METABOLISM OF 3,4-(METHYLENEDIOXY)-
METHAMPHETAMINE

by

Heng-Keang Lim

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SUPERVISORY COMMITTEE APPROVAL

This dissertation has been read by each member of the following supervisory committee and by majority vote has been found to be satisfactory.

October 10, 1989

[Signature]

October 10, 1989

[Signature]

October 10, 1989

[Signature]

October 10, 1989

[Signature]

October 10, 1989

[Signature]
To the Graduate Council of The University of Utah:

Heng-Keang Lim

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Date

Member, Supervisory Committee

Approved for the Major Department

James W. Gibb
Chairman, Dean

Approved for the Graduate Council

B. Gale Dick
Dean of The Graduate School
ABSTRACT

(RS)-3,4-(Methylenedioxy)methamphetamine (MDMA) is a ring-substituted amphetamine derivative, hence its common reference in the literature as a "designer drug." With respect to its pharmacology, MDMA is more closely related to amphetamine than to the hallucinogen mescaline. The structural similarity of MDMA to the neurotoxin, 3,4-(methylenedioxy)amphetamine (MDA), has led to its being listed as a Schedule I drug by the US Drug Enforcement Agency. MDMA is now an established serotonergic neurotoxin in several animal models such as rats, mice and nonhuman primates. Even though the metabolism of MDMA in the rat had not been determined at the time the research reported here was initiated, several investigators had postulated the involvement of metabolite(s) in MDMA-induced neurotoxicity on the basis of the similarity of its neurotoxicity profile to that of another neurotoxin, p-chloroamphetamine.

The objective of this research was to elucidate the metabolism of MDMA. Initially, four metabolic pathways of MDMA have been identified in the rat: N-demethylation, O-dealkylation, deamination, and conjugation (O-methylation, O-glucuronidation, and/or O-sulfation). The specific MDMA metabolites that have been identified are 3-hydroxy-4-methoxymethamphetamine, 4-hydroxy-3-methoxymethamphetamine, 3,4-dihydroxymethamphetamine, 4-hydroxy-3-methoxyamphetamine, 3,4-(methylenedioxy)amphetamine, (4-hydroxy-3-methoxyphenyl)acetone, [3,4-(methylenedioxy)phenyl]acetone, and (3,4-dihydroxyphenyl)acetone. Only MDMA and three of its metabolites (MDA, 4-hydroxy-3-methoxymethamphetamine, and 4-hydroxy-3-methoxyamphetamine) were consistently detected in urine, feces, plasma, liver, and brain. Metabolism of MDMA via O-dealkylation, N-demethylation, and O-methylation was also shown to occur in the rat brain.
Further investigation resulted in the identification of 6-hydroxy-MDMA in rat liver, plasma and brain. The 6-hydroxy-MDMA is a particularly significant metabolite in view of its structural similarity to such potent neurotoxins as 6-hydroxydopamine and 6-hydroxyserotonin.

Four metabolic pathways of MDMA in the rat (N-demethylation, O-dealkylation, deamination and conjugation) were also shown to occur in men. All but two [(3,4-dihydroxyphenyl)acetone and 6-OH-MDMA] of the metabolites previously reported in the rat were shown to be present in the urine from a MDMA user.

A capillary gas chromatography-positive ion chemical ionization mass spectrometry (GC-Cl/MS) assay, linear from 1 - 500 ng/g wet brain tissue, was successfully developed for MDMA, MDA, 4-hydroxy-3-methoxymethamphetamine (HMM) and 4-hydroxy-3-methoxyamphetamine (HMA) in brain tissues. The sensitivity of the assay was sufficient to permit monitoring MDMA and MDA concentrations in the rat brain for up to 24 hr after a subcutaneous injection of 10 mg/kg MDMA. HMM and HMA could be measured for up to 8 and 10 hr, respectively.

Stereoselective disposition of MDMA and MDA was observed in the rat as shown by the distortion of the mean enantiomeric ratio (R/S) of MDMA (1.661) and MDA (0.660) in 24-hr urine after administration of a racemic mixture of the drug.
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CHAPTER 1

INTRODUCTION

3,4-(Methylenedioxy)methamphetamine (MDMA), a ring-substituted amphetamine designer drug, which is commonly known on the street as "Ecstacy," "XTC" or "Adam." MDMA was first synthesized as an anorexic drug by E. Merck and Company (Germany) in 1914 but was never marketed. Because it has one asymmetric center, there are two enantiomers of R-(−) and S-(+) configurations. Structurally, MDMA is related to the psychomotor stimulant amphetamine, and to the hallucinogen mescaline (Figure 1). Despite its structural resemblance to mescaline, MDMA apparently lacks hallucinogenic effects in animal models (Glennon et al., 1982; Glennon and Young, 1984; Nichols et al., 1986) and in humans (Anderson et al., 1978; Downing, 1986; Peroutka et al., 1988); rather, its subjective effects are more amphetamine-like (Glennon and Young, 1984; Evans and Johanson, 1986; Kamien et al., 1986; Glennon et al., 1988; Peroutka et al., 1988). This, together with its empathy-enhancing properties, probably accounts for the increased abuse of MDMA as a recreational drug among college students (Peroutka, 1987). Death from abuse of MDMA is rare but has been reported in individuals with underlying cardiac disease (Dowling et al., 1987). The concern over MDMA's high abuse potential and its structural similarity to 3,4-(methylenedioxy)amphetamine (MDA) (Figure 1), a known neurotoxin (Ricaurte et al., 1985), prompted the US Drug Enforcement Agency to temporarily categorize MDMA as a Schedule I drug in 1985. This decision generated considerable opposition from some psychiatrists who found the drug to be an useful adjunct to psychotherapy because of its ability to ameliorate anxiety or fear during recall of emotionally painful events (Greer, 1983). A successful legal challenge to the scheduling of MDMA resulted in its removal
Figure 1. Chemical structures of MDMA, amphetamine, mescaline and MDA.
from the temporary Schedule I list (Barnes, 1988). However, based upon numerous recent reports of neurotoxicity of MDMA in animals (Schmidt, 1987; Stone et al., 1987a,b,c; Commins et al., 1987; O'Hearn et al., 1988; Slikker et al., 1988), the drug has now been placed on permanent Schedule I status (Drug Enforcement Agency, 1988).

**Pharmacokinetics**

There is only one report on the disposition of MDMA and its N-demethylated metabolite in the human (Verebey et al., 1988). Following a single 50-mg oral dose of MDMA in a 74-kg man, peak plasma concentrations of MDMA (105.6 ng/ml) and MDA (28.4 ng/ml) were observed 2 and 4 hr after administration, respectively. The half-life of MDMA was calculated to be 7.6 hr but no corresponding calculation of MDA's half-life was reported although the complete plasma concentration-time profile for MDA was available. Urinary excretion data show that MDMA and MDA accounted for 65% and 7% of the oral dose, respectively. The metabolic fate of the remaining 28% of the oral dose was not determined.

Enantiomeric dispositions of MDMA and MDA were recently reported in rats (Hiramatsu et al., 1989). Following an intravenous dose of 10 mg/kg of the respective MDMA enantiomers, the plasma half-lives of the R-(-) and S-(+) enantiomers were 86.3 and 121.7 min, respectively. Slight differences were also observed in the volumes of distribution of the R-(-) [1.4 l/kg] and S-(+) [2.3 l/kg] enantiomers. However, differences in the peak concentrations of the MDA enantiomers were marked; the S-(+) enantiomer concentration (1.95 nmol/ml) was 6.5 times that of the R-(-) antipode. Since MDMA is a neurotoxin, there is considerable interest in the disposition of MDMA and its metabolites in the rat brain. Recently, the disposition of MDMA and three of its metabolites (MDA, 4-hydroxy-3-methoxymethamphetamine, and 4-hydroxy-3-methoxyamphetamine) were reported for the first time in rat brain (Lim and Foltz, 1989b).
Metabolism

The absence of neurotoxicity following intracerebral injection of MDMA to rats led to the speculation that a peripherally produced metabolite of MDMA is involved in MDMA-induced neurotoxicity (Molliver et al., 1986). Consequently, there has been considerable interest in characterizing the metabolism of MDMA. At the beginning of our investigation, MDMA was known to undergo N-demethylation and N-hydroxylation in rats (Brady et al., 1986; Fitzgerald et al., 1988; Gollamudi et al., 1988); only the N-demethylation pathway had been reported for MDMA in humans (Verebey et al., 1988). It is surprising that only N-hydroxy-MDA was identified after incubation of MDMA with rat microsomes (Brady et al., 1986) because N-hydroxymethamphetamine and N-hydroxyamphetamine were observed following in vitro metabolism of methamphetamine by 9,000 x g rat liver supernatants (Baba et al., 1987). Furthermore, stereoselectivity in the N-demethylation pathway was observed following incubation of the individual enantiomers of MDMA with rat microsomes from female (but not from male) Sprague-Dawley rats (Gollamudi et al., 1988; Slikker et al., 1989). However, a later report provided evidence for stereoselectivity in the N-demethylation pathway, using liver microsomes from male Sprague-Dawley rats (Hiramatsu et al., 1989); the source of the discrepancy in results is not known. Recently, seven new metabolites of MDMA were reported in rats (Lim and Foltz, 1988). However, only three of the seven new metabolites of MDMA in rats were later confirmed: 3-hydroxy-4-methoxymethamphetamine, 4-hydroxy-3-methoxymethamphetamine (HMM) and 4-hydroxy-3-methoxyamphetamine (HMA) (Narasimhachari et al., 1989). The inability to detect the remaining metabolites in the latter report may be related to the different analytical instrument used in the identification of metabolites.

It is well documented that metabolism of amphetamine-type drugs is highly species-dependent (Caldwell, 1976). Therefore, the investigation of metabolism of MDMA has recently been extended to humans and all but one of the identified MDMA metabolites in rats (Lim and Foltz, 1988) were also observed in human (Lim and Foltz, 1989a).
Elucidation of the enzymatic systems responsible for metabolism of MDMA is also of considerable interest. N-Demethylation of MDMA is mediated by cytochrome P-450 and flavin-containing monoxygenase (Brady et al., 1986; Slikker et al., 1989). The specific cytochrome P-450 isozyme involved in N-demethylation of MDMA was investigated using inducers like phenobarbital and 3-methylcholanthrene. Phenobarbital-inducible forms (b + e) are more active than 3-methylcholanthrene-inducible forms (c + d) in N-demethylation of MDMA; however, neither inducible form exhibited any stereoselectivity in N-demethylation (Gollamudi et al., 1988; Slikker et al., 1989).

MDMA has two functionalities (amine and methylenedioxy moieties) that can interact with the active site of cytochrome P-450. In a binding study, a “metabolic-intermediate” complex spectrum (spectral peaks at 455 and 428 nm) was obtained in the presence of NADPH (Brady et al., 1986). This indicates the formation of either a methylenedioxy-heme complex or a combination of this and a nitroso-heme complex (Franklin, 1982). MDA was a minor metabolite of MDMA (Brady et al., 1986) and in addition to cytochrome P-450, the flavin-containing monoxygenase also contribute to the N-demethylation of MDMA.

**Pharmacology**

The biochemical pharmacology of MDMA is attributed mainly to its ability to release, and block active reuptake, of endogenous monoamine neurotransmitters like serotonin (Johnson et al., 1986; Nichols, 1986; Nichols et al., 1982), dopamine (Johnson et al., 1986; Nichols, 1986; Kalix et al., 1988) and norepinephrine (Nichols, 1986; Monti and Beaton, 1989). Stereoselectivity has been observed in these biochemical processes; in all instances, the S-(+) enantiomer is more potent than the R-(-) antipode. This probably accounts for the S-(+) enantiomer being mainly responsible for the in vivo pharmacodynamic effects of MDMA.
MDMA binds with low affinity to 5-hydroxytryptamine (5-HT) receptors in the brain; of the two enantiomers, the R-(−) enantiomer displays higher affinity for the 5-HT receptors (Lyon et al., 1986). This reverse in stereoselectivity, combined with observations that the release of neurotransmitter is calcium-independent (Johnson et al., 1986) and that intact neurons are not required for release of neurotransmitter (Nichols et al., 1982), argues against direct action of MDMA on postsynaptic receptors. However, direct receptor action of MDMA cannot be totally excluded, since specific binding sites for MDMA are present in the rat brain (Gehlert et al., 1985).

Observations like the influence of MDMA on catecholamine neurotransmission, the self-administration of MDMA by rhesus monkeys (Beardsley et al., 1986), and the production of amphetamine-like, discriminative-stimulus effects in animal models (Kamien et al., 1986; Glennon and Young, 1984; Glennon et al., 1988; Evans and Johanson, 1986) probably account for the amphetamine-like subjective effects in recreational users (Peroutka et al., 1988). The effective oral dose of MDMA ranges from 75 to 150 mg. Onset of drug effects occurs within 30 min; maximum response is reached in 1 to 1.5 hr (Davis et al., 1987). The amphetamine-like subjective effects, including side effects, have been described extensively in man (Peroutka et al., 1988; Davis et al., 1987). Tolerance to the "desirable" empathy-enhancing effects of MDMA develops rapidly, and the remaining stimulant effect is responsible for its abuse potential.

**Toxicology**

A study comparing the acute lethality of five amphetamine-type hallucinogens in five animal species suggested that MDMA was the second most toxic compound in the series (Hardman et al., 1973). There were species differences in the acute toxic response; the dog being the most susceptible (Hardman et al., 1973). The potential CNS toxicity of MDMA initially attracted considerable attention from researchers primarily because of its structural resemblance to the neurotoxin, MDA.
Indeed, MDMA has been shown to deplete selectively serotonin in the rat brain following both acute (Schmidt et al., 1986, 1987; Stone et al., 1986, 1987b; Schmidt, 1987; Commins et al., 1987) and multiple subcutaneous doses (Stone et al., 1986, 1987a,b; Commins et al., 1987). The depletion of serotonin was biphasic: the acute phase reached a maximum between 3-6 hr and the latter phase began 1 week after dosing (Schmidt, 1987) and continued for as long as 14 days (Stone et al., 1987b). A corresponding time-course reduction in tryptophan hydroxylase (the rate-limiting enzyme in biosynthesis of serotonin) activity was also observed following an acute subcutaneous dose of MDMA (Stone et al., 1987b; Schmidt and Taylor, 1987). The reversible, acute effects of MDMA may be related to inhibition of serotonin synthesis (Stone et al., 1986, 1987b; Schmidt and Taylor, 1987), blockade of serotonin reuptake (Nichols, 1986; Schmidt et al., 1987), and an increase in carrier-mediated release of serotonin (Schmidt, 1987; Schmidt et al., 1987). Accompanying these changes is a MDMA-induced loss in the number of serotonin uptake sites, as indicated by the decrease in the $V_{\text{max}}$ of the uptake carrier with no corresponding changes in the $K_m$ or the affinity of the carrier for serotonin (Schmidt, 1987). Only the latter phase of serotonin depletion exhibits stereoselectivity, mainly with respect to the $S$-$(+)$ enantiomer (Schmidt, 1987).

In addition to the biochemical evidence for neurotoxicity, studies have shown that MDMA is neurotoxic at the histological level in rats (Commins et al., 1987; O'Hearn et al., 1988; Slikker et al., 1988) and nonhuman primates (Ricaurte et al., 1988b). Immunocytochemical and silver staining methods have shown that MDMA causes profound structural damage to serotonergic nerve fibers in all species investigated. In general, the catecholaminergic nerve fibers were spared from the neurotoxic action of MDMA.

Under biochemical and histological criteria of neurotoxicity, species differences in susceptibility to MDMA-induced neurotoxicity have been reported. The descending order of susceptibility of animal species to MDMA-induced neurotoxicity is in the order: nonhuman primates $>$ rats $>$ mice (Ricaurte et al., 1988a,b; Slikker et al., 1988; Stone et al.,
1987c; Logan et al., 1988). Nonhuman primates are four to eight times more sensitive than rats to the neurotoxicity of MDMA. This different susceptibility among animal species may be the result of differences in metabolism of MDMA; metabolism of amphetamine-type drugs is highly species-dependent (Caldwell, 1976). The species-dependent neurotoxicity of MDMA prompted an investigation into the effect of MDMA on serotonin, dopamine and norepinephrine in the cerebrospinal fluid of recreational users (Peroutka et al., 1987). Recreational doses of MDMA in man did not result in any significant change in the serotonergic, dopaminergic or adrenergic activity in the central nervous system. However, no explanation was given for the discrepancy between these data and those derived from animal studies.

Concern over the influence of the route of administration of MDMA on development of neurotoxicity arose when no neurotoxicity was observed following intracerebral injection of MDMA to rats (Molliver et al., 1986). Only continuous intracerebroventricular infusion of MDMA depressed TPH activity and depleted brain serotonin; the implications of this observation with respect to development of neurotoxicity over the long term have not been established (Schmidt and Taylor, 1988). Neurotoxicity has now been demonstrated in rats and nonhuman primates after oral and subcutaneous administration (Slikker et al., 1988; Ricaurte et al., 1988a,b); however, the oral route is half as effective as the subcutaneous route in producing neurotoxicity in primates (Ricaurte et al., 1988a).

Despite the extensive MDMA research, the mechanism of MDMA-induced neurotoxicity remains unknown. Speculation that metabolite(s) of MDMA are involved in neurotoxicity rests on similarity between the neurotoxicity of MDMA and that of another neurotoxin, p-chloroamphetamine (Schmidt, 1987). It has been hypothesized further that the toxic metabolite undergoes cyclization and demethylation of the isopropylamine side-chain, to form a compound with a structure similar to the serotonergic neurotoxin, 5,6-dihydroxytryptamine (Schmidt, 1987). Others have speculated that MDMA causes neurotoxicity via formation of 5,6-dihydroxytryptamine from nonenzymatic oxidation of released serotonin.
There appears to be a common theme in both proposed mechanisms; that is, MDMA causes neurotoxicity via oxidative stress. The notion that oxidative stress may play a role in the neurotoxicity of MDMA came from a recent observation implicating the formation of disulfide linkages as the cause of inactivation of tryptophan hydroxylase following acute treatment of rats with MDMA (Stone et al., 1989).

**Quantitative Analytical Methods**

Few analytical methods have been reported for quantitation of MDMA and its metabolites in biological specimens. A method based on capillary gas chromatography with flame ionization detection has been used to quantitate MDMA in forensic samples without derivatization (Dowling et al., 1987). Recently, a gas chromatography-mass spectrometry (GC-MS) method was developed to measure trifluoroacetyl derivatives of MDMA and MDA in rat plasma extracts; deuterium-labelled analogs (d2) were the internal standards (Hiramatsu et al., 1989). A potential problem with that GC-MS assay is limited dynamic range due to the likelihood that M+2 isotopic peak from the unlabelled compounds, when present in high concentration, will interfere with the internal standards; this problem has been reported for standards having too few deuterium atoms (one or two) (Fentiman and Foltz, 1976). Recently, a GC-MS assay was developed for simultaneous quantitation of low-nanogram concentrations of MDMA and three of its metabolites in rat brain (Lim and Foltz, 1989b). Details of the GC-MS assay are described in this thesis. Another GC-MS assay for MDMA and MDA in human plasma and urine has been reported (Verebey et al., 1988) but details of the assay have not been published.

An enantioselective GC-MS assay has been developed for quantitation of enantiomers of MDMA and MDA, from 5 - 200 ng/ml, in whole blood (Fitzgerald et al., 1989a). The assay involved conversion of MDMA and MDA into diastereomers by derivatizing them with N-trifluoroacetyl-L-prolyl chloride prior to resolution on a capillary column.
High performance liquid chromatography (HPLC) has been employed for analysis of MDMA and its metabolites in plasma, urine and brain (Narasimhachari et al., 1989). This procedure uses an ultraviolet detector for quantitation of amine compounds (MDMA and MDA) and an electrochemical detector for phenolic amine metabolites.
CHAPTER 2

MATERIALS AND METHODS

Materials

MDMA, MDA and 3,4-(methylenedioxy)ethylamphetamine (MDE) were purchased from Alltech Applied Science (Deerfield, IL). [3,4-(Methylenedioxy)phenyl]acetone was purchased from Fluka Chemical Co. (Hauppauge, NY). These standards were checked for purity by GC-MS analyses. Trifluoroacetic anhydride (99% pure) was obtained from Pierce Chemical Co. (Rockford, IL). All solvents used were glass-distilled, HPLC grade, and were obtained from Burdick and Jackson (Muskegon, MI). The following reagents were obtained from Aldrich Chemical Co. (Milwaukee, WI): sodium bisulfite, sodium hydroxide (98%), sodium acetate trihydrate (99+%), anhydrous sodium sulfate (99+%), potassium chloride (99+%), anhydrous dibasic potassium phosphate, anhydrous monobasic potassium phosphate (99+%), magnesium chloride hexahydrate (99%), 4-hydroxy-3-methoxybenzaldehyde (99%), 3-hydroxy-4-methoxybenzaldehyde (99%), 3,4-dimethoxybenzaldehyde (99%), 2,4,5-trimethoxybenzaldehyde (98%), anhydrous ammonium acetate, nitroethane (96%), 325 mesh powdered iron (97%), lithium aluminium hydride (95%), potassium sodium tartrate tetrahydrate (99%), Celite 521, methylamine hydrochloride (98%), sodium cyanoborohydride (95%), anhydrous cesium fluoride (99%), and boron tribromide (1M solution in dichloromethane). β-Glucuronidase (Helix pomatia, type H-1), D-glucose 6-phosphate (monopotassium salt), β-nicotinamide adenine dinucleotide phosphate (monosodium salt), and glucose-6-phosphate dehydrogenase (Torula yeast, in ammonium sulfate solution) were purchased from Sigma Chemical Co. (St. Louis, MO). Ammonium hydroxide and glacial acetic acid were obtained from VWR Scientific Co. (San
Francisco, CA). Silica gel, 60-200 mesh, was purchased from American Scientific Products (McGaw Park, IL).

**Instrumentation**

Identifications of MDMA metabolites were carried out using a Hewlett-Packard Model 5890 gas chromatograph interfaced to a Finnigan MAT Model 800 ion trap detector (ITD). The data system for the ITD consisted of an IBM AT microcomputer. The software (version 3.31), which contained both automatic gain control (AGC) and automatic reaction control (ARC) programs, was made available to us by Finnigan MAT. The ion trap manifold temperature was maintained at 220 °C during analysis.

The ITD was autotuned according to the manufacturer's recommendations. Electron ionization (EI) mass spectra were acquired by repetitive scanning of the mass range m/z 50-649 at a rate of 1 scan/sec. Positive ion chemical ionization (CI) mass spectra were acquired by scanning the mass range m/z 100-649. Either methanol or acetone served as the CI reagent gas. The reagent gas pressure within the ion trap was adjusted by opening the reagent gas metering valve to give an ion current intensity of approximately 2500-4000 counts at the mass of the protonated reagent gas molecule. This adjustment was carried out with the ARC function turned off, and with the B value (sensitivity) set to the minimum. For methanol, the ionization and reaction radio frequency (RF) levels were set at 3.5 and 26 amu, respectively. However, the ionization and reaction RF levels were set at 5 and 40 amu, respectively, when acetone was used as the reagent gas. Other operating parameters were the same as previously reported for the acquisition of CI mass spectra using the ARC scan function (Lim et al., 1988c).

MDMA and metabolites were separated chromatographically with a fused silica capillary column coated with dimethylsilicone (12.5 m x 0.2 mm i.d., 0.3 μm film thickness, Hewlett-Packard, Palo Alto, CA). The GC column was interfaced directly to the ion trap through the standard transfer line connector. Hydrogen served as both carrier gas
(linear velocity = 60 cm/sec at 100 °C) and buffer gas. The injector and transfer line temperatures were 260 and 220 °C, respectively. The oven temperature was held at 80 °C for 0.5 min after injection and then temperature-programmed to 300 °C at 10 °C/min. N-Pentafluorobenzoyl-MDMA (1 ng/μl) was injected to evaluate the performance of the GC-MS prior to analysis of samples.

Identification of 6-hydroxy-MDMA (6-OH-MDMA) was carried out using a Hewlett-Packard Model 5890 gas chromatograph interfaced to a Finnigan MAT ion trap mass spectrometer (ITMS) via a modified triple stage quadrupole transfer line. The data system was an IBM AT microcomputer with revision B of the ITMS applications software. The ITMS was operated in the selective mass storage and tandem mass spectrometric (MS/MS) modes.

The ITMS manifold temperature was maintained at 120 °C during analysis. The pressure within the manifold was set at 5 x 10^{-5} Torr by adjusting the flow of the buffer gas. Tuning of the ITMS was carried out manually by adjusting the B value so that 10-15% valley resolution was obtained for m/z 414 from perfluorotributylamine. The ion intensity of CH₃OH₂⁺ (m/z 33) ions was maintained at about 4000 counts by bleeding in methanol to give a pressure of 1 x 10^{-5} Torr and ionizing for 1 msec. The RF level was then adjusted so that the qₓ value of m/z 33 was 0.786, which is the apex of the stability diagram. A DC pulse of -31 V was then applied to the ring electrode to cause selective mass storage of m/z 33. Ion-molecule reactions between protonated methanol molecule ion and analyte was allowed to proceed for 240 msec and the RF level was again adjusted so that the qₓ value of m/z 402 (MH⁺ of trifluoroacetyl derivative of 6-OH-MDMA) was 0.786. Selective mass storage of m/z 402 was then achieved by applying a DC pulse of -401 V. Collision-activated dissociation of m/z 402 to the daughter ions was effected by application of supplementary AC "tickle" voltage of 250 mV across the end-caps of the ion trap at a "tickle" frequency of 52,899 Hz and for 60 msec ("tickle" time). Under these conditions, the qₓ value was at 0.14. The RF was then ramped so that the resulting daughter ions (m/z
60 to 450) were ejected out of the ion trap and detected by the electron multiplier. Initially, optimum MS/MS conditions were determined for the trifluoroacetyl derivative of 4-hydroxy-3-methoxyethylamphetamine (HME, same molecular weight as 6-OH-MDMA) which was introduced into the ITMS by a solid probe. The same MS/MS conditions were then used for the analysis of the trifluoroacetyl derivative of 6-OH-MDMA, after slight modification of "tickled" voltage and time (270 mV and 58 ms).

The trifluoroacetyl derivatives of 6-OH-MDMA and HME were chromatographically separated on a fused silica capillary column coated with 5% phenyl/95% methylsilicone (12.5 m x 0.2 mm i.d., 0.25 µm film thickness; Lee Scientific, Salt Lake City, UT). The GC column was connected directly to the ITMS through the modified triple stage quadrupole transfer line. Helium was the carrier gas and its linear velocity was set at 30 cm/sec at 100 °C. The injector and transfer line temperatures were maintained at 260 and 250 °C, respectively. The oven temperature was held at 100 °C for 0.1 min after injection and then temperature-programmed to 310 °C at 10 °C/min. N, O-bis(trifluoroacetyl)-HME (100 pg/µl, freshly prepared) was injected to evaluate the performance of the GC-MS/MS prior to analysis of samples.

For investigation of human metabolism of MDMA, a fused silica capillary column coated with 5% phenyl/95% methylsilicone (30 m x 0.2 mm i.d., 0.25 µm film thickness; J & W Scientific, Folsom, CA) was used. The oven temperature was held at 100 °C for 0.5 min after injection and then temperature-programmed to 300 °C at 12 °C/min. Other chromatographic and mass spectrometric conditions were as described for identification of MDMA metabolites in the rat.

Quantitation of MDMA and three of its metabolites (MDA, HMM and HMA) was performed on the same GC-MS system used for identification of 6-OH-MDMA. In these experiments, the ITMS was used in the single stage mass analysis mode. The ITMS is capable of axial modulation, both for high-sensitivity analysis at low analyte concentration and for reduction in loss of mass resolution due to space-charging at high concentration of
analyte. Details on axial modulation have been reported elsewhere (Weber-Grabau et al., 1988). Isobutane was bled into the ITMS to give a pressure of $2.5 \times 10^{-5}$ Torr. With the ARC function turned off and B value set to minimum, an ion current intensity of 4000 counts was obtained for C$_4$H$_9^+$ ions (m/z 57). The ionization and reaction RF levels were set at 10 and 20 amu, respectively. Maximum ionization and reaction times were set at 500 µsec and 200 msec, respectively. During analysis the mass spectrometer was set to scan repetitively a narrow mass range of m/z 273-390, which encompasses the ion currents for the protonated molecule ions (MH$^+$) of the trifluoroacetyl derivatives of MDMA, MDA, HMM, HMA, and MDE. Other ITMS conditions were identical to those used for identification of 6-OH-MDMA.

Chromatographic separation of MDMA, MDA, HMM, HMA, and MDE was carried out on a fused silica capillary column coated with 5% phenyl/95% methylsilicone (25 m x 0.2 mm i.d., 0.25 µm film thickness; Lee Scientific, Salt Lake City, UT). The oven temperature was held at 100 °C for 2 min after injection and then temperature-programmed to 310 °C at 10 °C/min. N-Trifluoroacetyl-MDMA and metabolites (100 pg/µl, freshly prepared) was injected to evaluate the performance of the GC-MS prior to analysis of samples. Other GC conditions were identical to those as described for identification of 6-OH-MDMA.

Analysis of diastereomeric pairs of MDMA and MDA was carried out on a Hewlett-Packard Model 5890 gas chromatograph coupled to a Finnigan MAT Model 800 ITD. Acetonitrile was the CI reagent gas. The ionization and reaction RF levels were set at 4.6 and 31.7 amu, respectively. The maximum ionization and reaction times were 2 and 240 msec, respectively. Other ITD conditions used were identical to those described for identification of MDMA and metabolites. The diastereomeric pairs of MDMA and MDA were resolved on fused silica capillary column coated with 6% cyanopropylphenyl/94% methylsilicone (15 m x 0.2 mm i.d., 0.25 µm film thickness; J & W Scientific, Folsom, CA). The oven temperature was held at 100 °C for 0.5 min and then temperature-
programmed to 280 °C at 10 °C/min. Other GC conditions were identical to those described for identification of MDMA metabolites.

Proton nuclear magnetic resonance (1H NMR) spectra were recorded with a 200-MHz IBM NR-200 spectrometer, using tetramethylsilane as the internal standard. Chemical shifts are reported in ppm downfield from tetramethylsilane.

**In Vivo Rat Metabolism of MDMA**

Six male Sprague-Dawley rats (200-250 g) were used for the in vivo studies. The rats were administered either 5 or 20 mg/kg MDMA (calculated as free base and prepared in physiological saline solution) by gavage and were then placed in metabolic cages. Urine and feces were collected over 24 hr. Fecal homogenate was prepared in 3 volumes of 0.9% (w/v) sodium chloride solution and then centrifuged at 10,000 x g for 15 min. In this set of experiments, each rat served as its own control and was fed only the vehicle by gavage. In another set of experiments, rats were decapitated at either 6 or 24 hr after drug administration. Blood was collected immediately and centrifuged at 1,475 x g for 15 min to separate the plasma. Also, brains and livers were removed; these tissues were homogenized in 3 volumes of cold 0.4% (v/v) perchloric acid containing 0.05% (w/v) sodium bisulfite. The supernatant, obtained from centrifugation of the homogenate at 10,000 x g for 15 min, was stored at -20 °C until analysis. The blood, brains, and livers from saline-treated rats served as control specimens and were processed in the same manner.

For identification of 6-OH-MDMA, six male Sprague-Dawley rats (200-250 g) were orally administered 20 mg/kg MDMA (calculated as free base and prepared in physiological saline solution) by gavage. Four other male Sprague-Dawley rats received only the vehicle. The rats were all decapitated 6 hr after drug administration. Blood was collected immediately and centrifuged at 1,475 x g for 15 min; the plasma was stored at -20 °C until required. Brains and livers were also removed, and homogenized in 3 volumes of cold 0.4% (v/v) perchloric acid containing 0.05% (w/v) sodium bisulfite; the
supernatants, obtained from centrifugation of the homogenates at 10,000 x g for 15 min, were stored at -20 °C until analysis.

**In Vitro** Rat Metabolism of MDMA

Four male Sprague-Dawley rats (200-250 g) were used for the *in vitro* experiments. The 10,000 x g rat liver and brain supernatants were prepared by modification of previously reported procedures (Coutts et al., 1984; Lim et al., 1988a). Rat brain (33% w/v) and liver (50% w/v) homogenates were prepared in 1.15% (w/v) potassium chloride (KCl) solution. The 10,000 x g supernatant was stored at -80 °C until required. The incubation procedure for *in vitro* brain and liver metabolic studies is a modification of that reported for LSD (Lim et al., 1988a). All incubations, 4-ml total volume, contained 120 mM phosphate buffer, pH 7.4, 46 mM KCl, 2 mM magnesium chloride, 0.4 mM β-nicotinamide adenine dinucleotide phosphate (NADP+), 4 mM glucose-6-phosphate (G-6-P), 0.4 unit of G-6-P dehydrogenase, 12 μM MDMA, and 0.8 ml of 10,000 x g supernatant. After a 5-min preincubation, the reaction was started by addition of the NADPH-generating system and 10,000 x g supernatant. The reaction mixture was further incubated for 2 hr at 37 °C. At the end of the incubation period, the reaction was stopped by immersing the test tubes in an ice bath prior to extraction. Control incubations were performed in the same manner except that no MDMA was added.

**In Vivo** Human Metabolism of MDMA

Because MDMA is a schedule I drug, it is difficult to obtain approval to administer the drug to human subjects for metabolic studies. However, a urine specimen from a fatally injured motorcyclist was made available to us after it was found to contain MDMA. No information is available regarding the amount of MDMA ingested prior to the subject's death, nor the time lapse between ingestion and death. All the metabolites of MDMA that have been identified in the human were present in this urine specimen.
**Enzymatic Hydrolysis**

The pH of the biological specimens from rats (urine, plasma, feces, brain and liver; 3 ml each) was adjusted to about 5 prior to addition of 1.5 ml of 1M sodium acetate buffer (pH 4.8) containing β-glucuronidase (10,000 units/ml of sample). The samples were then incubated for 16 hr at 37 °C.

For identification of 6-OH-MDMA, 3.5 ml each of plasma, brain and liver supernatants were hydrolyzed by addition of 1.75 ml of 1M sodium acetate buffer, pH 4.8, containing 7,000 units of β-glucuronidase (the pH of the resultant solution was about 5). Incubation conditions were as described above.

Only 2 ml of human urine was available and the whole specimen was subjected to enzymatic hydrolysis by addition of 1 ml of 1M sodium acetate buffer, pH 4.8, containing 20,000 units of β-glucuronidase (the pH of the resultant solution was about 5). Incubation conditions were as described above.

**Extraction and Derivatization**

Each biological specimen in a 15-ml test tube with Teflon-lined screw cap was spiked with 2 μg of MDE as the internal standard. For identification of 6-OH-MDMA, 0.25 μg of HME was spiked as the internal standard. The sample was basified to pH 9 with 2M sodium hydroxide, saturated with sodium chloride, and then extracted twice with 5 ml of dichloromethane/2-propanol (3:1 v/v) by gentle rocking for 15 min. The liquid phases were separated by centrifugation at 1,025 x g for 15 min. The pooled organic extract was washed with 5 ml of 0.1M ammonium hydroxide solution and centrifuged as above. The organic extract was then dried over anhydrous sodium sulfate. Following centrifugation, the liquid phase was transferred to a 10-ml test tube with Teflon-lined screw cap, and 50 μl of glacial acetic acid was added. The organic phase was evaporated to dryness under a gentle stream of air at 60 °C.
Ethyl acetate (100 μl) and trifluoroacetic anhydride (TFAA, 200 μl) were added to the residue, and the tube was tightly capped, hand-vortexed (30 sec), and then heated for 20 min at 80 °C. Just before analysis, the excess organic solvent and TFAA were removed under vacuum or with a gentle stream of nitrogen at 40 °C. The residue was reconstituted in 100 μl of ethyl acetate and hand-vortexed for 30 sec prior to injection of a 1-μl aliquot into the GC-ITD or GC-ITMS. For identification of 6-OH-MDMA, the residue was instead reconstituted in 100 μl of 5% N-methyl-bis(trifluoroacetamide) [MBTFA] in ethyl acetate, heated for 10 min at 80 °C, and then allowed to cool to room temperature prior to analysis by GC-MS/MS.

Chemical Synthesis of Metabolites

The reference compounds were synthesized according to published synthetic routes for related compounds. The purified reference compounds were all characterized by 1H NMR and mass spectrometry. Only 6-OH-MDMA and its precursors were not purified; they were characterized by GC-CI/MS. No purification of 6-OH-MDMA was necessary since separation of 6-OH-MDMA from the reaction by-products was performed in the mass spectrometer itself when operated in the MS/MS mode.

(4-Hydroxy-3-methoxyphenyl)acetone (VII). Condensation of 4-hydroxy-3-methoxybenzaldehyde with nitroethane (Gairaud and Lappin, 1953) gave 4-hydroxy-3-methoxy-β-nitrostyrene. Recrystallization of the nitrostyrene compound from methanol-water gave golden yellow crystals: 1H NMR (CDCl3) 2.47 (s, 3 H, CCH3), 3.93 (s, 3 H, OCH3), 6.17 (b s, 1 H, OH), 7.01 (m, 3 H, aromatic), 8.04 (s, 1 H, CH); CI/MS (trifluoroacetyl [TFA] derivative), m/z 306 (MH+, base peak).

The nitrostyrene compound was treated with ferric chloride, electrolytic iron powder, and concentrated hydrochloric acid by modification of the previously reported procedure (Morgan and Beckett, 1975) to give (4-hydroxy-3-methoxyphenyl)acetone as an oil. The oil was purified by elution from a silica gel column (60-200 mesh) with toluene-
ldichloromethane (9:1). Fractions containing the purified product were pooled. Evaporation of the organic solvent with a rotary evaporator yielded a golden yellow oil: $^1$H NMR (CDCl$_3$), 2.12 (s, 3 H, CCH$_3$), 3.59 (s, 2 H, CH$_2$), 3.79 (s, 3 H, OCH$_3$), 6.31 (b s, 1 H, OH), 6.76 (m, 3 H, aromatic); CI/MS (TFA derivative), m/z 277 (MH$^+$, base peak).

4-Hydroxy-3-methoxymethamphetamine (III). This compound was prepared from the ketone VII by modification of the procedure reported elsewhere (Morgan and Beckett, 1975). The free base of methylamine was liberated from the hydrochloride salt by stirring together 0.24 mol of methylamine hydrochloride, 0.22 mol of sodium carbonate, and 250 ml of methanol at room temperature for 30 min; it was then filtered into a 500-ml three-neck round-bottom flask containing 0.04 mol of (4-hydroxy-3-methoxyphenyl)acetone. After the mixture was refluxed for 2 hr, 0.032 mol of sodium cyanoborohydride was added in small portions over 20 min at room temperature. Reflux was resumed for another 5 hr, and during this time, the pH of the solution was maintained at neutrality by addition of 4M methanolic hydrochloric acid as needed. The organic solvent was removed with a rotary evaporator, and residue was taken up in 200 ml of water. The solution was acidified to pH 2-3 with 6M hydrochloric acid, extracted with ethyl acetate, then basified to pH 9-10 with 6M sodium hydroxide, and finally saturated with sodium chloride. The mixture was extracted with ethyl acetate; the pooled organic extract was then dried over anhydrous sodium sulfate and the ethyl acetate was removed by evaporation to give an oil, which was purified by silica gel column chromatography (60-200 mesh) and eluted with a solvent mixture of toluene/dichloromethane/methanol (2:2:1). Fractions containing the 4-hydroxy-3-methoxymethamphetamine were pooled, and the organic solvent was evaporated to yield a dark brown oil: $^1$H NMR (CDCl$_3$) 1.10 (d, J = 6 Hz, 3 H, CCH$_3$), 2.4 (s, 3 H, NCH$_3$), 2.71 (m, 3 H, CH$_2$CH$_3$), 3.79 (s, 3 H, OCH$_3$), 5.06 (b s, 2 H, NH and OH), 6.71 (m, 3 H, aromatic); CI/MS (TFA derivative), m/z 388 (MH$^+$, base peak).

4-Hydroxy-3-methoxyamphetamine (IV). This amine was synthesized by reduction of 4-hydroxy-3-methoxy-β-nitrostyrene with lithium aluminium hydride
(LiAlH₄) by slight modification of the procedure reported elsewhere (Morgan and Beckett, 1975). The modifications were as follows: (1) The nitrostyrene was dissolved in a tetrahydrofuran/ether mixture (1:10), and the solution added to LiAlH₄ in a three-neck 500 mL round bottom flask. The excess LiAlH₄ was decomposed with a saturated solution of potassium sodium tartrate. After the pH of the resulting solution was adjusted to 9 with 4M HCl, the solution was filtered through Celite. The product was purified by silica gel column chromatography as described for compound III. Fractions containing the product were pooled, and the organic solvent was evaporated to yield a dark brown oil: ¹H NMR (CDCl₃) 1.14 (d, J = 6 Hz, 3 H, CCH₃), 2.55 (m, 2 H, CH₂), 3.14 (m, 1 H, CH), 3.71 (s, 3 H, OCH₃), 4.30 (b s, 3 H, NH₂ and OH), 6.71 (m, 3 H, aromatic); CI/MS (TFA derivative), m/z 374 (MH⁺, base peak).

(3,4-dihydroxyphenyl)acetone (IX). Synthesis of (3,4-dimethoxyphenyl)acetone was carried out according to the procedure reported elsewhere (Morgan and Beckett, 1975). The compound was purified by silica gel chromatography (60-200 mesh) and eluted with a mixture of toluene/dichloromethane (9:1). Removal of the organic solvent from pooled fractions containing the product left an amber color oil: CI/MS, m/z 195 (MH⁺, base peak).

The ketone was then demethylated with boron tribromide in dichloromethane, according to the previously reported method (McOmie et al., 1968). The reaction was quenched by addition of methanol (Musson et al., 1986); subsequent evaporation yielded a dark oil. Purification of (3,4-dihydroxyphenyl)acetone was by silica gel chromatography; the elution solvent was toluene/dichloromethane/ethyl acetate (2:5:1). Fractions containing the product were pooled and evaporated to give a light brown oil: CI/MS (TFA derivative), m/z 359 (MH⁺, base peak). The EI mass spectrum was consistent with that reported for the trifluoroacetyl derivative of the ketone IX (Midha et al., 1978).

3,4-Dihydroxymethamphetamine (V). The synthetic route for preparation of 3,4-dimethoxymethamphetamine was identical to that reported elsewhere (Morgan and Beckett,
1975). This amine was purified by silica gel column chromatography (60-200 mesh) and eluted with a solvent mixture of toluene/dichloromethane (9:1). Evaporation of the organic solvent gave an amber color oil: CI/MS (TFA derivative), m/z 306 (MH⁺, relative intensity = 4%). Demethylation of this amine was performed as previously described for compound IX. The dark oil obtained after evaporation was purified by flash chromatography and eluted with dichloromethane/methanol (3:1). The fractions containing the product were combined and evaporated to a light brown oil: ¹H NMR (DMSO-d₆) 1.09 (d, J = 6.4 Hz, 3 H, CCH₃), 2.53 (s, 3 H, NCH₃), 2.53 (m, 2 H, CH₂), 3.01 (m, 1 H, CH), 6.58 (m, 3 H, aromatic), 8.99 (b s, 3 H, NH and OH); CI/MS (TFA derivative), m/z 470 (MH⁺, base peak).

3-Hydroxy-4-methoxymethamphetamine (II). The procedures used for synthesis and purification of this amine were the same as that for compound III except that the starting material was 3-hydroxy-4-methoxybenzaldehyde. The product was purified by silica gel chromatography as described for compound III. The solvent was evaporated to a dark brown oil: ¹H NMR (DMSO-d₆) 0.92 (d, J = 6 Hz, 3 H, CCH₃), 2.29 (s, 3 H, NCH₃), 2.48 (m, 2 H, CH₂), 2.66 (m, 1 H, CH), 3.73 (s, 3 H, OCH₃), 5.50 (b s, 2 H, NH and OH), 6.65 (m, 3 H, aromatic); CI/MS (TFA derivative), m/z 388 (MH⁺, base peak).

6-Hydroxy-3,4-(methylenedioxy)methamphetamine (X). Demethylation of 2,4,5-trimethoxybenzaldehyde according to a previously reported procedure (McOmie et al., 1968) gave 2,4,5-trihydroxybenzaldehyde: GC-CI/MS (TFA derivative; methanol was the reagent gas), m/z 443 (MH⁺, base peak). Methylenation of 2,4,5-trimethoxybenzaldehyde using the method reported elsewhere (Clark et al., 1976) yielded 6-hydroxy-3,4-(methylenedioxy)benzaldehyde: GC-CI/MS (TFA derivative; acetone was the CI reagent gas), m/z 263 (MH⁺, base peak). Condensation of 6-hydroxy-3,4-(methylenedioxy)benzaldehyde with nitroethane (Gairaud and Lappin, 1953) gave the 6-hydroxy-3,4-(methylenedioxy)-ß-nitrostyrene: GC-CI/MS (TFA derivative; acetone was the CI reagent
gas), m/z 320 (MH\(^+\), base peak). Using the previously reported procedure (Morgan and Beckett, 1975), the nitrostyrene compound was then converted to [6-hydroxy-3,4-(methylenedioxy)phenyl]acetone: GC-CI/MS (TFA derivative; acetone was the CI reagent gas), m/z 291 (MH\(^+\), base peak). Reductive amination of [6-hydroxy-3,4-(methylenedioxy)phenyl]acetone by the method reported elsewhere (Morgan and Beckett, 1975) gave 6-hydroxy-3,4-(methylenedioxy)methamphetamine (6-OH-MDMA): GC-CI/MS (TFA derivative; methanol was the CI reagent gas), m/z 402 (MH\(^+\), base peak). Relevant ions, m/z (% relative abundance) in the EI mass spectrum of trifluoroacetyl derivative of 6-OH-MDMA: 402 (M\(^+\), 5), 275 (14), 274 (68), 178 (7), 155 (5), 154 (100), 147 (9), 121 (7), 79 (5), 69 (34), 63 (7), 57 (6), 56 (15), 53 (12), 51 (5), 42 (55).

**Development of the GC-CI/MS Analytical Method**

**Assay procedure.** The primary stock solutions of MDMA, MDA, HMM, HMA, and MDE were prepared by weighing out accurately and dissolving each compound in 10 ml of methanol; the concentration of each methanolic stock solution was then recorded. A working calibration solution containing the four compounds (MDMA, MDA, HMM, and HMA), at the concentration of 10 μg/ml each, was prepared from the primary stock solutions by appropriate dilution with deionized water. Additional calibration solutions, at concentrations of 1 μg/ml and 100 ng/ml, were prepared from the 10 μg/ml solution by serial dilution with deionized water. The working internal standard (MDE) solution of 10 μg/ml was prepared from the MDE primary stock solution.

Test tubes with Teflon-lined screw caps were washed and silanized according to a previously reported procedure (Lim et al., 1988a). After silylation, the test tubes were given a final rinse with methanol.

The complete rat brain was accurately weighed and then homogenized in two volumes of cold deionized water containing 1.6 mg ethylenediaminetetraacetic acid (EDTA). Homogenate equivalent to 1 g of whole brain tissue was weighed out and the
proteins were precipitated by addition of 3 ml of cold 0.4M perchloric acid which contained 0.05% (w/v) sodium metabisulfite. Following centrifugation at 1,133 x g for 15 min, the resultant supernatant was transferred to a 15-ml screw-capped test tube. MDE (200 ng) was added to the supernatant; the tube was capped, hand-vortexed for 30 sec and then allowed to equilibrate for 30 min. The pH of the supernatant was then adjusted to about 9 using 90 µl of 10M sodium hydroxide, 0.5 ml saturated sodium borate solution and finally saturated with sodium chloride. Extraction was carried out twice with 5 ml dichloromethane/n-butanol (3:1) by gentle rocking for 15 min, followed by centrifugation at 1,133 x g for 15 min. The pooled organic layer was then washed with 3 ml of 0.1M ammonium hydroxide for 10 min with gentle rocking. After separation of the two phases by centrifugation at 1,133 x g for 10 min, the aqueous layer was removed with a Pasteur pipet. The organic layer was subsequently dried over anhydrous sodium sulfate. Following centrifugation at 1,133 x g for 10 min, the dried organic layer was transferred to 10-ml screw-capped test tube. One percent concentrated hydrochloric acid in methanol (200 µl) was added to the tube and the extract evaporated under a gentle stream of air at 55 °C.

Derivatization was achieved by heating the residue in the tube with 100 µl ethyl acetate and 200 µl TFAA for 20 min at 80 °C. The tube was allowed to cool to room temperature and the solvent plus excess TFAA were removed by evaporation under a gentle stream of nitrogen at 40 °C. The residue was reconstituted in 50-100 µl of ethyl acetate containing 2% MBTFA and then heated for 10 min at 80 °C. When the tube had cooled to room temperature, an aliquot of 1 µl was injected into the GC-CI/MS.

** Calibration curves.** Calibration curves for MDMA, MDA, HMM, and HMA were constructed by analyzing aliquots of pooled rat brain supernatant, equivalent to 1 g of whole brain tissue, which had been spiked with the working calibration solutions to give concentrations over the range of 1-500 ng/g of brain tissue. The samples were then processed as described.
Recovery studies. Aliquots of rat brain supernatant, equivalent to 1 g whole brain tissue, were spiked with MDMA, MDA, HMM, and HMA to give concentrations of 5 and 250 ng/g of brain tissue. These samples were processed as described, except that 200 ng of MDE was added to the pooled organic extracts prior to the ammonium hydroxide wash as an external standard. In a parallel set of experiments, the same amounts of the four compounds (MDMA, MDA, HMM, and HMA) and MDE were spiked into the pooled organic extracts prior to the alkali wash. Each concentration was analyzed in quadruplicate. Recoveries were then calculated by comparison of the peak area ratios obtained from the extracted samples to those obtained when equal amounts of the compounds were derivatized without prior extraction.

Intraassay and interassay variability studies. Intraassay variation in the quantitation of MDMA, MDA, HMM, and HMA using this GC-CI/MS assay was determined by analyzing samples, in quadruplicate, at concentrations of 2 and 50 ng/g of brain tissue. Interassay variation in the quantitation of MDMA, MDA, HMM, and HMA was determined similarly except that the samples were analyzed on four separate days.

Applications of the GC-CI/MS Assay. The disposition in the rat brains of MDMA and its three metabolites (MDA, HMM, and HMA) was investigated following subcutaneous administration of 10 mg/kg of MDMA (calculated as free base, prepared in physiological saline solution) to male Sprague-Dawley rats (200-250 g) that had fasted overnight. The rats were decapitated at 0, 0.08, 0.25, 0.5, 0.75, 1, 2, 3, 6, 8, 10, 12, 16, 24, 36 and 48 hr after dosing. The intact brain was removed immediately, rinsed with cold physiological saline solution, patted dry with paper towels, wrapped in aluminium foil, and finally stored at -20 °C until analysis. Two brains were used for each time point. Three control rats had received the same volume of vehicle without drug.

The ability of these phenolic amine metabolites (HMM and HMA) to cross the blood-brain barrier in the rat was investigated by subcutaneous administration, individually, of equimolar amounts (51.8 µmol/kg, calculated as free base) of MDMA hydrochlo-
ride, HMM hydrochloride and HMA hydrochloride to male Sprague-Dawley rats (200-250 g) that had fasted overnight. The rats were decapitated at 3 hr after administration. Intact brains were immediately removed and processed as described above. Six brains were used for investigation of each compound. Three control rats had been injected subcutaneously with the same volume of vehicle without drug.

**Stereoselective Disposition**

**Chiral assay procedure.** Enzymatic hydrolysis of the 24-hr rat urine was carried out as described under the section on enzymatic hydrolysis. The procedure for extraction of MDMA and its N-demethylated metabolite, MDA, from hydrolyzed rat urine was similar to that described under the section on extraction and derivatization, except that addition of MDE was omitted. Enantiomers of MDMA and MDA were converted into their respective diastereomers by aqueous derivatization with the chiral reagent, N-heptafluorobutyryl-l-prolyl chloride (HPC), under Schotten-Baumann conditions as described elsewhere (Lim *et al.*, 1986). Finally the residue was reconstituted in 100 μl ethyl acetate, and an aliquot of 1 μl was analyzed by GC-CI/MS.

**Synthesis of chiral reagent, HPC.** The method for synthesizing and checking the optical purity of HPC was essentially the same as that reported elsewhere (Lim *et al.*, 1986).

**Animal studies.** After two male Sprague-Dawley rats (200-250 g) were administered 20 mg/kg MDMA (calculated as free base) by gavage, they were then placed in metabolic cages and urine was collected for 24 hr. Two control rats received the same volume of vehicle without drug.
CHAPTER 3

RESULTS

Rat Metabolism of MDMA

Identification of in vivo metabolites. The trifluoroacetylated extracts of hydrolyzed urine from dosed and control rats were analyzed by capillary column GC-MS under both EI and CI conditions. The CI total ion current chromatogram of hydrolyzed urine from one of the dosed rats (Figure 2) showed eight peaks that were not present in the CI total ion current chromatogram from hydrolyzed control urine (not shown). Full-scan CI mass spectra were obtained for all eight peaks. However, only compounds corresponding to peaks A, C, D, and E were present in sufficient quantity to permit the acquisition of interpretable full-scan EI mass spectra. The minor chromatographic peaks B, F, G, and H, which are barely detectable in Figure 2, were clearly evident in ion current chromatograms of selected ions (Figure 3). Tentative identification of the metabolites by interpretation of their mass spectra was confirmed by comparison of the mass spectra with the spectra obtained for synthetic reference compounds prepared as described under Materials and Methods.

The EI and CI mass spectra of peak A are shown in Figure 4. The mass spectra of peak A and its retention time relative to MDE were identical with those of the N-trifluoroacetyl derivative of MDMA (N-TFA-MDMA). The EI mass spectrum contains diagnostic fragment ions resulting from a McLafferty rearrangement (m/z 162) and cleavage β to the nitrogen (m/z 135 and 154). The molecular ion is weak, but detectable, as is the MH⁺ ion in the CI mass spectrum. The most abundant ion in the CI mass spectrum (m/z 163) is formed by loss of CH₃N=O(OH)CF₃ from the MH⁺ ion.
Figure 2. Total ion current chromatogram (positive ion chemical ionization) of a derivatized extract of urine collected from a rat dosed with 5 mg/kg MDMA (calculated as free base)
Figure 3. Positive ion current profiles of MH\(^+\) ions corresponding to the minor chromatographic peaks B (m/z 388), F (m/z 277), G (m/z 179), and H (m/z 359).
Figure 4. EI (top) and CI (bottom) mass spectra of the trifluoroacetyl derivative of MDMA (peak A in Figure 2).
Because the major fragmentation pathways observed in the mass spectrum of N-TFA-MDMA are evident in the mass spectrum of each of the nitrogen-containing metabolites of MDMA, a tentative structural identification is possible by interpretation of the mass spectra for each of these metabolites. For example, Figure 5 shows the EI and CI mass spectra of chromatographic peak C. The most abundant ion in the CI mass spectrum, at m/z 388, was presumed to be the MH⁺ ion. A molecular mass of 387 daltons indicated that the metabolite had an odd number of nitrogen atoms. Retention of the intact N-methyl-N-trifluoroacetamide group was further supported by the presence of an abundant ion at m/z 261 [MH⁺ - CH₃N=C(OH)CF₃] in the metabolite's CI mass spectrum corresponding to the same major fragmentation pathway observed in the CI mass spectrum of N-TFA-MDMA. The absence of structural change in the metabolite's side chain is further indicated by the abundant fragment ion at m/z 154 in the EI mass spectrum. Another EI fragment ion that will corroborate the presence of the N-methyl-N-trifluoroacetamide group is m/z 110, formed by skeletal rearrangement and loss of 44 daltons from m/z 154; that this fragment is derived from m/z 154 was shown by collision-activated dissociation of m/z 154 from N-TFA-MDMA using the ITMS (data not shown). This skeletal rearrangement and loss of 44 daltons requires the presence of a methyl group on the amine function since this fragmentation pathway is absent in all the EI mass spectra of primary amine metabolites of MDMA. Therefore, biotransformation must have occurred on the 3,4-methylenedioxy-substituted phenyl ring of the metabolite. The fragment ions at m/z 162 and 135 in the EI mass spectrum of N-TFA-MDMA are shifted to m/z 260 and 233 (98 daltons) in the EI mass spectrum of the metabolite. A mass shift of 98 daltons is consistent with the trifluoroacylation of a phenolic functional group formed by O-dealkylation of the (3,4-methylenedioxy)phenyl group and subsequent O-methylation of one of the phenolic groups. Tentative identification of the metabolite as 4-hydroxy-3-methoxymethamphetamine (III) was given further support by a report that the catechol O-methyltransferase that catalyzes the methylation reaction exhibits regioselectivity for the m-hydroxyl group (Mesnil et al.,
Figure 5. EI (top) and CI (bottom) mass spectra of the trifluoroacetyl derivative of MDMA metabolite III (peak C in Figure 2).
This structural assignment was subsequently shown to be correct by comparison of the mass spectra (EI and CI) and relative retention time of this metabolite with those of the TFA derivative of synthesized 4-hydroxy-3-methoxymethamphetamine.

The EI mass spectrum of the TFA derivative of metabolite III shows no molecular ion but a very weak protonated molecule (m/z 388), which is due to self-chemical ionization. Self-chemical ionization has been observed during acquisition of EI mass spectra with the ion trap detector when analyte concentrations are high (Eichelberger et al., 1987).

The EI mass spectrum of chromatographic peak D (Figure 6) shows the same m/z 260 and 233 fragment ions previously discussed in regard to the EI mass spectrum of chromatographic peak C, indicating that these two metabolites have the same phenyl ring substitution. However, as the CI mass spectrum of chromatographic peak D indicates a molecular weight of 373, this metabolite appears to have one less methyl group than the metabolite corresponding to chromatographic peak C. Also, chromatographic peak D gave an EI fragment ion at m/z 140 [CH3CH=NHCOCF3+] and a CI fragment ion at m/z 261 [MH+ - HN=C(OH)CF3], a clear indication that it had lost the methyl group attached to the nitrogen in MDMA. Confirmation that chromatographic peak D corresponded to the TFA derivative of 4-hydroxy-3-methoxyamphetamine (IV) was based on the similarity of its mass spectra (EI and CI) and its relative retention time to those of the TFA derivative of synthesized 4-hydroxy-3-methoxyamphetamine.

Chromatographic peak E was readily identified as the TFA derivative of MDA (VI). This metabolite's EI and CI mass spectra (Figure 7) show a molecular weight of 275 which suggests that it has one methyl group less than N-TFA-MDMA. The presence of an EI fragment ion at m/z 140 and of a CI fragment ion at m/z 261 [MH+ - HN=C(OH)-CF3] indicates that the methyl group has been lost from the nitrogen of MDMA. The two most abundant EI fragment ions (m/z 162 and 135) indicate that the 3,4-(methylenedioxy)phenyl group remains unaltered in this metabolite. Conclusive identification of the metabolite as
Figure 6. EI (top) and CI (bottom) mass spectra of the trifluoroacetyl derivative of MDMA metabolite IV (peak D in Figure 2).
Figure 7. EI (top) and CI (bottom) mass spectra of the trifluoroacetyl derivative of MDMA metabolite VI (peak E in Figure 2).
MDA was achieved by comparison of the EI and CI mass spectra of its TFA derivative and its relative retention time with those of a TFA derivative of authentic MDA.

CI mass spectra of uniformly high quality were obtained for the remaining MDMA metabolites identified in hydrolyzed rat urine, and they provided the basis for tentative structural identifications. For example, the methanol CI mass spectrum of the very small chromatographic peak B in Figures 2 and 3 is shown in Figure 8. This spectrum is nearly identical with the CI mass spectrum obtained for the TFA derivative of the metabolite identified as 4-hydroxy-3-methoxymethamphetamine (III, chromatographic peak C). We therefore concluded that it was isomeric with III and most likely corresponded to 3-hydroxy-4-methoxy-methamphetamine (II). This conclusion was verified by comparison of the metabolite's relative retention time and its CI mass spectrum with those of synthesized 3-hydroxy-4-methoxymethamphetamine.

The CI mass spectra of chromatographic peaks F, G, and H are each characterized by a single prominent ion corresponding to the MH+ ion of the metabolite or its TFA derivative (Figure 9). Because the molecular weights of these metabolites are even numbers, they must have lost their amine functional groups. Since oxidative deamination is a well-established metabolic process, we anticipated that these MDMA metabolites might be ring-substituted phenylacetones. Initial identification of chromatographic peak F as (4-hydroxy-3-methoxyphenyl)acetone (VII), peak G as [3,4-(methylenedioxy)phenyl]acetone (VIII), and peak H as (3,4-dihydroxyphenyl)acetone (IX) on the basis of their CI mass spectra, was confirmed by comparison of their respective relative retention times and their CI mass spectra with the retention times and CI mass spectra of the corresponding synthesized compounds (Table 1). The methanol CI mass spectra of the TFA derivatives of each of the MDMA metabolites exhibit prominent MH+ ions. The nitrogen-containing metabolites also show a prominent CI fragment ion corresponding to loss of the McLafferty rearrangement product from the MH+ ions. However, the MH+ ions are the only abundant ions in the methanol CI mass spectra of the oxidatively deaminated MDMA metabolites.
Figure 8. CI mass spectrum of the trifluoroacetyl derivative of MDMA metabolite II (peak B in Figures 2 and 3).
Figure 9. CI mass spectra of the trifluoroacetyl derivative of MDMA metabolites VII (top), VIII (middle) and IX (bottom) (peaks F, G and H, respectively, in Figures 2 and 3).
Table 1. Gas chromatographic relative retention times and CI-MS data for trifluoroacetyl derivatives of MDMA and its metabolites

<table>
<thead>
<tr>
<th>Drug and metabolites</th>
<th>Retention time relative to MDE</th>
<th>Prominent CI ions, m/z (% of total ion current)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDMA (I)</td>
<td>0.947</td>
<td>163 (100), 290 (MH⁺, 9)</td>
</tr>
<tr>
<td>3-Hydroxy-4-methoxymethamphetamine (II)</td>
<td>0.933</td>
<td>261 (25), 388 (MH⁺, 100)</td>
</tr>
<tr>
<td>4-Hydroxy-3-methoxymethamphetamine (III)</td>
<td>0.918</td>
<td>261 (13), 388 (MH⁺, 100)</td>
</tr>
<tr>
<td>4-Hydroxy-3-methoxyamphetamine (IV)</td>
<td>0.825</td>
<td>261 (8), 374 (MH⁺, 100)</td>
</tr>
<tr>
<td>3,4-Dihydroxymethamphetamine (V)</td>
<td>0.824</td>
<td>343 (3), 470 (MH⁺, 100)</td>
</tr>
<tr>
<td>3,4-(Methylenedioxy)amphetamine (VI)</td>
<td>0.813</td>
<td>163 (100), 276 (MH⁺, 90)</td>
</tr>
<tr>
<td>(4-Hydroxy-3-methoxyphenyl)-acetone (VII)</td>
<td>0.629</td>
<td>277 (MH⁺, 100)</td>
</tr>
<tr>
<td>[3,4-(Methylenedioxy)phenyl]-acetone (VIII)</td>
<td>0.602</td>
<td>179 (MH⁺, 100)</td>
</tr>
<tr>
<td>(3,4-Dihydroxyphenyl)-acetone (IX)</td>
<td>0.552</td>
<td>359 (MH⁺, 100)</td>
</tr>
</tbody>
</table>
The methanol CI mass spectrum of the TFA derivative of a particularly interesting metabolite of MDMA is shown in Figure 10. The most abundant ion in the CI mass spectrum, at m/z 402, was judged to be the protonated molecule. The odd molecular mass of 401 suggests that the metabolite has an odd number of nitrogen atoms, that is, the metabolite retains the amine functionality. Absence of structural change to the N-methyl-N-trifluoroacetamide moiety was further supported by the presence of an abundant ion at m/z 275 \([\text{MH}^+ - \text{CH}_3\text{N} = \text{C(OH)CF}_3]\) in the CI mass spectrum; this fragmentation pathway appeared to correspond to the major fragmentation pathway present in the CI mass spectrum of N-TFA-MDMA. This deduction was further corroborated by the base peak at m/z 154 in the EI mass spectrum (Figure 10) that was generated from pooled extracts of several rat brains. The fragment ion at m/z 162 in the EI mass spectrum of N-TFA-MDMA is shifted to m/z 274 (112 daltons) in the EI mass spectrum of the metabolite. A mass shift of 112 daltons is consistent with the trifluoroacetylation of a hydroxyl functional group, either formed via aromatic hydroxylation or \(\beta\)-hydroxylation.

The latter process is unlikely, since no fragment ion corresponding to the loss of trifluoroacetic acid was observed in the metabolite's CI mass spectrum. Loss of trifluoroacetic acid is a major fragmentation pathway in the CI mass spectra of compounds with \(\beta\)-hydroxyl functionality (that is, the TFA derivative of pseudoephedrine) (Lim et al., 1988b). Therefore, hydroxylation must have occurred on the 3,4-methylenedioxy-substituted phenyl ring. There are three potential locations for aromatic hydroxylation: positions 2, 5, or 6. Preferential aromatic hydroxylation \textit{para} to the methoxy substituent has been observed in the metabolism of methoxyphenamine in rat (McKay et al., 1983). On the basis of the similarity of the 3,4-methylenedioxy moiety to the methoxy substituent of methoxyphenamine, it seemed likely that aromatic hydroxylation of MDMA occurred at position 6. Hence, the metabolite was tentatively assigned the structure of 6-OH-MDMA.

The EI mass spectrum of the TFA derivative of the new metabolite was identical to that of the TFA derivative of synthetic 6-OH-MDMA. However, this did not constitute
Figure 10. EI (top) and CI (bottom) mass spectra of the trifluoroacetyl derivative of MDMA metabolite X, identified as 6-OH-MDMA.
proof of the chemical structure, since regioisomers generally cannot be readily distinguished on the basis of their EI mass spectra. Consequently, we based the confirmation of this metabolite's identity as 6-OH-MDMA on its retention time relative to HME and co-injection with a synthetic standard of 6-OH-MDMA, as shown in Figure 11. The positive daughter ion current profile from analysis of rat brain tissues clearly shows the presence of a peak, in the daughter ion mass chromatogram at m/z 275, that eluted at a relative retention time (1.038) identical to that of synthetic 6-OH-MDMA. This identification was further supported by the co-elution of synthetic 6-OH-MDMA with the metabolite that resulted in enhancement of peak intensity when the synthetic reference compound was co-injected with the rat brain extracts containing the metabolite (Figure 11).

Identification of in vitro metabolites. An additional metabolite of MDMA was detected in extracts of the 10,000 × g rat brain and liver incubates. The CI mass spectrum of the metabolite's TFA derivative contained a single prominent ion (m/z 470) that was assumed to be the MH$^+$ ion (Figure 12). The derivative's molecular weight of 469 and the EI fragment ion at m/z 154 [CH$_3$CH=N+(CH$_3$)COCF$_3$] indicate that the metabolite's side chain remains unaltered from that of MDMA. That biotransformation must have occurred on the 3,4-methylenedioxy-substituted phenyl ring of this metabolite is supported by the structurally diagnostic EI fragment ions at m/z 315 and 342, which are shifted 180 daltons from the fragment ions in the spectrum of N-TFA-MDMA at m/z 135 and 162, respectively (Figure 4). A mass shift of 180 daltons can be accounted for by trifluoroacetylation of two phenolic functionalities formed by O-dealkylation of the 3,4-(methylenedioxy)phenyl group. The structure of this metabolite, tentatively identified as 3,4-dihydroxymethamphetamine (V), was confirmed by agreement of the relative retention time, and the EI and CI mass spectra of the TFA derivative of the metabolite with those of the TFA derivative of synthesized 3,4-dihydroxymethamphetamine.

In addition to 3,4-dihydroxymethamphetamine, we identified 4-hydroxy-3-methoxymethamphetamine, MDA, and [3,4-(methylenedioxy)phenyl]acetone in the
Figure 11. Positive daughter ion current profiles of HME (m/z 261) and 6-OH-MDMA (m/z 275) from analysis of rat brain tissues at 6 hr after an oral dose of 20 mg/kg MDMA (top) and analysis of the same sample with coinjection of synthetic 6-OH-MDMA (bottom).
Figure 12. EI (top) and CI (bottom) mass spectra of the trifluoroacetyl derivative of MDMA metabolite V, identified as 3,4-dihydroxymethamphetamine.
10,000 x g liver incubate (data not shown). The identification of metabolites, other than 3,4-dihydroxymethamphetamine, in extracts of the rat brain incubate was based solely on comparison of the relative retention times of the metabolites with those of synthesized compounds. We were unable to obtain high-quality CI or EI mass spectra for these metabolites because of their very low concentrations and because interfering substances were present in the biological matrix. We detected these metabolites by displaying, in a similar manner as in Figure 3, profiles of ion currents at m/z values corresponding to the MH\(^+\) ions of the TFA derivatives of each of the metabolites (data not shown). As a result, 4-hydroxy-3-methoxymethamphetamine, 4-hydroxy-3-methoxyamphetamine, and MDA were identified in the 10,000 x g brain incubate.

**Human Metabolism of MDMA**

**Identification of in vivo metabolites.** The total ion chromatogram obtained from the trifluoroacetylated extract of the human urine specimen previously described in Chapter 2 is shown in Figure 13; the locations of the peaks corresponding to metabolites of MDMA are indicated by arrows. Each of these peaks is clearly evident in the reconstructed ion current profile for the corresponding MH\(^+\) ion (Figure 14). Good quality EI and CI mass spectra were obtained for MDMA (I), 3-hydroxy-4-methoxymethamphetamine (II), 4-hydroxy-3-methoxymethamphetamine (III), 4-hydroxy-3-methoxyamphetamine (IV), 3,4-dihydroxy-methamphetamine (V), and [3,4-(methylenedioxy)phenyl]acetone (VII). Because both the EI and the CI mass spectra obtained for (4-hydroxy-3-methoxyphenyl)acetone (VII) contained interfering ions due to coeluting compounds, its identification was based primarily on its retention time relative to the internal standard, MDE. The mass spectra of the other metabolites are in good agreement with those of the synthetic metabolites and the relative retention times of the metabolites were identical with those of reference standards run after the sample.
Figure 13. Total ion chromatogram (positive ion chemical ionization, reagent gas is methanol) of a trifluoroacetylated extract of human urine.
Figure 14. Positive ion current profiles of MH⁺ ions of the TFA derivatives of each of the MDMA metabolites identified in the human urine.
Derivatization of MDMA and three of its metabolites (MDA, HMM and HMA) was carried out for two purposes: conversion of these compounds into less polar derivatives improved their chromatographic properties, and the shift in the molecular weights of these compounds to higher masses conferred additional selectivity to the assay by reducing the likelihood of interference from the biological matrices.

Various derivatives for MDMA and its metabolites were investigated, namely the perfluoroacyl (TFA, pentafluoropropionyl [PFP], heptafluorobutyryl [HFB], and pentafluorobenzoyl [PFB]) and trimethylsilyl [TMS] derivatives. The many peaks obtained when the TMS derivatives of MDMA and metabolites were analyzed by GC-MS rendered this class of derivatives unsuitable for trace analysis of these compounds. This result was expected since all ions formed are trapped and scanned out of the analyzer when the ion trap is operated in the RF-only mode. The bis-PFB derivatives of the phenolic amine metabolites eluted from the capillary column at around 300 °C; at this temperature, there was considerable column bleed which drastically reduced the ionization and reaction times under the ARC mode. Consequently, quantitation of these metabolites at low ng/g concentrations was not feasible and utilization of this PFB derivative for development of the assay was abandoned. In general, the aliphatic perfluoroacyl (TFA, PFP and HFB) derivatives of MDMA and metabolites were low-boiling compounds; all eluted at below 240 °C. However, better chromatographic resolution was obtained with the TFA derivative as compared to either PFP or HFB derivatives, so the TFA derivative was selected for the development of the assay.

Considerable fragmentation was obtained with the TFA derivatives of MDMA, MDA and MDE (Table 1). In each case, the base peak (m/z 163) was due to the loss of a neutral fragment formed by McLafferty rearrangement of the MH⁺ ion. In comparison, the PFB derivatives of the amine compounds show little fragmentation (Lim et al., 1988b).

Since monitoring the MH⁺ ion normally provides the best specificity, the effect of various
reagent gases on the intensity of the MH\(^+\) ion of the TFA derivatives was investigated. Substantial fragmentation occurred even when a mild CI reagent gas like isobutane was used. Also, greater fragmentation of the MH\(^+\) ion was observed with longer carbon chain of the N-alkyl substituent. However, the MH\(^+\) ion became the base peak for all compounds when isobutane was used in combination with lower manifold temperature (< 140 °C, Figure 15). The TFA derivatives of HMM and HMA were relatively unaffected by manifold temperature (data not shown). Therefore, a manifold temperature of 120 °C was selected for subsequent analysis in order to minimize fragmentation of MH\(^+\) ions, while providing narrow chromatographic peaks.

The choice of the internal standard is critical to the successful development of any quantitative assay using ion trap mass spectrometers (ITD and ITMS). The addition of a high concentration of coeluting deuterated internal standard reduces the ionization time, thereby decreasing the number of analyte ions formed (Strife et al., 1989). The consequence is a higher limit of detection and quantitation. The problem can be overcome by using a homolog, which is chromatographically separable from the analyte, as internal standard. The internal standard employed in this assay (MDE, the ethyl homolog of MDA) fulfilled the above criterion.

The extraction scheme did not incorporate enzymatic hydrolysis, since hydrolysis with B-glucuronidase made only a slight difference in recoveries of phenolic amine metabolites (HMM and HMA), and none in the case of MDMA and MDA. The recoveries of MDMA and metabolites were investigated with many solvent systems; efficient recoveries for all compounds, including the amphoteric metabolites (HMM and HMA), were only achieved using a highly polar solvent mixture like dichloromethane : isopropanol (3:1). However, the high recoveries of these compounds with this solvent system were accompanied by coelution of interfering peaks from biological matrices, with the result that MDMA and its metabolites could not be measured reliably at concentrations below 5 ng/g. However, a lower limit of quantitation for MDMA and metabolites was achieved with a
Figure 15. Intensities of MH⁺ ions of the TFA derivatives of MDMA (m/z 290), MDA (m/z 276), and MDE (m/z 304) as a function of the manifold temperature. The CI reagent gas was isobutane.
solvent mixture of dichloromethane : n-butanol (3:1) without a substantial reduction in recoveries. Recovery data for MDMA, MDA, HMM and HMA from fortified brain tissues are shown in Table 2. The mean recoveries of MDMA, MDA, HMM and HMA over the concentration range examined (5 and 250 ng/g) were 84, 86, 87, and 83%, respectively. In each case, the coefficient of variation (C.V.) was around 12%.

Calibration curves for MDMA, MDA, HMM, and HMA were linear from 1 to 500 ng/g wet brain tissue (Figures 16 and 17). Correlation coefficients greater than 0.99 were obtained for all compounds.

Table 3 shows the precision and accuracy of the assay for quantitation of MDMA and its three metabolites in fortified brain tissues. The measured concentrations of MDMA, MDA, HMM, and HMA in fortified brain tissues were within 10% of the target concentrations. At each concentration, the intra- and interassay coefficients of variation obtained for MDMA, MDA, HMM and HMA were about 10%. The lowest concentration of MDMA, MDA, HMM, and HMA that could be reliably measured by this assay was 1 ng/g wet brain tissue.

Application of the Assay

The analysis of a rat brain tissue from a pilot disposition study, at 3 hr after a subcutaneous dose of 10 mg/kg MDMA (calculated as free base), is shown in Figure 18. With the exception of MDA and HMA, baseline resolution was observed for all of the compounds. No endogenous materials coeluted with the analytes in control rat brain tissue (data not shown). The brain concentration-time profiles for MDMA and its three metabolites (MDA, HMM and HMA) following a subcutaneous dose of 10 mg/kg MDMA (calculated as free base) are displayed in Figure 19. MDMA reached a peak concentration of 9715 ng/g wet brain tissue within 1 hr; however, there was a delay in the time to peak concentration for the metabolites; the peak concentrations of MDA (947 ng/g), HMM (9.3 ng/g) and HMA (2.8) occurred at 3, 2 and 2 hr, respectively. The brain profile of HMM
Table 2. Extraction recoveries of MDMA and its metabolites from fortified rat brain tissues

<table>
<thead>
<tr>
<th>Amount of MDMA and metabolites added to rat brain tissues, ng/g</th>
<th>% mean recovery from fortified rat brain tissues (% C.V.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MDMA</td>
</tr>
<tr>
<td>5.0</td>
<td>73 (10.5)</td>
</tr>
<tr>
<td>250.0</td>
<td>94 (4.5)</td>
</tr>
</tbody>
</table>

C.V. = coefficient of variation; n = 4
Figure 16. Calibration curves for quantitation of MDMA (top) and MDA (bottom) in rat brain tissues.
Figure 17. Calibration curves for quantitation of HMM (top) and HMA (bottom) in rat brain tissues.
Table 3. Precision and accuracy for determination of MDMA and its metabolites in fortified rat brain tissues

<table>
<thead>
<tr>
<th>Target concentration (ng/g wet brain tissue)</th>
<th>Measured concentration, ng/g wet brain tissue (% C.V.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MDMA</td>
</tr>
<tr>
<td></td>
<td>Intraassay variation (n = 4)</td>
</tr>
<tr>
<td>2.0</td>
<td>2.2 (11.8)</td>
</tr>
<tr>
<td>50.0</td>
<td>49.5 (10.3)</td>
</tr>
<tr>
<td></td>
<td>Interassay variation (n = 4)</td>
</tr>
<tr>
<td>2.0</td>
<td>2.0 (8.6)</td>
</tr>
<tr>
<td>50.0</td>
<td>50.1 (7.6)</td>
</tr>
</tbody>
</table>

C.V. = coefficient of variation
Figure 18. Ion current profiles of MH⁺ ions of MDMA and its metabolites from analysis of brain tissue at 3 hr after a subcutaneous dose of 10 mg/kg MDMA (calculated as free base).
Figure 19. Rat brain concentration-time profiles of MDMA and its metabolites after a subcutaneous dose of 10 mg/kg MDMA (calculated as free base). Each point is an average of two analyses.
roughly follows that of MDMA. In comparison, the profile of HMA is very similar to that of MDA; however, the MDA profile shows slow elimination from the brain from 3 to 6 hr but the profile from 12 to 24 hr follows that of MDMA. In general, the brain concentrations of the two phenolic amine metabolites, HMM, and HMA, never exceeded 10 ng/g and the concentrations dropped below the limit of quantitation within 10 hr after administration of MDMA. In contrast, the brain concentrations of MDMA and MDA ranged from microgram to low-nanogram per gram wet brain tissue. The initial accumulation of high concentrations of MDMA and MDA in the brain, and slow elimination of both compounds, probably account for their brain profiles being observable for up to 24 hr.

Since both phenolic amine metabolites (HMM and HMA) were present in the brain following a subcutaneous dose of MDMA, rats were administered an equimolar amount of each of the metabolites to ascertain the ability of these metabolites to pass the blood-brain barrier. Data are presented in Table 4. The brain concentration of HMM from a subcutaneous dose of HMM is 28 times that resulting from administration of an equimolar amount of MDMA. In comparison, a 96-fold difference was observed for HMA using similar experimental protocols.

Stereoselective Disposition

The optical purity of the synthesized chiral reagent, HPC, was determined by derivatization of racemic alpha-methylbenzylamine and its optically pure S-(-) enantiomer. Following derivatization of racemic alpha-methylbenzylamine with HPC, the proportion of R-(+) and S-(-) enantiomers was found to be 49.7 : 50.3 which is very close to the expected value of 50 : 50 for a racemic mixture. The experimentally determined optical purity for the S-(-) enantiomer of 97% differs by 1% from the reported purity of 98%. On the basis of these data, the optical purity of HPC was judged to be ≥99%. Furthermore, because only one major peak was obtained, respectively, when optically pure enantiomers of MDMA and MDA were converted to their respective diastereomers, apparently no
### Table 4. Rat whole brain concentrations of HMM and HMA

<table>
<thead>
<tr>
<th>Compound administered</th>
<th>Metabolite (nmol/g of wet brain tissue), Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDMA</td>
<td>HMM: 0.033 ± 0.008</td>
</tr>
<tr>
<td>HMM</td>
<td>HMM: 0.933 ± 0.089</td>
</tr>
<tr>
<td>HMA</td>
<td>HMM: 1.346 ± 0.388</td>
</tr>
</tbody>
</table>

Rats were administered an equimolar amount of each compound, 51.8 μmol/kg (free base), by s.c. route and decapitated at 3 hr after dosing. Whole rat brains were removed and analyzed. (n = six rats for each compound, D = detected but below limit of quantitation).
inversion of configuration occurred during derivatization or during chromatography (data not shown).

Table 5 shows the retention times and resolution factors for the diastereomeric pairs of N-heptafluorobutyryl-l-prolyl derivatives of MDMA and MDA. Baseline resolution is obtained when the resolution factor is greater than or equal to 1.5. Hence, there was baseline resolution of the diastereomeric pairs of MDMA and MDA since the resolution factors were very much greater than 1.5. Greater chromatographic resolution was observed for N-HP-MDA than for N-HP-MDMA. In each case, the earlier eluting peak had the R-(−) configuration.

The ion current profiles corresponding to the diastereomeric pairs of MDMA and MDA in an extract of hydrolyzed 24 hr rat urine is shown in Figure 20. However, the diastereomeric pairs of the phenolic amine metabolites (N-HP-HMM [MH+ = 782] and N-HP-HMA [MH+ = 768]) were not detected, since the molecular weights of these derivatives exceed the mass range of the ITD. No interfering peaks from the biological matrices eluted at the same retention times as N-HP-MDMA or N-HP-MDA (data not shown).

The ion current profiles clearly show distortion in the enantiomeric ratio of MDMA and MDA in hydrolyzed 24 hr urine. The mean enantiomeric ratios (R/S) of N-HP-MDMA and N-HP-MDA in hydrolyzed 24 h urine were 1.661 and 0.660, respectively, considerably different from values of 1.039 and 0.995 that would correspond to racemic N-HP-MDMA and N-HP-MDA, respectively (Table 6). The enantiomeric ratio of N-HP-MDMA was more distorted than that of N-HP-MDA. Also, the configurations of the major enantiomers of MDMA and MDA excreted in the urine were reversed; S-(+)-MDA and the R-(−) MDMA were the major enantiomers excreted in the urine.
Table 5. GC retention times and resolution factors of N-heptafluorobutyryl-l-prolyl derivatives of MDMA (N-HP-MDMA) and MDA (N-HP-MDA).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Retention time (min)</th>
<th>Resolution factor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R(-)</td>
<td>S(+/-)</td>
</tr>
<tr>
<td>N-HP-MDMA</td>
<td>14.98</td>
<td>15.13</td>
</tr>
<tr>
<td>N-HP-MDA</td>
<td>13.92</td>
<td>14.27</td>
</tr>
</tbody>
</table>

Resolution factor \( = \frac{[T(S{-}) - T(R{-})] + [(W(S{+}) + W(R{-}))]}{2} \); \( T \) is the retention time and \( W \) is the peak base width (Lee et al., 1984).

Figure 20. Ion current profiles corresponding to the MH\(^{+}\) ions of N-heptafluorobutyryl-l-prolyl derivatives of MDA (top) and MDMA (bottom) in an extract of hydrolyzed 24 hr rat urine.
Table 6. Enantiomeric composition of MDMA and MDA in hydrolyzed 24 hr rat urine.

<table>
<thead>
<tr>
<th>Sample</th>
<th>MDMA</th>
<th>MDA</th>
<th>Ratio (R/S)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(RS)-Standards</td>
<td>45434</td>
<td>43711</td>
<td>50813</td>
</tr>
<tr>
<td>Rat urine, 24 hr:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rat # 1</td>
<td>12684</td>
<td>7585</td>
<td>2182</td>
</tr>
<tr>
<td>Rat # 2</td>
<td>34484</td>
<td>20902</td>
<td>4497</td>
</tr>
</tbody>
</table>
CHAPTER 4

DISCUSSION

The mass spectrometric analyses described in this thesis were performed on either an ITD or an ITMS. These mass spectrometers were used because of their high sensitivity; interpretable full-scan EI and CI mass spectra could be obtained on as little as a few picograms of N-pentafluorobenzoyl derivatives of MDMA with the ITD (Lim et al., 1987, 1988b,c). Also, high CAD efficiency (about 50%) was achieved for MDMA and its metabolites with the ion trap tandem mass spectrometer (data not shown). Consequently, less than 5 ml each of the biological specimens was routinely used for the identification of MDMA metabolites. This degree of sensitivity and selectivity has proven invaluable in the investigation of brain metabolism and disposition of MDMA, since the drug-metabolizing enzymes are present in extremely low quantities in the rat brain (Sasame et al., 1977; Naslund et al., 1988).

Metabolism of MDMA in Rat

Identification of metabolites. The GC-MS is an invaluable tool for metabolic studies. The capillary column not only resolves the metabolites efficiently from endogenous materials, but also concentrates the metabolites to facilitate their detection by MS. The ion trap mass spectrometer is particularly suitable for detecting trace amounts of metabolites; a good signal-to-noise ratio can be easily obtained because it is an ion trapping device where an optimum population of ions can be achieved for EI by using longer ionization time and for CI by longer ionization and reaction of the reagent gas with the analytes. All MDMA
metabolites discussed here have been identified unambiguously through comparison of their mass spectra and relative retention times with those of synthetic reference metabolites.

At the beginning of this study of MDMA metabolism in rats, only two metabolites (MDA and N-hydroxy-MDA) had been reported in the literature. Since then, seven new metabolites of MDMA have been identified in rats (Lim and Foltz, 1988). The *in vivo* and *in vitro* investigation of metabolism of MDMA reported in Chapter 3 identified the following metabolites in rats: 3-hydroxy-4-methoxymethamphetamine, 4-hydroxy-3-methoxymethamphetamine, 3,4-dihydroxymethamphetamine, 4-hydroxy-3-methoxyamphetamine, 3,4-(methylenedioxy)amphetamine, (4-hydroxy-3-methoxyphenyl)acetone, \([3,4-(methylenedioxy)phenyl]acetone\) and \((3,4-dihydroxyphenyl)acetone\). Recently, 4-hydroxy-3-methoxymethamphetamine, 3-hydroxy-4-methoxymethamphetamine and 4-hydroxy-3-methoxyamphetamine have also been reported as metabolites of MDMA in rats (Narasimhachari *et al.*, 1989). To account for the persistence of unchanged MDMA in rats, MDMA was speculated to be resistant to ring hydroxylation but no experimental data were provided to support this hypothesis (Logan *et al.*, 1988).

Some reports in the literature suggest that catecholamines may undergo aromatic hydroxylation at position six to produce neurotoxic "6-hydroxylated" metabolites such as 6-hydroxydopamine (6-OH-DA) (Kostrzewa and Jacobowitz, 1974; Seiden and Vosmer, 1984). Consequently, there has been considerable interest in identifying a "6-hydroxylated metabolite" of MDMA in an effort to better understand the mechanism of MDMA-induced neurotoxicity. An initial attempt to detect a 6-hydroxylated metabolite was not successful, probably because the concentration of this metabolite in the brain was too low. Only when extracts from several brains were pooled was it possible to acquire reasonably good quality EI and CI mass spectra for interpretation (Figure 10). Another explanation for the failure in the initial attempt to identify this metabolite was the instability of the TFA derivative of this compound. This instability was confirmed by the observation that the peak area was enlarged following rederivatization in 5% MBTFA and immediate analysis without prior
evaporation. Furthermore, the phenolic ester bond of this metabolite was more susceptible to hydrolysis than that of HMM or HMA.

Despite the fact that both EI and CI mass spectra were obtained, the peak corresponding to the 6-hydroxylated metabolite was not readily discernible in the normalized ion current profile due to the presence of many larger peaks from endogenous materials present in brain tissues. However, only a major peak at relative retention time of 1.038 was observed when the same sample was analyzed by capillary GC-ion trap tandem mass spectrometer operated in the CI-RF/DC-MS/MS mode. This was expected since tandem mass spectrometry has now been established as an invaluable analytical tool in the identification of trace quantities of metabolites in complex biological matrices (Straub, 1986; Johnson et al., 1986). That this peak was conclusively identified as 6-OH-MDMA by coinjection with synthetic reference metabolite and subsequent analysis by GC-MS/MS (Figure 11). This technique of coinjection with the authentic reference compound and analysis by GC-MS/MS has recently been used successfully to identify leukotriene B4 at low pg/ml concentrations (Hughes et al., 1988).

Unlike the tandem analyzer mass spectrometers (triple quadrupoles for example), the ITMS is normally capable of very high collision-activated dissociation efficiency of 30 to 100% (Simms and Strife, 1989; Strife et al., 1989). Collision-activated dissociation efficiencies of 47 and 46% were obtained for the TFA derivatives of 6-OH-MDMA and HME, respectively. That the ITMS is capable of high selectivity and sensitivity is shown by its ability to detect two minor peaks, in addition to 6-OH-MDMA, at relative retention times of 1.003 and 1.138 in the positive daughter ion current profile from rat liver (Figure 21). Each of the daughter ion mass spectra of these two minor peaks is identical to the daughter ion mass spectrum of authentic 6-OH-MDMA; therefore, they probably correspond to other aromatic hydroxylated metabolites of MDMA, such as 2-hydroxy-MDMA and 5-hydroxy-MDMA (Figure 22). This observation further corroborates the assignment of the structure of 6-OH-MDMA to the major peak at the relative retention time
Figure 21. Positive daughter ion current profiles (402 --> 275) from analysis of rat liver tissue 6 hr after an oral dose of 20 mg/kg MDMA. Peak B identified as 6-OH-MDMA and peaks A and C tentatively identified as the other aromatic hydroxylated metabolites of MDMA.
Figure 22. Positive CI - CAD daughter ion mass spectrum of $\text{MH}^+$ (m/z 402) of synthetic 6-OH-MDMA (top) and of minor peak C (bottom) at relative retention time of 1.138 in Figure 21.
of 1.038 in Figures 11 and 21, and disproves the hypothesis that MDMA is resistant to aromatic hydroxylation. However, the exact mechanism by which the hydroxylated metabolites are formed remains undetermined; they are either formed from epoxides, or are formed through a direct insertion mechanism. So far, a total of eight previously unreported metabolites of MDMA has been identified in rats. All of them, except 3,4-dihydroxy-methamphetamine and 6-hydroxy-3,4-(methylenedioxy)methamphetamine, were excreted in the urine. The hydroxylated metabolites appeared to be excreted in urine primarily as O-glucuronide and/or O-sulfate conjugates based on the observation that only trace quantities of free 4-hydroxy-3-methoxymethamphetamine and 4-hydroxy-3-methoxyamphetamine were detected in unhydrolyzed urine.

In conclusion, the proposed metabolic pathways for MDMA in rat shown in Figure 23 are based on the metabolites identified from in vivo and in vitro metabolic studies. The metabolite in brackets has not been detected, but is a postulated intermediate in the formation of 4-hydroxy-3-methoxyamphetamine. This assumption is reasonable, since 3,4-dihydroxymethamphetamine has been reported as an intermediate in the metabolism of MDA to 4-hydroxy-3-methoxyamphetamine (Midha et al., 1978). Furthermore, the metabolic pathway from metabolite C to D was recently established experimentally by detection of 4-hydroxy-3-methoxyamphetamine following subcutaneous injection of 4-hydroxy-3-methoxymethamphetamine (Table 4). Since primary amines are more readily deaminated than secondary or tertiary amines (Timbrell, 1982) the deamination pathways were assumed to proceed from metabolites VI to VIII and from IV to VII. Therefore, MDMA is metabolized in the rat via O-dealkylation, N-demethylation, aromatic hydroxylation, deamination, and conjugation (O-methylation, O-glucuronidation, and/or O-sulfation). It has also been shown that some of these biotransformations occur in the brain (O-dealkylation, N-demethylation, and O-methylation).

**Distribution of metabolites.** Since the toxicodynamic effects of a drug are related to the drug and/or metabolite concentrations at the receptor or target site, better understanding
Figure 23. Metabolism of MDMA in the rat. Dotted arrows indicate metabolism occurring in the rat brain. The letters in parentheses correspond to the chromatographic peak labels shown in Figure 2.
of the neurotoxicology of MDMA requires investigation of the distribution of MDMA and its metabolites in various fluids and tissues of the rat. The qualitative data on distribution of MDMA and all the identified metabolites are summarized in Table 7. Knowing the metabolic profile in the target organ will help to reduce the number of metabolites that need to be tested further to determine their contribution to the neurotoxicology of MDMA.

All the identified metabolites were detected in 24-hr urine except for 3,4-dihydroxy-3-methoxymethamphetamine. Our inability to find this metabolite in urine, even after increasing the MDMA dose from 5 to 20 mg/kg, may be due to its total metabolism to 4-hydroxy-3-methoxymethamphetamine via 3-O-methylation. Recently, MDA was reported as the major urinary metabolite of MDMA in rat urine (Fitzgerald et al., 1988). In the present study, comparison of the relative peak sizes of MDMA metabolites in both EI and CI total ion chromatograms suggested that 4-hydroxy-3-methoxymethamphetamine, not MDA, is the major metabolite in the basic extract of rat urine. This discrepancy may be due to the omission of enzymatic hydrolysis in the previous study and by the fact that the other metabolites were unknown at the time.

Of the known metabolites, only 4-hydroxy-3-methoxymethamphetamine, 4-hydroxy-3-methoxyamphetamine, and MDA were detected in the feces of rats. The metabolic profile of MDMA in the plasma was similar to that of the 24-hr feces, except for two additional deaminated metabolites, (4-hydroxy-3-methoxyphenyl)acetone and [3,4-(methylenedioxy)phenyl]acetone. With the exception of [3,4-(methylenedioxy)phenyl]acetone, all of the metabolites were detected in the plasma 24 hr after administration of 20 mg/kg MDMA. In liver, the metabolic profile of MDMA was similar to that in the urine except for the absence of (3,4-dihydroxyphenyl)acetone. However, only the N-demethyl and the 3-O-methyl phenolic amine metabolites of MDMA remained in the liver 24 hr after administration of 20 mg/kg MDMA. Judging from the relative peak sizes, the major metabolite in both feces and plasma was MDA and in liver was 4-hydroxy-3-methoxymethamphetamine.
Table 7. Distribution of MDMA and its metabolites in the rat

<table>
<thead>
<tr>
<th>Drug and metabolites</th>
<th>urine</th>
<th>feces</th>
<th>plasma</th>
<th>liver</th>
<th>brain</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDMA (I)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3-Hydroxy-4-methoxymethamphetamine (II)</td>
<td>+</td>
<td></td>
<td></td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>4-Hydroxy-3-methoxymethamphetamine (III)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>4-Hydroxy-3-methoxyamphetamine (IV)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3,4-Dihydroxymethamphetamine (V)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>3,4-(Methylenedioxy)amphetamine (VI)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>(4-Hydroxy-3-methoxyphenyl)acetone (VII)</td>
<td>+</td>
<td></td>
<td></td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>[3,4-(Methylenedioxy)phenyl]acetone (VIII)</td>
<td>+</td>
<td></td>
<td></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>(3,4-Dihydroxyphenyl)acetone (IX)</td>
<td></td>
<td></td>
<td></td>
<td>+</td>
<td></td>
</tr>
</tbody>
</table>
Since MDMA is known to cause neurotoxicity in rats for up to 14 days after a single dose (Stone et al., 1987b), it is important to determine the metabolic profile of MDMA in the brain and also to examine the capacity of the brain to metabolize MDMA. All of the MDMA metabolites found in the rat brain (Table 7), except [3,4-(methylenedioxy)phenyl]acetone, were still detected in the brain 24 hr after dosing with 20 mg/kg MDMA. The similarity in the profiles of the MDMA metabolites in brain and plasma suggests that these compounds are derived from peripheral metabolism of MDMA, and that the unconjugated metabolites can penetrate the blood-brain barrier. This conclusion is supported by the observation that 4-hydroxy-3-methoxymethamphetamine and 4-hydroxy-3-methoxyamphetamine were detected in the rat brain following subcutaneous injection of 4-hydroxy-3-methoxymethamphetamine and 4-hydroxy-3-methoxyamphetamine, respectively (Table 4). However, metabolism within the brain may contribute to the concentrations of these metabolites in the brain, since the brain tissue has been shown to convert MDMA in vitro to each of the identified metabolites except for [3,4 (methylenedioxy)phenyl]acetone. This suggestion is consistent with a report that the brain is able to cleave the methylenedioxy bridge of the prodrug of the potent dopamine agonist 10,11-(methylenedioxy)-N-propylnorapomorphine, via O-dealkylation (Mesnil et al., 1984). On the basis of the relative peak sizes of the metabolites in the brain, MDA appears to be the major metabolite, a conclusion supported by the quantitative data in Chapter 3.

6-Hydroxy-MDMA has only recently been identified conclusively, so only limited investigation into its distribution has been carried out. Nevertheless, the available data show that 6-OH-MDMA was present in plasma, liver, and brain of rats 6 hr after subcutaneous injection of 10 mg/kg MDMA. However, the other two aromatic hydroxylated metabolites were only found in the liver.

In conclusion, the qualitative distribution study shows that most of the known metabolites of MDMA are present in the brain and one or more of them may contribute to the neurotoxicity of MDMA.
Metabolism and neurotoxicity. Several researchers have proposed that MDMA-induced neurotoxicity is caused by its metabolites (Schmidt, 1987; Molliver et al., 1986; Slikker et al., 1989). Demonstrations in vitro that the rat brain is able to metabolize MDMA to MDA, a known neurotoxin (Ricaurte et al., 1985) and to at least some of the phenolic amine metabolites (Lim and Foltz, 1988) have provided further support for this hypothesis. Furthermore, the in vitro brain metabolites, plus 6-OH-MDMA and [3,4-(methylenedioxy)phenyl]acetone, are present in the rat brain after subcutaneous injection of MDMA. With the exception of MDA, none of the metabolites has been tested for neurotoxicity. Nevertheless, on the basis that 4-hydroxy-3-methoxyamphetamine is a major metabolite of MDA, and that protection against MDA-induced neurotoxicity has been observed after pretreatment with SKF 525-A, 4-hydroxy-3-methoxyamphetamine has been postulated to contribute to some of the neurotoxic effects of MDA (Marquardt et al., 1978). The toxicodynamic effects of the 4-hydroxy-3-methoxyamphetamine appear to be stereoselective; only the S-enantiomer has been shown to produce the neurotoxicity (Marquardt et al., 1978). Conceivably, the neurotoxicity of MDMA may be mediated by the phenolic amine metabolites found in the rat brain, such as 3,4-dihydroxymethamphetamine, 4-hydroxy-3-methoxymethamphetamine, 4-hydroxy-3-methoxyamphetamine, and 6-OH-MDMA. 6-OH-MDMA is unlikely to be neurotoxic by itself but it could be neurotoxic following O-dealkylation to form the 3,4,6-trihydroxymethamphetamine, which is structurally related to 6-hydroxydopamine (Figure 24). This is not an unreasonable supposition since O-dealkylation has been established as a metabolic route for MDMA.

The bioactivation of phenolic compounds is well documented, and two general mechanisms have been advanced to explain their cytotoxicity: 1) covalent binding of oxidation products to macromolecules, through sulphhydryl groups; 2) production of reactive oxygen species such as superoxide anion, hydrogen peroxide, hydroxyl radical, and singlet oxygen (Ito et al., 1988; Irons and Sawahata, 1985). Accordingly, neurotoxicity may result from initial reduction of 3,4-dihydroxymethamphetamine, 4-hydroxy-3-
Figure 24. Postulated metabolism of 6-OH-MDMA to 3,4,6-trihydroxymethamphetamine (top) and chemical structure of 6-hydroxydopamine (bottom).
methoxymethamphetamine, 4-hydroxy-3-methoxyamphetamine, and 3,4,6-trihydroxy-
methamphetamine to their respective semiquinone species followed by reoxidation to the
quinones with reduction of molecular oxygen to superoxide anions (Figure 25).
Subsequent reactions of the superoxide anions can result in the formation of other reactive
oxygen species such as hydrogen peroxide, hydroxyl radical, and singlet oxygen. The
hydroxyl radical may initiate lipid peroxidation; this mechanism probably explains the
observation of lipofuscin within the raphe nuclei in the nerve cell bodies of monkeys treated
with 5 mg/kg MDMA (Ricaurte et al., 1988b). The semiquinones of these metabolites can
also bind covalently to highly nucleophilic sulphydryl groups in proteins, causing
inactivation. Therefore, these phenolic amine metabolites may cause neurotoxicity by
damaging the neurons, via lipid peroxidation of the neuronal membranes and inactivation of
important macromolecules in the neurons. Also, production of reactive oxygen species and
covalent binding of semiquinones during redox cycling of these metabolites may be
responsible for the observed inactivation of tryptophan hydroxylase in the brains of
MDMA-treated rats (Stone et al., 1989).

Another pathway of metabolism of MDMA capable of damaging neurons is
deamination by monoamine oxidase (MAO), an enzyme that has recently gained importance
as a producer of neurotoxins (Youdim, 1989). One of the many reaction products of the
deamination reaction is hydrogen peroxide; it can react with superoxide from redox cycling
of phenolic amine metabolites or iron, to form the hydroxyl radical. It is the hydroxyl
radical that causes lipid peroxidation of neuronal membranes and ultimately leads to
neuronal degeneration.

In summary: neuronal degeneration in the brains of MDMA-treated rats has been
hypothesized to result from the activity of reactive oxygen species and semiquinone
produced from deamination of primary amine metabolites such as MDA and 4-hydroxy-3-
methoxyamphetamine, and from redox cycling of the phenolic amine metabolites. Whether
the chemical insult to the neurons is expressed as neuronal degeneration depends on the
Figure 25. Proposed bioactivation pathways for 4-hydroxy-3-methoxymethamphetamine (R = CH₃) and 4-hydroxy-3-methoxyamphetamine (R = H) (top), 3,4-dihydroxymethamphetamine (middle) and 3,4,6-trihydroxymethamphetamine (bottom).
balance between chemical insult and status of the detoxification mechanisms (catalase, glutathione, glutathione peroxidase, etc.) present in the brain.

**Metabolism of MDMA in the Human**

The data in Chapter 3 indicate that all but three of the metabolites identified in the rat are also formed in man following MDMA ingestion. The human metabolites that have been identified include 3-hydroxy-4-methoxymethamphetamine, 4-hydroxy-3-methoxymethamphetamine, MDA, 4-hydroxy-3-methoxyamphetamine, 3,4-dihydroxymethamphetamine, (4-hydroxy-3-methoxyphenyl)acetone, and [(3,4-methylenedioxy)phenyl]acetone. The human metabolic pathways of MDMA that have been identified are N-demethylation, O-dealkylation, deamination, and O-methylation, as shown in Figure 26.

In conclusion, the metabolic data in man and rat show that qualitatively the two species are similar with respect to metabolism of MDMA. As in the rat, the major metabolite in the basic extract of human urine is 4-hydroxy-3-methoxymethamphetamine, judging from the relative peak sizes in the total ion chromatogram (Figure 12). In view of this metabolic similarity, since MDMA is a neurotoxin in animal models including nonhuman primates, caution must be exercised in the recreational use of MDMA by humans.

**Development and Application of the GC-CI/MS Assay**

An analytical method for quantitation of compounds in the low ng/g range requires complete derivatization of the analytes, since incomplete derivatization is automatically translated into smaller signal-to-noise ratio. The consequence is an increase in the limit of detection and quantitation. Complete derivatization of MDMA and metabolites appears to be achieved when trifluoroacetic anhydride was used, since in the experiments reported here no underivatized compounds were detected by the mass spectrometer. Unfortunately, the trifluoroacetate moieties of the TFA derivatives of phenolic amine metabolites are susceptible to hydrolysis in the presence of a trace amount of moisture. For this reason the
Figure 26. Proposed metabolic pathways of MDMA in human.
reproducibility of the assay was initially poor. However, rederivatization with 2% MBTFA in ethyl acetate reduced the magnitude of this problem, as indicated by the tighter coefficient of variations obtained for all TFA derivatives (< 12%). The base peaks in the CI mass spectra of the TFA derivatives of MDMA and its amine metabolite are fragment ions at low m/z values, even when isobutane is the reagent gas. Furthermore, there was increased fragmentation of the quasi-molecular ions of the TFA derivatives of these amine compounds with higher manifold temperature as shown in Figure 15. This observation is consistent with the fact that input of additional thermal energy can have a noticeable effect on the fragmentation of quasi-molecular ions in the presence of milder reagent gases like isobutane or ammonia (Chapman, 1985). In this assay, the mass spectrometer was set to scan, at 1 sec/scan (4 μscans), a narrow mass range that included the masses of all the quasi-molecular ions. The consequence was greater selectivity and sensitivity of the assay, since these ions are diagnostic of the analytes. Under the same mass spectrometric conditions for CI but now scanning a single mass in the pseudo-selected ion monitoring (SIM) mode, the only scan speed available, 2 sec/scan (1 μscan), yielded data points sampled across the narrow chromatographic peak that were inadequate for accurate description of the shape of the mass peak.

Unlike conventional mass spectrometric assays that employ a deuterated analog of the analyte as internal standard, a homolog that is chromatographically separable from the analyte is preferable for an assay using mass spectrometers based on ion trapping technology. The reasons for this have been discussed in Chapter 3; the linear calibration curves for MDMA and metabolites achieved in the assay developed here clearly support the conclusion that a homolog is a suitable internal standard for assays using ion trap mass spectrometers.

Development of a selective and efficient extraction scheme for MDMA and its metabolites was challenging due to the amphoteric properties of the phenolic amine metabolites (HMM and HMA), which are normally difficult to extract efficiently from
aqueous solution. Furthermore, many endogenous materials present in brain tissue can be coextracted with the analytes. Initial purification by precipitation of proteins with cold perchloric acid solution resulted in cleaner extract and also improved recoveries of MDMA and its metabolites. Of the many solvent systems investigated, good overall recoveries of MDMA and metabolites were obtained with a moderately polar extraction solvent mixture of dichloromethane:n-butanol (3:1) and considerable amounts of endogenous materials were also coextracted. These endogenous materials must be resolved chromatographically from the analytes before reliable quantitation can be obtained.

Adequate chromatographic separation among analytes, and from endogenous materials was achieved with a 25-m narrow-bore capillary column and with a temperature ramp of 10 °C/min. For best results, the capillary column should be flushed by injection of 1 ul organic solvent, and the temperature program ramped from 100 to 300 °C after every two to three injections of biological samples. The purpose of flushing the capillary column is to remove accumulations of high-boiling polar compounds that can coelute irreproducibly with the analytes as a very broad peak. The population of the reagent gas species formed in the ion trap mass spectrometer is dependent on the reagent gas pressure when the other CI parameters as described in Chapter 2 are kept constant. Since the number of the MH+ ions formed is dependent on the population of reagent gas species, it is important to ensure that the reagent gas pressure is stable throughout analysis. Finally, nonlinearity and poor quantitative reproducibility can easily arise if space-charging occurs due to high concentration of analyte in the ion trap. This problem of space-charging can be readily detected as mass missassignment of the MH+ ions through scanning a narrow mass range instead of a single mass in the pseudo-SIM mode. This problem can be remedied by either dilution of the sample prior to addition of internal standard or by decreasing the sample size for extraction.

With the above considerations in mind, a capillary GC-CI/MS assay, linear from 1 to 500 ng/g wet brain tissue, was successfully developed for simultaneous quantitation of
MDMA and three of its metabolites (MDA, HMM, and HMA). Even with calibration curves covering such a wide linear dynamic range, smaller samples had to be extracted for quantitation of MDMA and MDA in brain tissues for the disposition study, when the concentrations of these compounds were outside the calibration curves. The sensitivity of the GC-CI/MS assay was adequate to permit quantitation of MDMA and MDA in the rat brain for up to 24 hr, and HMM and HMA for 8 and 10 hr, respectively, after subcutaneous injection of 10 mg/kg MDMA (Figure 19). Five minutes after subcutaneous injection of this dose, the parent drug, MDA and HMM were found in the brain. The lag phase in appearance of HMA in the brain, 1 hr after subcutaneous injection, could not be accounted for by the difference in its rate of penetration into the brain; rather, it must reflect a different rate of metabolism. This conclusion was based on the observation that no lag phase occurred in the appearance of the N-methyl analog of HMA, HMM, in the brain. Furthermore, comparable amounts of HMA and HMM were found in the brain following subcutaneous equimolar injection of each compound individually to rats (Table 4) lend support to the conclusion that penetration of HMA and HMM across the blood-brain barrier does not differ significantly. The rapid penetration of MDMA and metabolites into the rat brain probably explains the previously reported rapid onset of decline in tryptophan hydroxylase activity and of decrease in concentration of serotonin in brain within 15 min of a single injection of 10 mg/kg MDMA (Stone et al., 1987b).

The previously reported maximum depletion of cortical serotonin content in rat brain 3-6 hr after administration of MDMA (Schmidt, 1987) correlates better with the brain profiles of HMM and HMA than with those of MDMA or MDA (Figure 19). This correlation is corroborated by the recovery of the cortical serotonin concentrations in 6-24 hr, which coincides with the complete elimination of HMM and HMA from the brain. In contrast, the concentrations of MDMA and MDA remain high during this time period. Since the administration of fluoxetine at 12 hr after MDMA was found to be ineffective in blocking the development of MDMA-induced neurotoxicity, this suggests that the putative
neurotoxic metabolite is formed during the first 12 hr after subcutaneous injection (Schmidt, 1987). The present data suggest that the phenolic amine metabolites (HMM and HMA) are better candidates for the above mentioned putative neurotoxic metabolites. The postulated mechanisms of neuronal degeneration by these phenolic metabolites are summarized in Figure 25. In an effort to better understand the potential contribution of peripheral metabolism to the concentrations of the phenolic amine metabolites in the brain, brain concentrations of HMM and HMA were measured following equimolar subcutaneous injection of HMM and HMA, respectively, to rats. The low concentrations of HMM and HMA detected in the brain after subcutaneous injection of equimolar quantities of each compound to rats suggests a low permeability of the brain to these phenolic amine metabolites. This conclusion is consistent with the report of the low permeability of the rat brain to phenolic amines such as p-hydroxyamphetamine (Brien et al., 1977). At present, the possibility that peripherally-formed phenolic amine metabolites contribute to the neurotoxicity of MDMA cannot be ruled out merely on the basis of the low permeability of the brain to these compounds, because high neuronal concentrations of these metabolites may achieved during selective uptake into serotonergic neurons. Finally, it is not known whether the acute effect of MDMA is a prerequisite for the development of long term neurotoxic effects, or whether the short term and long term events are caused by separate processes.

In conclusion, the GC-CI/MS assay developed for MDMA and three of its metabolites in brain tissues has been applied successfully to disposition studies of MDMA in the rat brain; the brain concentration-time profiles of MDMA, MDA, HMM and HMA are shown in Figure 19. Another successful application of the assay demonstrated low permeability of the rat brain to the phenolic amine metabolites of MDMA.
Stereoselective Disposition of MDMA

It is now well recognized that many biochemical processes at the molecular level exhibit stereoselectivity. This is not at all surprising, since animal studies have shown that L-amino acids in the diet are preferentially incorporated into biological proteinaceous macromolecules (Rose et al., 1954; Rose et al., 1955). Hence, proteinaceous macromolecules in the biological systems should be viewed as asymmetric entities that can have different interactions with the enantiomers of a racemic drug. The stereoselectivity in interactions at the molecular level in biological systems has been traced to stereospecific interactions with corresponding receptors, an observation that explains the different pharmacodynamic or toxicodynamic effects seen with drug enantiomers; differences in pharmacokinetics of drug enantiomers may be related to stereospecific differences in transport mechanisms or metabolic pathways (Koroklovas, 1974; Testa, 1989; Hubbard et al., 1986). That enantiomers of a racemic drug can have different biological activities is now well documented; an illustration was the discovery that the devastating teratogenic effect of thalidomide is associated only with the S-(−) enantiomer (Blaschke et al., 1979). Despite this body of evidence, it is still a common practice to treat a racemic drug as though it were a single pure compound, an assumption that leads to the neglect of stereoselectivity in clinical pharmacology and pharmacokinetics (Ariens, 1982).

MDMA is a chiral drug whose enantiomers have different biological activities, as discussed in Chapter 1. With this in mind, investigation into stereoselectivity of disposition of MDMA was carried out in rats. One of the prerequisites for the study was an enantioselective assay. The previously reported enantioselective assay based on gas chromatography with nitrogen-phosphorus detection (Lim et al., 1986) was successfully adapted for analysis of MDMA and MDA with only slight modification as described in Chapter 2.

This enantioselective GC-CI/MS method is based on conversion of the enantiomers of MDMA and MDA into their respective diastereomers following aqueous derivatization with N-heptafluorobutyryl-l-prolyl chloride prior to separation by capillary column.
Because the enantiomeric ratio of R-(-) to S-(+) of close to unity was used as an index of the absence of stereoselectivity in disposition, there was a critical need to determine accurately the enantiomeric ratio. An initial attempt to express the enantiomeric ratio using the peak areas of the quasi-molecular ions of each diastereomeric pairs resulted in values that deviated considerably from unity for both racemic MDMA (1.141) and racemic MDA (0.909). However, enantiomeric ratios close to unity were obtained for MDMA (1.039) and MDA (0.995) when the combined peak areas of the quasi-molecular and fragment ions at m/z 163 were used in the calculation (Table 6). Even though the CI mass spectra of the diastereomers were indistinguishable, subtle differences appeared in the intensity of the fragment ion at m/z 163 arising from loss of a McLafferty rearrangement product from the quasi-molecular ion. As with the TFA derivative, greater fragmentation was observed with MDMA diastereomers than with those of MDA. Finally, consistency in the procedure used in calculation of the enantiomeric ratios is important in the qualitative determination of stereoselectivity in disposition of MDMA.

That stereoselectivity exists in disposition of MDMA and MDA in the rat is clearly shown by the deviation of the enantiomeric ratios from unity for MDMA and MDA in Table 6. The present results are identical to those reported for MDMA and MDA in rat plasma (Fitzgerald et al., 1989b). In both plasma and urine of rats, R>S and S>R were observed for MDMA and MDA, respectively. In view of the similarity in the enantiomeric ratios for MDMA and MDA in plasma and urine, it is unlikely that the distortion in the enantiomeric ratio is due to stereoselective excretion into urine. The present study ruled out stereoselective absorption also, because the racemic MDMA was administered by subcutaneous injection. The present results, combined with the in vitro data (Hiramatsu et al., 1989) which showed that the S-(+) enantiomer of MDMA was more extensively metabolized to MDA and 4-hydroxy-3-methoxymethamphetamine, suggest that the distortion in the enantiomeric ratios of MDMA and MDA is most likely due to stereoselective metabolism in
the rat; that is, preferential N-demethylation of S-$(+)$-MDMA to S-$(+)$-MDA over the R-$(–)$ antipode.

In conclusion, there is stereoselectivity in disposition of MDMA and MDA in rat. This stereoselective disposition of MDMA and MDA is very likely due to stereoselective metabolism of the parent drug.
REFERENCES


CURRICULUM VITAE

I. Personal Data

A. Name: Heng-Keang Lim
B. Birthdate: August 25, 1958
C. Birthplace: Kampar, Malaysia
D. Citizenship: Malaysia

E. Home Address: Apartment 316, Medical Plaza
Salt Lake City, Utah 84112
Phone: 801-583-4603

F. Business Address: Center for Human Toxicology
Research Park
417 Wakara Way, Room 290
Salt Lake City, Utah 84108
Phone: 801-581-5117

II. Education

A. Graduate degrees:
   Ph.D. in Pharmacology, 1989
   University of Utah

   M.Sc. in Toxicology, 1985
   University of Saskatchewan

B. Undergraduate degree:
   B.Sc. (First Class Honours) in Biochemistry, 1982
   Memorial University of Newfoundland

III. Honors

A. 1985 - 1989: Research Fellowship from Center for Human Toxicology

B. 1982 - 1985: University of Saskatchewan Graduate Student Scholarship

C. 1982: Department of Biochemistry Gold Medal for Academic Excellence in Biochemistry
D. 1981: Government of Newfoundland Centennial Scholarship

IV. Professional Experiences

A. 1985 - present Graduate Student, Ph.D. Candidate

B. 1983 - 1984: Teaching Assistant in Pharmaceutical Analysis and Forensic Toxicology

V. Professional Organization

American Society for Mass Spectrometry

VI. Publications

A. Published Papers


B. Abstracts


