these drugs will require consideration of other important factors, including also stereochemical factors.

Acknowledgements

This study was supported financially by a Project Grant from the National Health and Medical Research Council of Australia. The skilled technical assistance of Mr B. T. Wood is acknowledged with gratitude.

References

BHARGAVA, V. O., SOINE, W. H., and GARRETTSON, L. K., 1985, High-performance liquid chromatographic analysis of 1-(-beta-D-glucopyranosyl)phenobarbital in urine. *Journal of Chromatography*, **343**, 219–223.

EADIE, M. J., and TYRER, J. H., 1989, *Anticonvulstant Therapy: Pharmacological Basis and Practice*, 3rd edn (Edinburgh: Churchill Livingstone).

GIBALDI, M. K., 1977, *Biopharmaceutics and Clinical Pharmacokinetics*, 2nd edn (Philadelphia: Lea and Febiger), p. 46.

HAKY, J. E., and YOUNG, A. M., 1984, Evaluation of a simple HPLC correlation method for the estimation of the octanol–water partition coefficients of organic compounds. *Journal of Liquid Chromatography*, **7**, 675–689.

HOOPER, W. D., KUNZE, H. E., and EADIE, M. J., 1981, Qualitative and quantitative studies of methylphenobarbital metabolism in man. *Drug Metabolism and Disposition*, **9**, 381–385.

HOOPER, W. D., TRESTON, A. M., JACOBSEN, N. W., DICKINSON, R. G., and EADIE, M. J., 1983, Identification of *p*-hydroxyprimidone as a minor metabolite of primidone in rat and man. *Drug Metabolism and Disposition*, **11**, 607–610.

KAPETANOVIC, I. M., STRONG, J. M., and MIEYAL, J. J., 1979, Metabolic structure-activity relationship for a homologous series of phenacetin analogs. *Journal of Pharmacology and Experimental Therapeutics*, **209**, 20–24.

KUPFER, A., and BRANCH, R. A., 1985, Stereoselective mephobarbital hydroxylation cosegregates with mephenytoin hydroxylation. *Clinical Pharmacology and Therapeutics*, **38**, 414–418.

LIM, W. H., and HOOPER, W. D., 1989, Stereoselective metabolism and pharmacokinetics of racemic methylphenobarbital in humans. *Drug Metabolism and Disposition*, **17**, 212–217.

MCMAHON, R. E., and EASTON, N. R., 1961, Demethylation studies III. The *in vitro* demethylation of dialkymethylamines. *Journal of Medicinal and Pharmaceutical Chemistry*, **4**, 437–445.

MANIARA, W., LEVIN, S. S., SCHLEYER, H., and COOPER, D. Y., 1988, Biophysical and catalytic properties of the phenobarbital *p*-hydroxylase—a cytochrome P-450 dependent mixed function oxidase. *Xenobiotica*, **18**, 545–550.

SOINE, W. H., BHARGAVA, V. O., and GARRETTSON, L. K., 1984, Direct detection of 1-(beta-D-glucopyranosyl)phenobarbital in human urine. *Drug Metabolism and Disposition*, **12**, 792–794.

SOINE, W. H., SOINE, P. J., ENGLAND, T. M., OVERTON, B. W., and MERAT, S., 1989, Synthesis of *N*-beta-D-glucopyranosyl derivatives of barbital, phenobarbital, metharbital and mephobarbital. *Carbohydrate Research*, **193**, 105–113.

SOINE, W. H., SOINE, P. J., MONGRAIN, S. E., and ENGLAND, T. M., 1990, Stereochemical characterization of the diastereomers of the phenobarbital *N*-beta-D-glucose conjugate excreted in human urine. *Pharmaceutical Research*, **7**, 402–406.

TANG, B. K., KALOW, W., and GREY, A. A., 1979, Metabolic fate of phenobarbital in man: *N*-glucoside formation. *Drug Metabolism and Disposition*, **7**, 315–318.

TOON, S., and ROWLAND, M., 1983, Structure–pharmacokinetic relationships among the barbiturates in the rat. *Journal of Pharmacology and Experimental Therapeutics*, **225**, 752–763.

TRESTON, A. M., and HOOPER, W. D., 1985, *N*-Alkyl-barbiturates: a series of compounds for the study of metabolic structure-activity relationships. *Arzneimittel-Forschung* (*Drug Research*), **35**, 1627–1629.

TRESTON, A. M., and HOOPER, W. D., 1990, Metabolic studies with phenobarbitone, primidone, and their *N*-alkyl derivatives: quantification of substrates and metabolites using chemical ionization gas chromatography-mass spectrometry. *Journal of Chromatography*, **526**, 59–68.

TRESTON, A. M., PHILIPPIDES, A., JACOBSEN, N. W., EADIE, M. J., and HOOPER, W. D., 1987, Identification and synthesis of *O*-methylcatechol metabolites of phenobarbital and some *N*-alkyl derivatives. *Journal of the Pharmaceutical Sciences*, **76**, 496–501.

UNGER, S. H., COOK, J. R., and HOLLENBERG, J. S., 1978, Simple procedure for determining octanol-aqueous partition, distribution, and ionization coefficients by reversed-phase high-pressure liquid chromatography. *Journal of the Pharmaceutical Sciences*, **67**, 1364–1367.

WHYTE, M. P., DEKABAN, A. S., 1977, Metabolic fate of phenobarbital: a quantitative study of *p*-hydroxyphenobarbital elimination in man. *Drug Metabolism and Disposition*, **5**, 63–70.

XENOBIOTICA, 1992, VOL. 22, NO. 4, 395–403

Aromatic hydroxylation of methylenedioxybenzene (MDB) and methylenedioxymethamphetamine (MDMA) by rabbit liver microsomes

Y. KUMAGAI†, D. A. SCHMITZ and A. K. CHO‡

Department of Pharmacology, UCLA School of Medicine, Center for the Health Sciences, Los Angeles, 90024, USA

Received 16 April 1991; accepted 18 October 1991

1. Metabolites formed during incubation of methylenedioxybenzene (MDB) and methylenedioxymethamphetamine (MDMA) with rabbit liver microsomes were examined by h.p.l.c.–electrochemical detection and g.l.c.–mass spectrometry.

2. The trifluoroacetyl derivative of metabolite M-1, obtained from MDB, had a molecular ion at m/z 234 and was identified as 3,4-methylenedioxy-6-hydroxybenzene (sesamol) by comparison with authentic material.

3. The trifluoroacetyl derivative of metabolite M-2, obtained from MDMA, exhibited a molecular ion at m/z 401. Experiments with the deuterium substituted variants of MDMA indicated that the product was hydroxylated on the aromatic ring.

4. The formation of these hydroxylated metabolites required NADPH and was inhibited by carbon monoxide, indicating the possible participation of cytochrome P-450. Phenobarbital (PB) induction caused a marked enhancement of MDP hydroxylase activity whereas MDMA hydroxylation was not affected.

5. The aromatic hydroxylation of MDB and MDMA was also observed in a reconstituted system with cytochrome P-450 isozyme IIB4.

Introduction

Methylenedioxymethamphetamine (MDMA, figure 1) has neurotoxic actions indicated by lesions in 5-hydroxytryptamine (5-HT) neurons and reduction of 5-HT concentration after systematic administration (Ricaurte *et al.* 1985, Schmidt 1987). The exact mechanism for this effect is not known, but it is thought to be due, in part, to metabolites (Schmidt and Taylor 1988, Gollamudi *et al.* 1989).

In studies on MDMA metabolism, Lim and Foltz (1988) identified the *N*-demethylated, deaminated, demethylenated and *O*-methylated metabolites in rat urine and in rat brain homogenate preparations. We have demonstrated that, with rat liver microsomes, MDMA is demethylenated to dihydroxymethamphetamine (DHMA) by cytochrome P-450 and that DHMA is further oxidized to a quinone species which reacts easily with sulphydryl compounds such as glutathione (Hiramatsu *et al.* 1990). The metabolic routes described above were also found after incubation with rabbit liver preparations (Kumagai *et al.* 1991a). However, at that time, additional unknown metabolites, evidenced by electrochemical detection, were observed in the incubation mixtures of MDA and MDMA. This paper describes the identification of these metabolites and characterization of the responsible enzyme system.

† Merck postdoctoral Fellow (1990–1991).
‡ To whom correspondence should be addressed.

0049–8254/92 $3·00

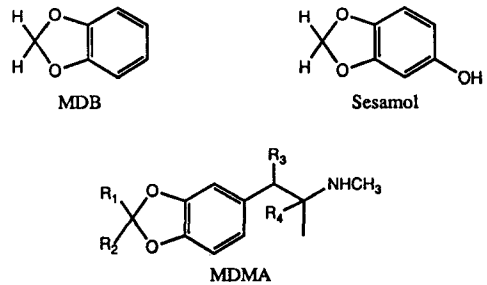


Figure 1. Structures of 3,4-methylenedioxybenzene (MDB), 3,4-methylenedioxy-6-hydroxybenzene (sesamol), 3,4-methylenedioxymethamphetamine (MDMA) and its deuterium substituted variants.

D_0-MDMA, $R_1 = R_2 = R_3 = {}^1H$; D_1-MDMA, $R_1 = R_2 = {}^2H$, $R_3 = R_4 = {}^1H$; D_2-MDMA, $R_1 = R_2 = {}^1H$, $R_3 = R_4 = {}^2H$.

Materials and methods

Chemicals

MDB, sesamol, and trifluoroacetic anhydride (99% pure) were obtained from Aldrich Chemical Co., Inc. (Milwaukee, WI, USA) and MDMA HCl from the Research Technology Branch of the National Institute of Drug Abuse (Rockville, MD, USA). 3,4-[$^2H_2$] Methylenedioxybenzene and 3,4-[$^2H_3$]methylenedioxy-1-phenylisopropyl-*N*-methylamine (D_1-MDMA) were synthesized from the corresponding catechols according to the method of Clark *et al.* (1976). 3,4-Methylenedioxy-1-phenyl-[1,2-^{2}H] isopropyl-*N*-methylamine (D_2-MDMA) was synthesized by reduction of 3,4-methylenedioxy-nitrostyrene with lithium aluminium deuteride. The structures of these deuterium substituted compounds are shown in figure 1. Dihydroxymethamphetamine (DHMA) was synthesized according to the method of Smissman and Borchardt (1971). Catechol was obtained from Sigma Chemical Co. (St Louis, MO, USA). All other chemicals were the highest grades available (99% pure).

Incubation and extraction of metabolite

Microsomes were prepared from livers of male white New Zealand Rabbits (2·5–3·0 kg) according to procedures described previously (Hiramatsu *et al.* 1990). For induction studies, phenobarbital (PB) 60 mg/kg, i.p. was injected every 24 h for 3 days. A typical incubation mixture contained 1 mM MDB or MDMA, the microsomal preparation (1–2 mg of protein), a NADPH-generating system consisted of 0·5 mM NADP, 8 mM glucose 6-phosphate, 1 unit of glucose 6-phosphate dehydrogenase, and 5 mM $MgCl_2$, in a final volume of 1·0 ml, 0·1 M Hepes buffer, pH 7·6. The reaction was carried out at 37°C for 10 min unless otherwise noted and terminated by addition of perchloric acid (final concentration 2·5%). After centrifugation at 13 500*g* for 5 min, one portion (10 μl) was injected into the h.p.l.c. instrument as described below. The remainder was transferred to an extraction tube. When M-1 was extracted the tube contained 6 ml of 1 M potassium phosphate buffer, pH 7·0, 13 ml of dichloromethane and 100 mg of NaCl. In the case of M-2 the tube contained 1 ml of 1·5 M Na carbonate buffer, pH 9·5, 5 ml of ethyl acetate and 100 mg of NaCl. These mixtures were shaken for 15 min. Samples of the dichloromethane (12 ml) or ethyl acetate solution (4·5 ml) were evaporated to 100 μl or to dryness in the case of ethyl acetate, under a stream of N_2. Trifluoroacetic anhydride (100 μl) was added to the dichloromethane solution and excess trifluoroacetic anhydride was allowed to evaporate at room temperature. The residue obtained from the M-2 sample was mixed with 50 μl of acetonitrile and 50 μl of trifluoroacetic anhydride and heated at 60°C for 15 min. Each derivatized sample (1 μl) was injected into the g.l.c.–mass spectrometer.

Reconstituted enzyme system

Cytochrome P450IIB4 was purified from liver microsomes of PB-treated rabbits according to the method of Coon *et al.* (1978). The specific content of the isozyme was 16·5 nmol/mg. Cytochrome P-450 was determined by the method of Omura and Sato (1964). NADPH-cytochrome P-450 reductase was purified as described previously (Duncan and Cho 1982). NADPH-cytochrome P-450 reductase activity was assayed by cytochrome c reduction according to Phillips and Langdon (1962). One unit of reductase activity is defined as the amount of enzyme catalysing the reduction of cytochrome c at an initial rate of 1 nmol/min at 22°C in 0·3 M KPi (pH 7·7), 0·1 mM EDTA. The specific activity of the final preparation was 64·9 units/mg. Both purified isozyme IIB4 and NADPH-cytochrome P-450 reductase gave a single major band on sodium dodecyl sulphate–polyacrylamide gel electrophoresis. Protein concentration was determined by the Bio-Rad assay (Bio-Rad Laboratories, Richmond, CA, USA) with bovine serum albumin as standard. The reaction mixture for reconstitution consisted of cytochrome P450-IIB4 (0·2 nmol), NADPH-cytochrome P-450 reductase (1·5 units), dilauroylphosphatidylcholine (30 μg), the NADPH-generating system, MDB or MDMA (1 mM) and 0·1 M Hepes, pH 7·6. Reaction was initiated by the addition of the NADPH-generating system and was performed at 37°C for 2 min. Under the conditions as described above, the rates of oxidation of MDB and MDMA were linear and the cytochrome was the rate-limiting component.

H.p.l.c.–electrochemical detection

Separation and quantification of metabolites was performed by the same conditions described previously (Kumagai *et al.* 1991b). Electrochemical measurements were made using a glassy carbon electrode (LC-4, Bioanalytical System, Inc., West Lafayette, IN, USA) set at +0·7 V (vs Ag/AgCl reference electrode). Signals were recorded with a Hewlett-Packard 3390A recording integrator and the peak heights of metabolites were compared with those of the standard samples. Under the conditions as described in figure 2, lower limits of detection for sesamol, DHMA, and catechol were about 10 pmol/ml. The good reproducibilities for retention time and peak height of each compound were indicated by coefficient of variation of less than 5%.

G.l.c.–mass spectrometry

A Hewlett-Packard 5971A g.l.c.–mass spectrometry system fitted with a methyl silicone capillary column (12 m × 0·2 mm int. diam.) was employed in the electron impact mode. Analysis for M-1 and M-2 was carried out with a temperature program from 70 to 195°C at a rate of 10°C (M-1)/min and from 100 to 195°C at a rate of 15°C (M-2)/min. Under these conditions the retention times of M-1 and M-2 were 3·3 and 6·7 min, respectively. The ionizing voltage was 70 eV.

Results

Identification

Figures 2A and 2B show typical h.p.l.c. elution patterns of the MDB and MDMA metabolites produced by rabbit liver microsomes in the presence of a NADPH-generating system. Formation of M-1 (peak 2) and M-2 (peak 6) with retention times of 22·4 min and 23·5 min, respectively, was dependent on substrate, enzyme concentration, and NADPH. To check the electrochemical properties of these metabolites the response of the electrochemical detector was monitored as the detector potential was varied over the range 0·5–0·9 V (figure 3). The peak heights of M-1 and M-2 markedly increased with potential and reached a plateau at about 0·9 V. The electrochemical potentials of M-1 and M-2 were very similar to those of catechol and sesamol, suggesting that each metabolite had a readily oxidizable group. When the MDB metabolite, M-1 was compared with the 6-hydroxy derivative, sesamol, h.p.l.c. and g.l.c. retention times were identical, as were the mass spectra of the trifluoracetyl (TFA) derivatives.

Figure 4 shows the relative mass spectra of the TFA derivative of sesamol and that of the metabolite formed from MDB. Both compounds showed a molecular ion peak at m/z 234 (100%). No apparent differences in fragmentation pattern (i.e. m/z 137 and 107) between the two mass spectra were seen. Furthermore, incubation of 3,4[$^2H_2$] methylenedioxybenzene gave a shifted peak at 236 (figure 4C). These results and the identity of h.p.l.c. retention times indicated that M-1 is 6-hydroxy-MDB.

The structure of M-2, formed from MDMA, was also assessed by g.l.c.–mass spectrometry (table 1). As reported previously by Lim and Foltz (1988), the TFA derivative of MDMA has a molecular ion at $m/z = 289$ major fragment ions at $m/z = 162$ (resulting from a McLafferty rearrangement) at $m/z = 154$ and $m/z = 135$ (resulting from cleavage β to the nitrogen) (figure 4). The TFA derivative of M-2 exhibited a molecular ion at $m/z = 401$ and other fragment ions at $m/z = 274$ and 247. A mass shift of 112 daltons of these ions in the metabolite is consistent with a

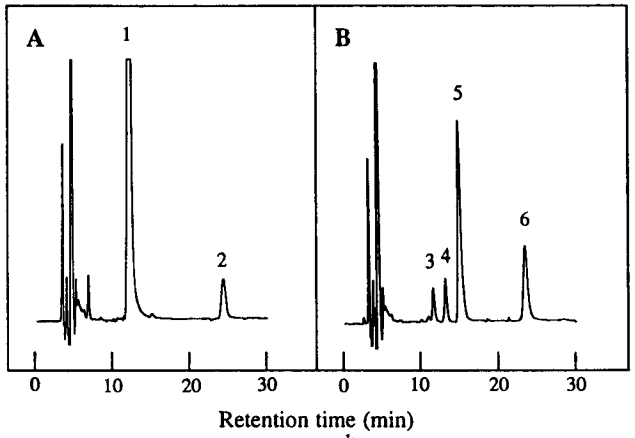


Figure 2. High-performance liquid chromatograms of MDB (**A**) and MDMA (**B**) oxidation products formed in an incubation mixture in the rabbit liver microsomes.

Based on coelution with authentic samples, peak 1, 3, 4 and 5 were identified as catechol, dihydroxyphenylacetone (DHPA), dihydroxyamphetamine (DHA), and dihydroxymethamphetamine (DHMA), respectively (Kumagai *et al.* 1991a). The chromatographic conditions are as follows: column, Biophase ODS column (4·6 × 250 mm, particle size 5 μm); mobile phase, 0·1 M citrate (pH 3·5), containing 1 mM sodium octyl sulphate–methanol–acetonitrile (8 : 1 : 1 by vol.); flow rate, 0·75 ml/min; ECD + 0·7 V.

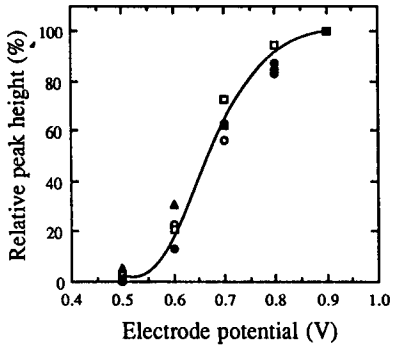


Figure 3. Electrochemical response curves of metabolites formed from MDB and MDMA.

○, Catechol; □, sesamol; ●, M-1; ▲, M-2. Each plot is expressed relative to its height at 0·9 V.

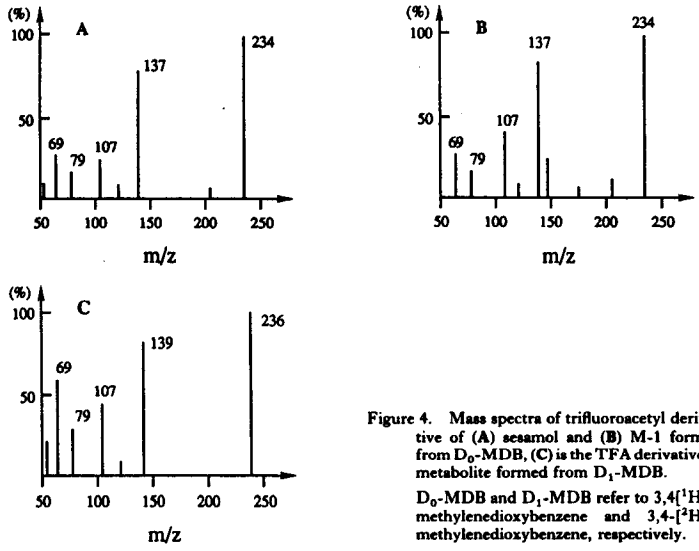
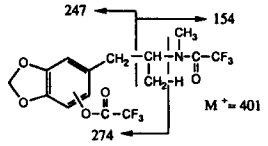


Figure 4. Mass spectra of trifluoroacetyl derivative of (**A**) sesamol and (**B**) M-1 formed from D_0-MDB, (**C**) is the TFA derivative of metabolite formed from D_1-MDB.

D_0-MDB and D_1-MDB refer to 3,4-[$^1H_2$]-methylenedioxybenzene and 3,4-[$^2H_2$]-methylenedioxybenzene, respectively.

Table 1. Mass spectral characteristics of the trifluoroacetyl derivative of MDMA metabolite.



Substrate	(*m/z*)	403	401	276	275	274	249	248	247	155	154	110	69
		Relative abundance (%)											
D_0-MDMA		—	3	—	—	41	—	—	3	5	100	16	33
D_1-MDMA		2	—	33	4	—	2	—	—	5	100	18	26
D_2-MDMA		2	—	26	12	—	—	2	—	100	2	14	24
D_0-MDMA + D_1-MDMA		1	1	15	4	16	2	—	2	5	100	17	19
D_0-MDMA + D_2-MDMA		1	1	15	12	35	—	2	2	53	100	25	23

D_0-, D_1- and D_2-MDMA refer to 3,4-[$^1H_2$]-methylenedioxy-1-phenyl-[1,2-^{1}H]isopropyl-*N*-methylamine, 3,4-[$^2H_2$]-methylenedioxy-1-phenyl-[1,2-^{1}H]isopropyl-*N*-methylamine and 3,4-[$^1H_2$]-methylenedioxy-1-phenyl-[1,2-^{1}H]isopropyl-*N*-methylamine, respectively.

trifluoroacetoxy group. Structural changes to the *N*-methyl-α-methyl group in the metabolite were excluded by the abundant fragment ion at m/z 154. The aromatic ring as the site of hydroxylation in M-2 was confirmed by experiments with an equimolar mixture of MDMA and D_1 or D_2-MDMA. Incubation of the mixture of the protio- and deuterio-substrates resulted in metabolites with peaks at two mass units higher in both cases (table 1). As the deuterium atoms of D_1- and D_2-MDMA were present on the methylenedioxy group and the α,β-carbon atoms of the side-chain, respectively, the persistent presence of $m/z+2$ fragment indicated an intact methylenedioxy group and side-chain. Thus, we concluded that metabolite M-2 was hydroxylated on benzene ring.

Enzyme responsible for ring hydroxylation

The enzymic nature of the hydroxylation reaction was investigated next (table 2). The ring hydroxylation of MDB and MDMA required NADPH which could not be replaced with hydrogen peroxide, indicating that the reaction will not proceed by the peroxidase pathway of cytochrome P-450. SKF-525 A (0·1 mM) suppressed metabolite formation minimally. However, the hydroxylation was strongly inhibited by carbon monoxide, indicating a cytochrome P-450 reaction. Desmethylimipramine (desipramine), a inhibitor of amphetamine metabolism (Dingell and Bass 1969), also had an inhibitory effect (50%) on metabolite formation. Superoxide dismutase (SOD) and glutathione (GSH) caused slight enhancement of MDMA hydroxylation but had no effect on sesamol formation. Neither catalase (CAT) nor thiourea, a hydroxyl radical scavenging agent (at concentrations equal to substrate), influenced ring hydroxylation. Each hydroxylation was dependent on substrate concentration. In the case of MDMA the hydroxylation reached saturation at 0·2 mM and no substrate inhibition was observed at 10 mM (data not shown). When the substrate was fixed at 1 mM, sesamol and hydroxy-MDMA formation were linear with time for 2 min and 5 min, respectively, and linearly dependent on protein concentration up to 1 mg. The rate of aromatic hydroxylation of MDB by untreated

Table 2. Effects of cytochrome P-450 inhibitors and scavenging agents for active oxygen species on the formation of the ring-hydroxylated metabolite of MDB and MDMA.

Conditions	Aromatic hydroxylation (percentage of control)	
	With MDB	With MDMA‡
Complete	100	100
+Hydrogen peroxide (5 mM)†	n.d.	n.d.
+SKF-524A (0·1 mM)	90±3	70±4
+CO/O_2 (9:1 v/v)	47±1	16±2
+EDTA (1 mM)	99±1	118±4
+Desmethylimipramine (0·1 mM)	52±2	56±4
+SOD (100 units)	106±1	121±4
+CAT (200 units)	112±4	101±2
+NaN_3 (0·2 mM)	107±3	113±1
+Thiourea (1 mM)	91±1	111±4
+GSH (1 mM)	100±2	122±4

n.d., Not detectable—the lower limit for detection (10 pmol/ml). Methylenedioxyphenyl compounds (1 mM) were incubated with untreated microsomes of rabbit liver (2 nmol of P-450) for 10 min. Under these conditions sesamol formed was 77 ±0·13 nmol. Each value is the mean ± SD of four determinations.
† Incubation was performed in the absence of the NADPH-generating system.
‡ Each value was expressed as relative peak height.
SOD, superoxide dismutase; CAT, catalase; GSH, glutathione.

liver microsomes was 0·80 nmol/min per mg protein and the turnover value was 4% of that of the demethylenation reaction. The effects of PB induction on sesamol and hydroxy-MDMA formation are shown in figure 5. The enzyme activity for sesamol formation was enhanced about 5 times by PB pretreatment. By contrast, PB did not drastically affect ring hydroxylation of MDMA. Metabolite formation was also examined in a reconstituted system with cytochrome P450IIB4, a major PB-inducible isozyme (Coon *et al.* 1978). The enzyme activity of aromatic hydroxylation of MDB was 0·57 nmol/min per nmol P-450 and the isozyme exhibited a turnover for demethylenation of 30·6 nmol/min per nmol P-450 (Kumagai *et al.* 1991a). Hydroxy-MDMA formation by cytochrome P450IIB4 was also observed, but with very low activity. Formation of hydroxy-MDMA was only 13% of that of DHMA, which was 0·55 nmol/min per nmol P-450 (estimated by peak heights).

Discussion

The results presented indicate that the ring hydroxylation of MDB and MDMA occurs in rabbit liver microsome preparations. The reaction product of MDB was identified as sesamol by comparison of h.p.l.c.–electrochemical detection and g.l.c–mass spectrometry data with that of authentic compound. The tentative identification of hydroxy MDMA is based on the following observations. The electrochemical properties of M-2, like those of M-1, indicated a readily oxidizable functionality, whereas MDMA itself or a side-chain hydroxylated species would be electrochemically inactive under these conditions. In related studies, Roston and Kissinger (1982) reported that phenol and catechol are both oxidized at electrochemical detectors, with phenol requiring much higher potentials. As shown in figure 3, the methylenedioxyphenyl metabolites exhibited electrochemical activity similar to the dihydroxyphenyl compound. Methylenedioxyphenyl compounds, being difficult to oxidize, were not detected under these conditions. However, as sesamol is readily oxidized, it was highly likely that M-2 was also an aromatic-ring-hydroxylated methylenedioxy compound. This notion was consistent with the mass spectral evidence in which changes in the m/z values of fragments attributable only to the aromatic ring were noted. Substitution of deuterium atoms on both the side-chain and methylenedioxy group resulted in the appropriate $m/z+2$ fragments as well. This evidence does therefore strongly support the aromatic ring as the site of MDMA hydroxylation but does not provide any evidence for the position of the hydroxyl group, although by analogy with MDB it is tempting to assign it to the 6 position.

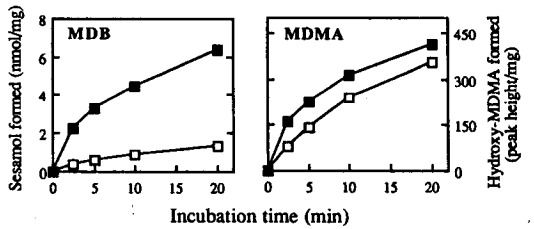


Figure 5. Effects of PB of the aromatic hydroxylation of MDB and MDMA.
□, untreated; ■, PB-treated microsomes. Each point is the mean of two determinations.

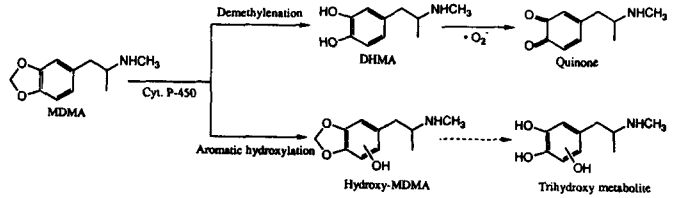


Figure 6. Proposed metabolic pathway of MDMA by rabbit liver.

Experiments with NADPH and carbon monoxide indicate that cytochrome P-450 mediated this aromatic hydroxylation of MDB and MDMA. The results with a reconstituted enzyme IIB4 further supported the notion of participation of cytochrome P-450 in sesamol and hydroxy-MDMA formation. The reaction was also observed with rabbit lung microsomes which contain isozyme IIB4 as about 40% of the total cytochrome P-450 (Wolf *et al.* 1978) and rat liver microsomes (Kumagai *et al.* unpublished). Since PB induction, and incubation with microsomes of untreated lung instead of that of untreated liver, resulted in an enhancement of sesamol formation, isozyme IIB4 appears to play an important role in the hydroxylation of MDB. The difference in the effect of PB between MDB and MDMA hydroxylation indicates that each reaction may be catalysed by different isozymes.

Previous studies from this laboratory (Hiramatsu *et al.* 1990) suggested that an *o*-quinone could be generated in the course of MDMA metabolism that would form covalent bonds with nucleophiles or undergo redox chemistry to generate active oxygen species. These reactions would then lead to biochemical lesions (figure 6). However, the present data raise another possibility resulting from the demethylenation of M-2. The product of this reaction is a 6-hydroxydopamine-like compound that would also exhibit a neurotoxicity (Sachs and Jonsson 1975). Thus, this alternative pathway for neurotoxicity must be considered.

Note in proof: During the review of this manuscript, Lim and Foltz [*Biological Mass Spectrometry*, **20**, 677–686 (1991)] isolated ring hydroxylated metabolites from tissues of MDMA treated rats and identified 6-hydroxy MDMA as the major isomer.

Acknowledgement

We thank Mrs Emma W. Di Stefano for synthesizing the deuterium substituted MDB and MDMA. This work was supported by grants from the USPHS (DA 04206) and NSF (EET-8807418) and the UC Biotechnology Research Program.

References

Clark, J. H., Holland, H. L., and Miller, J. M., 1976, Hydrogen bonding in organic synthesis IV: a simple high-yield method for the methylenation of catechols. *Tetrahedron Letters*, **38**, 3361–3364.

Coon, M. J., Van der Hoeven, T. A., Dahl, S. B., and Haugen, D. A., 1978, Two forms of liver microsomal cytochrome P-450, P-450 LM_2 and P-450 LM_4 (rabbit liver), in *Methods in Enzymology*, vol. 52: Biomembranes, edited by S. Fleischer and L. Packer, (New York: Academic Press), pp. 109–117.

Dingell, J. V., and Bass, A. L., 1969, Inhibition of the hepatic metabolism of amphetamine by desipramine. *Biochemical Pharmacology*, **18**, 1535–1538.

Duncan, J. D., and Cho, A. K., 1982, *N*-oxidation of phentermine to *N*-hydroxyphentermine by a reconstituted cytochrome P-450 oxidase system from rabbit liver. *Molecular Pharmacology*, **22**, 235–238.

Gollamudi, R., Ali, S. F., Lipe, G., Newport, G., Webb, P., Lopez, M., Leakey, J. E. A., Kolta, M., and Slikker, W., 1989, Influence of inducers and inhibitors on the metabolism *in vitro* and neurochemical effects *in vivo* of MDMA. *Neurotoxicology*, **10**, 455–466.

Hiramatsu, M., Kumagai, Y., Unger, S. E., and Cho, A. K., 1990, Metabolism of methylenedioxymethamphetamine (MDMA): formation of dihydroxymethamphetamine and a quinone identified as its glutathione adduct. *Journal of Pharmacology and Experimental Therapeutics*, **254**, 521–527.

Kumagai, Y., Wickham, K. A., Schmitz, D. A., Fukuto, J. M., and Cho, A. K. 1991a, Metabolism of methylenedioxyphenyl compounds by rabbit liver preparations: participation of different isozymes in the demethylenation reaction. *Biochemical Pharmacology*, **42**, 1061–1067.

Kumagai, Y., Lin, L. Y., Schmitz, D. A., and Cho, A. K., 1991b, Hydroxyl radical mediated demethylenation of methylenedioxyphenyl compounds. *Chemical Research in Toxicology*, **4**, 330–334.

Lim, H. K., and Foltz, R. L., 1988, *In vivo* and *in vitro* metabolism of 3,4-(methylenedioxy)methamphetamine in the rat: identification of metabolites using an ion trap detector. *Chemical Research in Toxicology*, **1**, 370–378.

Omura, T., and Sato, R., 1964, The carbon monoxide-binding pigment of liver microsomes. *Journal of Biological Chemistry*, **239**, 2370–2378.

Phillips, A. H., and Langdon, R. G., 1962, Hepatic triphosphopyridine nucleotide-cytochrome c reductase isolation, characterization, and kinetic studies. *Journal of Biological Chemistry*, **237** (8), 2652–2660.

Ricaurte, G., Bryan, G., Strauss, L., Seiden, L., and Schuster, C., 1985, Hallucinogenic amphetamine selectively destroys brain serotonin nerve terminals. *Science*, **229**, 986–988.

Roston, D. A., and Kissinger, P. T., 1982, Isolation and identification of benzene metabolites *in vitro* with liquid chromatography/electrochemistry. *Analytical Chemistry*, **54**, 1798–1802.

Sachs, C., and Jonsson, G., 1975, Mechanisms of action of 6-hydroxydopamine. *Biochemical Pharmacology*, **24**, 1–8.

Schmidt, C. J.,1987, Neurotoxicity of the psychedelic amphetamine, methylenedioxymethamphetamine. *Journal of Pharmacology and Experimental Therapeutics*, **240** (1), 1–7.

Schmidt, C. J., and Taylor, V. L., 1988, Direct central effects of acute methylenedioxymethamphetamine on serotonergic neurons. *European Journal of Pharmacology*, **156**, 121–131.

Smissman, E. E., and Borchardt, R. T., 1971, Conformational study of catecholamine receptor sites. 7. Synthesis of erythro- and threo-3-amino-2(3,4-dihydroxyphenyl)-2-butanol hydrochlorides and erythro- and threo-2-amino-3-(3,4-dihydroxyphenyl) butane hydrochlorides. *Journal of Medicinal Chemistry*, **14**, 702–707.

Wolf, C. R., Szutowski, M. M., Ball, L. M., and Philpot, R. M., 1978, The rabbit pulmonary monooxygenase system: characteristics and activities of two forms of pulmonary cytochrome P-450. *Chemico-Biological Interactions*, **21**, 29–43.