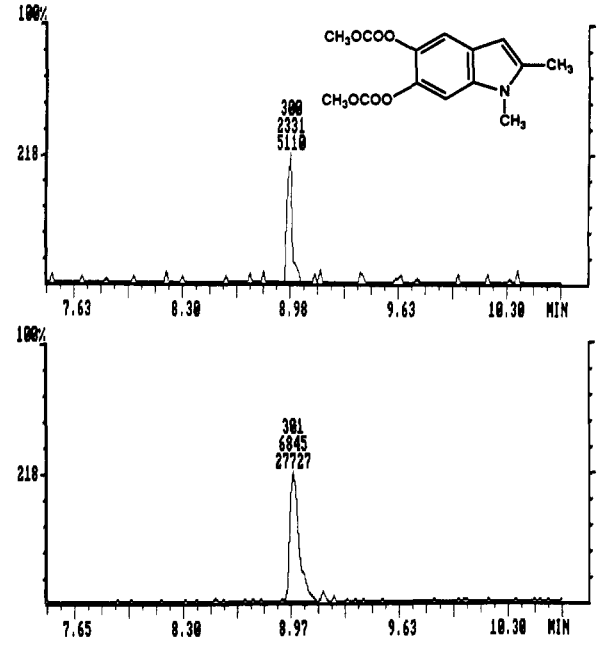

Figure 3. GC–CI–MS/MS analysis of derivatized extract from the incubation of 6-HO-MDMA with fortified 9000*g* rat liver supernatant. Top panel: Daughter ion current profile (m/z 294 → 218) showing a chromatographic peak (8.98 min) tentatively identified as 5,6-dihydroxy-1,2-dimethylindole. Bottom panel: Conclusive identification of this metabolite by coinjection with derivatized synthetic 5,6-dihydroxy-1,2-dimethylindole.

CID of the protonated molecules of derivatized 5,6-dihydroxy-1,2-dimethylindole, tri-HO-MA, and tri-HO-A at m/z 294, 430, and 458, followed by selected reaction monitoring of the daughter ions at m/z 218, 398, and 383, provided a specific and sensitive method for the detection of these metabolites in biological matrices. Application of the selected reaction monitoring technique to analysis of the in vitro metabolism of 6-HO-MDMA in rat liver incubate yielded a chromatographic peak with a retention time of 9.96 min in the m/z 398 daughter ion current profile (Figure 2, top), which coeluted with the derivatized synthetic tri-HO-MA when the same sample was coinjected with the synthetic standard. Furthermore, the compound's daughter ion mass spectrum was identical to that of the derivatized tri-HO-MA standard (Figure 2, bottom). Therefore, tri-HO-MA was established as an in vitro metabolite of 6-HO-MDMA, an identification which was further supported by the presence of a chromatographic peak at a retention time of 8.98 min in the m/z 218 daughter ion current profile (Figure 3, top) that coeluted with the derivatized synthetic 5,6-dihydroxy-1,2-dimethylindole (Figure 3, bottom) when the same sample was coinjected with the standard. The daughter ion mass spectrum of this compound agreed well with that of synthetic 5,6-dihydroxy-1,2-dimethylindole.

In view of the finding that 6-HO-MDMA is O-dealkylated to a potent neurotoxin, tri-HO-MA, it seemed likely that 6-HO-MDA would metabolize to tri-HO-A. To test this possibility, we incubated 6-HO-MDA in a similar manner as that used for the in vitro metabolism of 6-HO-MDMA. Analysis of the in vitro rat liver incubate of 6-HO-MDA by selected reaction monitoring produced a chromatographic peak with a retention time of 8.52 min (Figure 4, top) that was absent in the control. This compound coeluted with the derivatized synthetic tri-HO-A when the sample was coinjected with the synthetic standard. In addition, the daughter ion mass spectrum of the compound (Figure 4, bottom) was identical to that

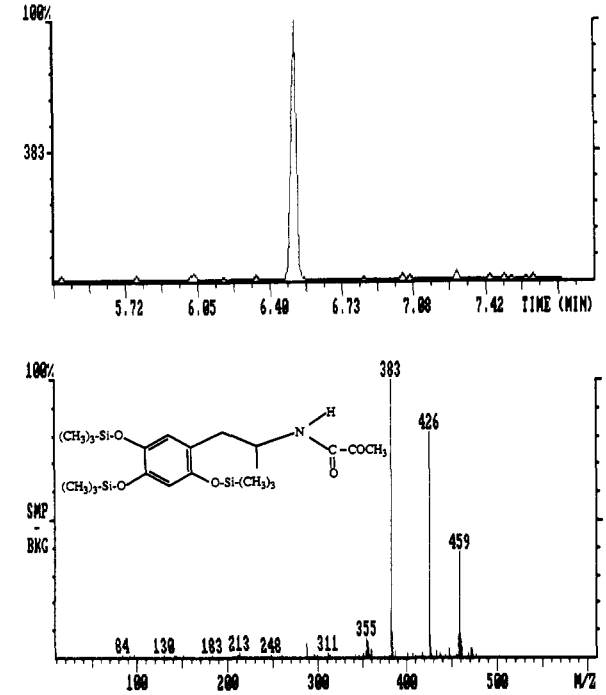

Figure 4. GC–CI–MS/MS analysis of derivatized extract from the incubation of 6-HO-MDA with fortified 9000*g* rat liver supernatant. Top panel: Daughter ion current profile (m/z 458 → 383) showing a chromatographic peak (8.50 min) identified as the *N*-(methoxycarbonyl)-*O,O,O*-tris(trimethylsilyl) derivative of tri-HO-A. Bottom panel: Daughter ion mass spectrum corresponding to the derivatized tri-HO-A.

of the derivatized synthetic tri-HO-A. These results provided conclusive evidence for the identification of tri-HO-A as a metabolite of 6-HO-MDA in vitro.

Since we have already shown that 6-HO-MDMA and 6-HO-MDA are minor metabolites of MDMA in the rat, it is reasonable to suggest the likelihood that this species also metabolizes MDMA to tri-HO-MA and tri-HO-A in

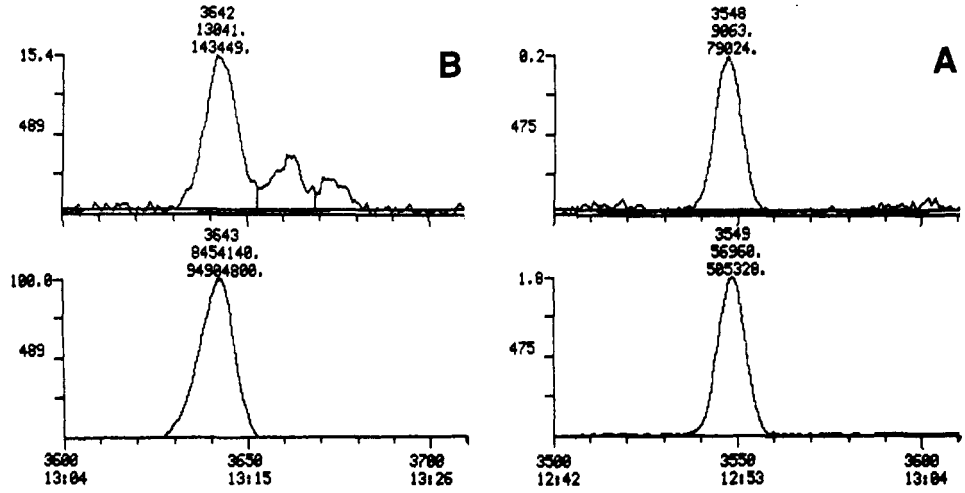

Figure 5. Selected ion monitoring analysis of derivatized liver extract from a rat injected subcutaneously with 20 mg/kg MDMA by GC–CI/MS. (A) Reconstructed ion current profile (m/z 475) showing a chromatographic peak (12.53 min, top) identified as the *N*-(methoxycarbonyl)-*O,O,O*-tris(trimethylsilyl) derivative of tri-HO-A by coinjection with derivatized synthetic tri-HO-A (bottom). (B) Reconstructed ion current profile (m/z 489) showing a chromatographic peak (13.13 min, top) identified as the *N*-(methoxycarbonyl)-*O,O,O*-tris(trimethylsilyl) derivative of tri-HO-MA by coinjection with derivatized synthetic tri-HO-MA (bottom).

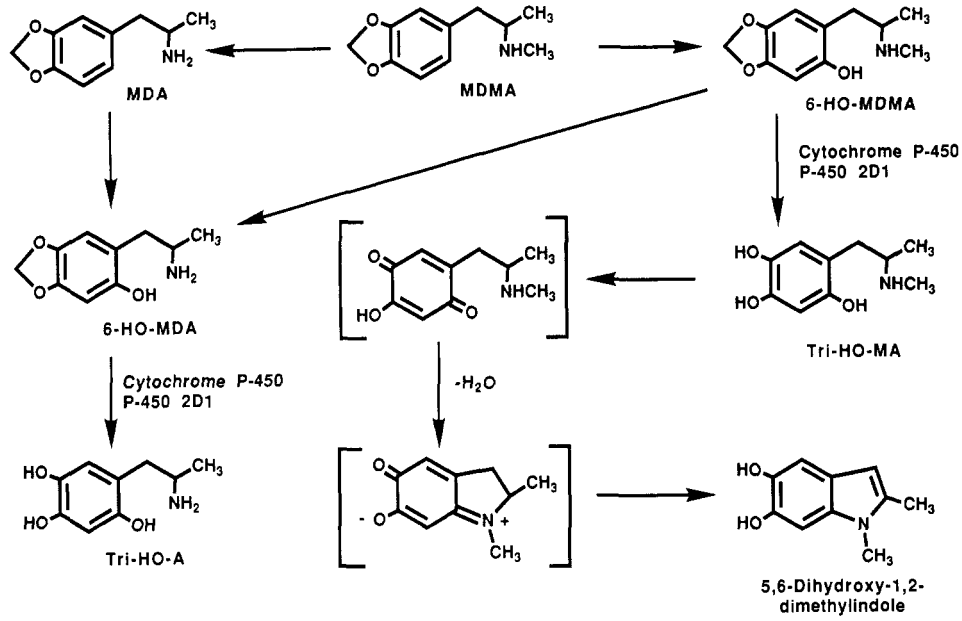

Figure 6. Proposed biotransformation pathways for the formation of the potent neurotoxins, tri-HO-MA and tri-HO-A, from MDMA in the rat.

vivo. In view of the potent neurotoxicity of these trihydroxy compounds and their potential roles in MDMA-induced neurotoxicity, it was important to demonstrate their existence in vivo in rats after administration of MDMA. Therefore, we injected rats subcutaneously with 20 mg/kg MDMA using the protocol for neurotoxicity testing of MDMA (*12*); livers were used for identification of these trihydroxy metabolites. The neurotransmitter serotonin was measured in brain homogenates from the same rats by an assay employing GC–MS with negative ion chemical ionization.[4] This analysis was done to ensure that neurotoxicity was elicited in each rat administered with MDMA. Selected ion monitoring analysis of derivatized liver extracts from rats dosed with MDMA by GC–CI/MS produced two chromatographic peaks at retention times of 12.53 (Figure 5A, top) and 13.13 min (Figure 5B, top) which were absent in liver extracts fron control rats. Coinjection of the sample with derivatized synthetic tri-HO-MA and tri-HO-A showed that the chromatographic peaks at retention times of 12.53 and 13.13 min coeluted with tri-HO-A and tri-HO-MA, respectively. Therefore, tri-HO-MA and tri-HO-A were conclusively identified as in vivo metabolites of MDMA in rats. All the MDMA-treated rats that were positive for these trihydroxy compounds also exhibited neurotoxicity, as indicated by reduction of serotonin by more than 80% of the control value in the frontal cortex and hippocampus.[4] The proposed biotransformation pathways leading to the formation of these potent neurotoxic trihydroxy metabolites from MDMA in the rat are summarized in Figure 6.

Investigation of the enzymatic formation of the trihydroxy metabolites may provide insight into the mechanism of MDMA-induced neurotoxicity. No tri-HO-MA was detected when 6-HO-MDMA was incubated either with boiled 9000*g* rat liver supernatant or in the absence of NADPH. Similar results were obtained with 6-HO-MDA except that formation of tri-HO-A was reduced by 54% when NADPH was omitted. Also, the metabolism of 6-HO-MDMA to tri-HO-MA was inhibited by known

[4] H. K. Lim, S. Zeng, R. Rupper, and R. L. Foltz, unpublished results.

Table I. Effect of Cytochrome P-450 Inhibitors on O-Dealkylation of 6-HO-MDMA[a]

	% inhibition[b]			
concn, μM	SKF-525A	1-amino-benzotriazole	quinine	quinidine
10000		76		
1000	>99			>99
100			86	15
50			56	8
2			57	
0.2			37	

[a] Each concentration was examined in duplicate. Estimated percent (%) inhibition = [mean peak area (with inhibitor)/mean peak area (without inhibitor)] × 100. [b] These preliminary inhibition data were semiquantitative and were obtained from the integrated peak areas corresponding to 5,6-dihydroxy-1,2-dimethylindole. The purpose of these data is to indicate the specific cytochrome P-450 enzyme involved in the O-dealkylation of 6-HO-MDMA.

inhibitors of cytochrome P-450, such as SKF-525A (>99%), quinidine (>99%), and 1-aminobenzotriazole (76%) (Table I). Only SKF-525A was evaluated as an inhibitor of 6-HO-MDA metabolism, and this compound inhibited the formation of tri-HO-A from 6-HO-MDA by more than 90%. Therefore, the O-dealkylations of 6-HO-MDMA and 6-HO-MDA to tri-HO-MA and tri-HO-A, respectively, are dependent upon cytochrome P-450 enzymes and NADPH. Since both hydroxy compounds are metabolized by the same pathway, further characterization of the cytochrome P-450 enzymes was carried out only with 6-HO-MDMA.

Preliminary data on inhibition by quinidine suggest that the cytochrome P-450 2D1 enzyme may participate in the O-dealkylation pathway. However, the involvement of other cytochrome P-450 enzymes cannot be excluded since other enzymes may be inhibited at the concentration of quinidine used in this study (*17*). Moreover, in the rat the cytochrome P-450 2D1 enzyme is more sensitive to inhibition by quinine than by its diastereomer, quinidine (*18*). Further investigation of the potential contribution of cytochrome P-450 2D1 enzyme to O-dealkylation of 6-HO-MDMA, using several concentrations of either quinine or quinidine, revealed concentration-dependent inhibition of the formation of tri-HO-MA; quinine was a more potent inhibitor of the O-dealkylation of 6-HO-MDMA than was quinidine (Table I). The relative potencies of this pair of diastereomers on the inhibition of formation of tri-HO-MA parallel data previously reported for the inhibition of cytochrome P-450 2D1 enzyme in the rat by these compounds (*18*) and therefore strongly suggest the participation of this enzyme in the O-dealkylation of 6-HO-MDMA. This conclusion is further supported by the partial inhibition of this reaction by low concentrations of quinine (concentrations used were less than or equal to the reported IC_{50} value); at low concentrations quinine has been shown to be a highly specific inhibitor of this enzyme (*18*). As cytochrome P-450 2D1 enzyme is present in the rat brain (*19*) and the O-dealkylation pathway is active in the same tissue (*2*), O-dealkylation of these hydroxy compounds to trihydroxy compounds can presumably occur in the rat brain. Furthermore, the human orthologue of cytochrome P-450 2D1 enzyme (cytochrome P-450 2D6) is present in the human brain (*19*); the genetic polymorphism associated with P-450 2D6 (*20*) would suggest that a slow metabolizer with defective functional expression of this enzyme might be protected to some degree from the neurotoxicity of MDMA.

Apart from the aromatic hydroxylated metabolites, metabolites of MDMA which are potential precursors of the trihydroxy compounds include the catecholamine metabolites such as di-HO-MA and its N-demethylated analogue. We were particularly interested in the potential aromatic hydroxylation of di-HO-MA, because this compound is also the precursor of the major urinary metabolite of MDMA (4-hydroxy-3-methoxymethamphetamine) in the rat (*21*). Consequently, we investigated the potential formation of tri-HO-MA and tri-HO-A from catecholamine metabolites like di-HO-MA and its N-demethylated analogue. Incubation of di-HO-MA under the same conditions as 6-HO-MDMA produced no detectable amounts of tri-HO-MA, and we did not attempt the same experiment with its N-demethylated analogue. This lack of metabolism may reflect the hydrophilic nature of di-HO-MA which limits its access to the hydrophobic cleft of cytochrome P-450 enzymes.

Demonstration that 6-HO-MDMA and 6-HO-MDA can undergo metabolic conversion in vitro to tri-HO-MA and tri-HO-A, respectively, and, even more important, identification of these trihydroxy compounds in livers from MDMA-treated rats constitute significant advances in our understanding of MDMA-induced neurotoxicity in view of the potent neurotoxicities of tri-HO-MA and tri-HO-A (*8*, *9*). The fact that one of the precursors of the trihydroxy compounds, 6-HO-MDMA, is not neurotoxic (*22*) does not preclude the involvement of these trihydroxy compounds in the neurotoxicity of MDMA. It is possible that the selective neurotoxic effects of MDMA may be due to active uptake of MDMA into the serotonergic neurons (*12*) and subsequently metabolism, by cytochrome P-450 enzymes present within the serotonergic neurons, to trihydroxy metabolites which can cause damage and eventually death to the neurons.

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Registry No. Tri-HO-MA, 136706-32-6; tri-HO-A, 136706-33-7; 6-HO-MDMA, 136706-34-8; 6-HO-MDA, 136706-35-9; MDA, 42542-10-9; NADPH, 53-57-6; 5,6-dihydroxy-1,2-dimethylindole, 136735-97-2; 5,6-dihydroxy-2-methylindole, 4821-01-6; cytochrome P-450, 9035-51-2; 2,4,5-trimethoxybenzaldehyde, 4460-86-0; 2,4,5-trimethoxymethamphetamine, 136779-31-2; 2,4,5-trimethoxyamphetamine, 1083-09-6.

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Structure of Formamidopyrimidine Adducts As Determined by NMR Using Specifically ^{15}N-Labeled Guanosine

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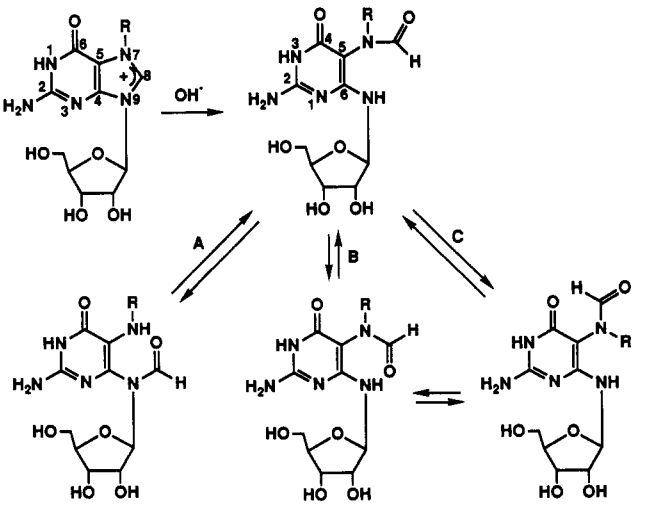
Chemical carcinogens are generally electrophilic species that interact with nucleophilic sites on cellular DNA (*1*). The specific site of interaction is governed by several factors, two of the most important being carcinogen reactivity and the reactivity of the site in question. Of the many nucleophilic sites on DNA the one that is most nucleophilic and thus generally most reactive is the N^7-position of guanine. Although in many cases the N^7-guanyl adducts have been shown to be very weakly mutagenic, there are several cases where these adducts are thought to be important, e.g., the adducts formed by aflatoxins (*2*) and the ethylene dibromide derived GSH adducts (*3*, *4*).

Two of the most common fates that N^7-deoxyguanosyl adducts typically undergo are depurination and imidazole ring opening. Acid-catalyzed depurination reactions yield N^7-guanyl adducts and apurinic sites, and base-catalyzed imidazole ring opening reactions yield FAPY[1] adducts. Under physiological conditions the relative rates with which specific N^7-guanyl adducts follow each pathway are quite dependent on the chemical nature of the substituent at the N^7-position (*5*). Thus for aflatoxin adducts a major species thought to be present is the FAPY adduct (*2*, *6*) while for the ethylene dibromide–GSH adduct, *S*-[2-(N^7-deoxyguanosyl)ethyl]GSH, this does not appear to be

Scheme I. FAPY Adduct Structure and Possible Explanations for the Interconversion Observed upon Adduct Isolation[a]



[a] Note the change from the purine to the pyrimidine numbering scheme upon FAPY adduct formation.

[1] Abbreviations: FAPY, formamidopyrimidine; GSH, reduced glutathione; GSH-FAPY-base, *S*-[2-[*N*-formyl-*N*-(2,6-diamino-4-oxo-3,4-dihydropyrimidin-5-yl)amino]ethyl]glutathione; GSH-FAPY-glycoside, *S*-[2-[*N*-formyl-*N*-[2-amino-6-(*N*-glycosylamino)-4-oxo-3,4-dihydropyrimidin-5-yl]amino]ethyl]glutathione; Me-FAPY-base, 2,6-diamino-5-(*N*-formyl-*N*-methylamino)-4-oxo-3,4-dihydropyrimidine; Me-FAPY-glycoside, 2-amino-5-(*N*-formyl-*N*-methylamino)-6-(*N*-glycosylamino)-4-oxo-3,4-dihydropyrimidine.

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