seem to be the key to a new type of field-induced ionization which produces molecular ions exclusively—even from highly thermolabile and nonvolatile organic materials.

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Application of Chemical Ionization Mass Spectrometry to the Study of Stereoselective in vitro Metabolism of 1-(2,5-Dimethoxy-4-methylphenyl)-2-aminopropane

Robert J. Weinkam,* Joseph Gal,[1] Patrick Callery,[2] and Neal Castagnoli, Jr.

Department of Pharmaceutical Chemistry, School of Pharmacy, University of California, San Francisco, Calif. 94143

The stereoselective metabolism of the chiral psychotomimetic amine 1-(2,5-dimethoxy-4-methylphenyl)-2-aminopropane has been studied using direct sample insertion isobutane chemical ionization mass spectrometric analysis of incubate extracts with the aid of deuterium labeled compounds as substrates and internal standards. Information has been obtained on deuterium isotope effects, stereoselective substrate disappearance and metabolite formation. Mass spectrometric identification of 8 metabolites is reported for each enantiomer. Quantitative estimations of 16 incubate constituents was achieved by total ion current summation during the exhaustive evaporation of the incubate extracts into the ion source. The results suggest that direct sample insertion CIMS is an effective technique for the initial analysis of in vitro metabolic reactions.

The dramatic increase in the need for information concerning the biological disposition of foreign compounds has stimulated major advances in bioanalytical methodology. Radioisotopic labeling, thin layer and gas–liquid chromatography and, most recently, gas–liquid chromatography coupled to mass spectrometry with or without selected ion monitoring have proved invaluable in the qualitative and quantitative analysis of small molecules present in complex biological systems (*1*).

Analytical problems encountered in in vitro drug biotransformation studies result from the limited substrate capacity of the enzyme systems employed and the complexity of metabolic mixtures formed. Furthermore, important metabolites may be formed in nanomolar quantities and represent only a small fraction of the substrate incubated. Nevertheless, a thorough metabolic study requires the determination of the amount of substrate consumed, the identification and quantification of the metabolites formed, and, in some cases, the determination of the rates of substrate disappearance and product formation.

This report describes the use of stable isotopes and direct sample insertion chemical ionization mass spectrometry (CIMS) to study these aspects of the metabolism of the psychotomimetic amine 1-(2,5,-dimethoxy-4-methylphenyl)-2-aminopropane (1) (*2*) by the 10000 × *g* supernatant and microsomal fractions obtained from rabbit liver. This compound, first described in 1969 (*3*), has been reported to be one of the most potent of a known series of 1-phenyl-2-aminopropanes. Racemic 1 is 80 times more active than mescaline in man (*3*). This activity, however, is dependent on the configuration about the chiral center. Shulgin has reported the (*R*)-(−)-1 isomer to be as active as twice its weight of racemic 1 while the (*S*)-(+)-1 isomer was inactive as a psychotogen at the doses studied (*4*). Analogous stereochemical selectivity in the action of 1 has been noted in mouse (*5*), cat (*6*), rabbit (*6*), and isolated smooth muscle tissue (*7*).

In view of the stereoselective biological activity demonstrated for amine 1, we have investigated the extent to which the metabolism of 1 is influenced by the configuration about the asymmetric center of the 2-aminopropyl side chain through the use of isotopically labeled resolved substrates and CIMS analysis. Additionally, we have characterized and quantified mass spectrometrically several in vitro metabolites of 1 as part of an effort to evaluate the possible role (*3, 8*) of these substances in the overall pharmacological activity of the parent drug. Information on deuterium isotope effects associated with the formation of some of these metabolites has also been gained.

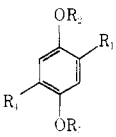
EXPERIMENTAL

Apparatus. NMR spectra were taken on a Varian Associates A-60A instrument and chemical shifts are reported in parts per million (δ) downfield relative to TMS (for $CDCl_3$ or DMSO-d_6) or to DSS (for D_2O).

Chemical ionization mass spectra were taken using an Associated Electrical Industries MS-902 high resolution mass spectrometer which has been modified to operate under chemical ionization

[1] Present address, Department of Pharmacology, School of Medicine, University of California, Los Angeles, Calif. 90024.
[2] Present address, Department of Medicinal Chemistry, School of Pharmacy, University of Maryland, Baltimore, Md. 21201.

Table I. Isobutane CIMS Peak Positions and Relative Intensities of Possible Incubate Constituents

	No.	R_4	$-OR_2$	$-OR_5$	R_1	MH^+	$-NH_3$	$-H_2O$	Other
Amines:	1	CH_3	CH_3	CH_3	$CH_2CHNH_2CH_3$	210(100)	193(7)		165(3)
	1-d_2	CH_3	CH_3	CH_3	$CHDCDNH_2CH_3$	212(100)	195(7)		166(3)
	1-d_6	CH_3	CD_3	CD_3	$CH_2CHNH_2CH_3$	216(100)	199(7)		171(3)
	2	CH_2OH	CH_3	CH_3	$CH_2CHNH_2CH_3$	226(100)	209(7)	208(7)	181(5), 182(8), 165(69)
	2-d_6	CH_2OH	CD_3	CD_3	$CH_2CHNH_2CH_3$	232	215	214	187, 188, 171
Neutrals:	3	CH_3	CH_3	CH_3	CH_2COCH_3	209(100)			165(1)
	3-d_6	CH_3	CD_3	CD_3	CH_2COCH_3	215			171
	4	CH_2OH	CH_3	CH_3	CH_2COCH_3	225		207	181
	4-d_6	CH_2OH	CD_3	CD_3	CH_2COCH_3	231		213	187
	5	CH_3	CH_3	CH_3	$CH_3CHOHCH_3$	211(100)		193(37)	165(1)
	5-d_1	CH_3	CH_3	CH_3	$CH_2CDOHCH_3$	212(100)		194(32)	165(1)
	5-d_6	CH_3	CD_3	CD_3	$CH_2CHOHCH_3$	217		199	171
	6	CH_2OH	CH_3	CH_3	$CH_2CHOHCH_3$	227		209	182, 166
	6-d_6	CH_2OH	CD_3	CD_3	$CH_2CHOHCH_3$	233		215	188, 172
Aminophenols	7a	CH_3	H	CH_3	$CH_2CHNH_2CH_3$	196(100)	179(6)		151(2)
	7a-d_3	CH_3	H	CD_3	$CH_2CHNH_2CH_3$	199	182		154
	7b	CH_3	CH_3	H	$CH_2CHNH_2CH_3$	196(100)	179(4)		151(1)
	7b-d_3	CH_3	CD_3	H	$CH_2CHNH_2CH_3$	199	182		154
	8	CH_2OH	$H(CH_3)$	$CH_3(H)$	$CH_2CHNH_2CH_3$	212	195	194	167, 151
	8-d_3	CH_2OH	$H(CD_3)$	$CD_3(H)$	$CH_2CHNH_2CH_3$	215	198	197	170,154
Phenols:	9	CH_3	$H(CH_3)$	$CH_3(H)$	$CH_2CHOHCH_3$	197		179	151
	9-d_3	CH_3	$H(CD_3)$	$CD_3(H)$	$CH_2CHOHCH_3$	200		182	154
	10	CH_3	$H(CH_3)$	$CH_3(H)$	CH_2COCH_3	195			151
	10-d_3	CH_3	$H(CD_3)$	$CD_3(H)$	CH_2COCH_3	198			154
Oxime:	11	CH_3	CH_3	CH_3	$CH_2CNOHCH_3$	224(100)		206(1)	165(14)
	11-d_6	CH_3	CD_3	CD_3	$CH_2CNOHCH_3$	230		212	171
Acid:	12	CH_3	CH_3	CH_3	COOH	197(100)		179(18)	
	12-d_6	CH_3	CD_3	CD_3	COOH	203		185	

conditions (*9*). Spectra were taken using isobutane reagent gas at 0.5 Torr and 200 °C.

Samples were introduced into the ion source via a direct insertion probe in which the sample was carried into the ionization chamber by the reagent gas flow. The insertion probe was warmed by induction from the heated source block.

Liver Preparations. Male Dutch rabbits (6 months, 1.5–2.0 kg) were killed by a blow to the neck. The liver was immediately removed and washed in cold isotonic KCl. Liver tissue, 5 g net weight, was homogenized in 30 ml of isotonic KCl with 0.01 *M* sodium phosphate buffer at pH 7.4 and 0.25 *M* in sucrose with a Potter–Elvehjem Teflon-pestle homogenizer. The homogenate was centrifuged at 10000 × *g* at 3 °C for 20 min and the supernatant used for incubations. The microsomal fraction was obtained by further centrifugation of the supernatant at 3 °C and 94000 × *g* for 1 hr. The microsomal pellet was suspended in ice cold isotonic KCl to make a total volume of 10 ml.

Incubations. The 10000 × *g* supernatant incubation mixture was made up with 14 ml supernatant fraction (corresponding to 2.0 g liver), 1.0 mg amine 1-HCl (4.1 μmol), 17.0 mg NADPH (17.8 μmol) and 1.52 mg $MgCl_2$ (16 μmol) in a final volume of 16.6 ml. Incubations were carried out at 37 °C in air using a metabolic shaker.

The microsomal incubations were carried out with 2 ml of the microsomal suspension (equivalent to 2 g liver), ~600 nmol substrate amine 1, 34 mg NADPH (35.6 μmol), 1 ml of 0.6 *M* potassium phosphate buffer (pH 7.4), and 1.8 mg $MgCl_2$ (19 μmol) in a final volume of 3 ml. Incubations were carried out at 37 °C in air using a metabolic shaker.

After 1 hr, the incubation flask was cooled in ice and appropriate internal standards (see below) were added. The pH of the incubation mixture was successively adjusted to 2.0, 13.0, and 9.5 and extracted with two volumes of benzene at each pH. In some experiments, the pH 2 benzene-soluble components (neutral and acid fractions) were further separated through the back-extraction of the benzene solution into pH 6.5 and pH 13 buffered water, adjustment of each aqueous solution to pH 2, and reextraction with two volumes of benzene to obtain neutral, carboxylic, and phenol acid fractions. The benzene solutions were evaporated to dryness in a nitrogen stream at room temperature. Each residue was then transferred to the probe with a minimum volume introduced into the CI ion source.

The following compounds were available from previous work (*10–13*): (*R,S*)-1-(2,5-dimethoxy-4-hydroxymethylphenyl)-2-aminopropane (**2**) (*11*); 1-(2,5-dimethoxy-4-methylphenyl)-2-propanone (**3**) (*10*); 1-(2-hydroxy-4-methyl-5-methoxyphenyl)-2-aminopropane hydrochloride (**7a**–HCl) (*13*); 1-(2-methoxy-4-methyl-5-hydroxyphenyl)-2-aminopropane hydrochloride (**7b**–HCl) (*13*); 2,5-dimethoxy-4-methylbenzoic acid (**12**) (*11, 12*).

1-(2,5-Dimethoxy-4-methylphenyl)-2-nitropropene (**15**) and (*R,S*)-1-(2,5-dimethoxy-4-methylphenyl)-2-aminopropane hydrochloride (**1**·HCl) were prepared according to the procedure previously described for the ^{14}C analogues (*11*). (*R,S*)-1-(2,5-Dimethoxy-4-methylphenyl)-1,2-$^2H_2$-2-aminopropane hydrochloride (**1-d_2**HCl) was prepared from **15** by the procedure of (*11, 12*) using LiAlD4 in 2.5-fold excess as the reducing agent.

(*R,S*)-1-(2,5-Di-$^2H_3$-methoxy-4-methylphenyl)-2-aminopropane Hydrochloride, (1-d_6 HCl). Following the procedure for the preparation of **1**, nitropropene **15-d_6** (11.5 g, 47.5 mmol) was reduced with $LiAlH_4$ (7.2 g) in dry tetrahydrofuran to yield crude 1-d_6 (12.2 g). Distillation (85–105 °C/0.25 mm) followed by HCl salt formation gave 8.1 g (79%) of pure product 1-d_6 HCl: pmr (D_2O) δ 1.41 (d, *J* = 6.5 Hz, $CHCH_3$), 2.30 (s, $ArCH_3$), 2.83–3.20 (comp m, CH_2), 3.45–4.15 (comp m, CH), 7.01 (bd s, C_6H_2); CIMS (MH^+) 99% isotope incorporation.

Resolution of 1-d_2 and 1-d_6. The resolution of amines 1-d_2 and 1-d_6 were accomplished with (+) and (−)-*O*-nitrotartranilic acids to yield the pure (*R*)(−) an*S*)(+) enantiomers. In each case, enantiomeric purity was confirmed by glc analysis of the amides obtained upon derivatization with the chiral reagent (*S*)-(−)-*N*-pentafluorobenzoylprolyl-1-imidazolide (11).

(*R,S*)-1-(2,5-Dimethoxy-4-methylphenyl)-2-propanol (5). A mixture of 1-(2,5-dimethoxy-4-methylphenyl)-2-propanone (**3**) (*11*) (0.2 g, 0.96 mmol), $NaBH_4$ (0.06 g, 1.6 mmol) and absolute ethanol (10 ml) was stirred at room temperature for 1 hr and then evaporated to dryness. The residue was stirred with water (10 ml) and extracted with ether (2 × 20 ml). The ether layers were dried ($MgSO_4$) and evaporated to give a white solid. Recrystallization from hexane provided the analytical sample; mp 80–82 °C; pmr ($CDCl_3$) δ 1.20 ppm (d, *J* = 6.0 Hz, CH_3), 2.22 (s, Ar CH_3), 2.63–2.92 (comp m, CH_2) 3.76 (s, OCH_3), 3.80–4.40 (comp m, CH), 6.99 (s, Ar), and 7.02 (s, Ar). *Anal.* Calcd for $C_{12}H_{18}O_3$: C, 68.55; H, 8.63. Found: C, 68.69; H, 8.53.

(*R,S*)-1-(2,5-Dimethoxy-4-methylphenyl)-2-propanol-2-^2H (5-d_1). A solution of ketone **3** (*11*) (1.91 g, 9.2 mmol) in dry THP (50 ml) was added dropwise to a stirred suspension of $LiAlD_4$ (99%, 1.0 g, 24 mmol) in dry tetrahydrofuran (50 ml). The mixture was stirred at room temperature for 7 hr and the reaction was terminated by the careful addition of water (1.0 ml) followed by 10% NaOH (1.5 ml) and water (3 ml). The salts were filtered and the

filter cake was digested for 30 min with ether. The organic layers were combined, dried ($MgSO_4$), and concentrated to give the alcohol **5**-d_1 (1.90 g, 98.5%, mp 80–81 °C). Recrystallization from hexane gave 1.76 g (8.4 mmol, 91%): mp 80–82 °C; pmr ($CDCl_3$) δ 1.20 ppm (s, $CDCH_3$), 2.22 (s, $ArCH_3$), 2.76 (s, CH_2), 3.76 (s, OCH_3), 7.70 (s, Ar); CIMS (MH^+) 99% isotope incorporation.

1-(2,5-Dimethoxy-4-methylphenyl)-2-propanone oxime (11). Powdered NaOH (1.1 g, 27.5 mmol) was added with stirring to a solution of ketone **3** (*11*) (1.15 g, 5.5 mmol) and NH_2OH·HCl (2.6 g, 8.6 mmol) in 75% aq ethanol. The mixture was heated and held at reflux for 10 min. After cooling, the contents were poured into 10% HCl (20 ml) and the light yellow oil which separated was extracted into ether (2 × 30 ml). Evaporation of the ether layers gave 1.1 g (90%) of an oil: pmr ($CDCl_3$) δ 1.78 and 1.85 (s, $CNCH_3$). Crystallization from hexane gave 1.01 g (82%): mp 72.74 °C. Recrystallization from hexane gave pure **11** (0.46 g, 37%): mp 73–75 °C, a second crop gave an additional 0.13 g, mp 64–72 °C, total yield 48%: pmr ($CDCl_3$) δ 1.85 (s, $CNCH_3$), 2.24 (s, $ArCH_3$), 3.54 (s, CH_2), 3.78 (s, OCH_3), 3.81 (s, OCH_3), 6.70 (s, Ar), and 7.75 (s, Ar). *Anal.* Calcd for $C_{12}H_{17}NO_3$: C, 64.55; H, 7.67; N, 6.27. Found: C, 64.69; H, 7.67; N, 6.41.

2,5-Di-$^2H_3$-methoxytoluene (13-d_6). Into a 3-neck 500-ml flask cooled in an ice bath and equipped with a magnetic stirrer, reflux condenser, and N_2 system was added 200 ml anhyd MeOH and KOH (19.7 g, 352 mmol). After the system was flushed with N_2 and cooled in an ice bath for 10 min, methylhydroquinone (22.0 g, 176 mmol) in 120 ml MeOH was added slowly followed by the dropwise addition of methyl iodide-d_3 (99%, 50.0 g, 352 mmol). The mixture was heated under reflux for 24 hr and then cooled. After removing solvent under vacuum, the residue was extracted with 3 × 50 ml ether and the combined ether extracts washed 3 × 25 ml 1 *N* NaOH. The ether was dried ($MgSO_4$), removed and the residue 13.7 g (51%) distilled (43–45 °C/0.25 mm) to yield pure **13**-d_6: 12.6 g (47%); pmr ($CDCl_3$) δ 2.23 (s, $ArCH_3$), 6.77 (s, Ar).

2,5-Di-$^2H_3$-methoxy-4-methylbenzaldehyde (14-d_6). Formylation of **13**-d_6 (12.5 g, 79 mmol) proceeded according to the literature (*11*) with *N*-methylformanilide (11.8 g, 87 mmol) and $POCl_3$ (13.3 g, 86 mmol) to give **14**-d_6 (19.9 g, 74%): mp 82–84 °C [lit. (*9*) mp 85–87 °C]; pmr ($CDCl_3$) δ 2.77 (s, $ArCH_3$), 6.83 (s, Ar), 7.27 (s, Ar), 10.43 (s, CHO).

1-(2,5-Di-$^2H_3$-methoxy-4-methylphenyl)-2-nitropropene (15-d_6). Following the literature (*11*) procedure, the aldehyde **14**-d_6 (10.9 g, 59 mmol) was condensed with nitroethane to yield the nitropropene **15**-d_6 (11.5 g, 81%): mp 83–90°C [lit (*11*) mp 85–87 °C] pmr ($CDCl_3$) δ 2.30 (s, $ArCH_3$), 2.42 (s, $CNCH_3$), 6.81 (S, Ar), 8.33 (s, ArCH).

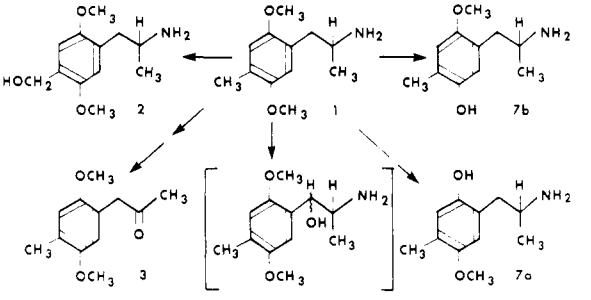
RESULTS AND DISCUSSION

Small molecule ionization occurs under chemical ionization conditions through proton transfer from a CI reagent ion to the organic molecule. When using the reagent gas isobutane, as in this study, proton transfer occurs with little energy transfer and fragmentation of the protonated molecular ion, MH^+, is minimized (*14*). The resulting simplicity of the individual compound CI spectrum makes it possible to use CIMS to analyze compound mixtures. When used in this way, the MS yields data which are analogous to those of a mass discriminating chromatography system.

Extracts of in vitro enzyme preparations are compatible with direct CI analysis as they are comparatively free of small organic molecule constituents. A CI scan of the incubate extract may show ions resulting from unreacted substrate and metabolites formed during the incubation as well as any compounds extracted from the enzyme preparation. In this study, benzene extracts of 10000 × *g* rabbit liver supernatant and microsomal fractions showed no significant ions in the mass range of *m/e* 150 to 250, although large peaks were observed for cholestanol, MH^+ 389, and cholestanone, MH^+ 387. The absence of detectable interfering peaks in the liver preparation extracts enabled the CI analysis to be carried out at the limits of CI scan sensitivity and reproducibility.

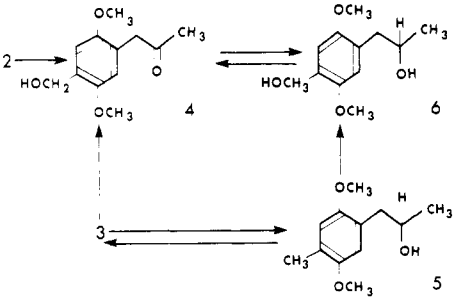
Table I lists the structure and isobutane CI spectral peak positions for the drug 1-(2,5-dimethoxy-4-methylphenyl)-2-aminopropane (**1**), its deuterio analogs **1**-d_2 and **1**-d_6 and possible metabolites. Relative peak intensities have been included for those compounds which have been synthesized and characterized. The table includes several entries for compounds which have not been isolated but which are postulated metabolites. The CI peaks indicated are based on analogies to the CI spectra of related compounds (*15*). Such predictions may be made with confidence because of the low energy and spectral simplicity involved in the CI processes. The only compound showing an unusual fragmentation pattern was the hydroxymethylamine **2**. In addition to a major fragment ion corresponding to loss of water from the protonated molecular ion, a second fragment ion appeared at *m/e* 165 corresponding to loss of water and C_α–C_β bond cleavage of the aminopropyl side chain.

The compounds listed in Table I were proposed as they correspond to expected metabolites of **1** which would have CI spectral peaks at the *m/e* values observed in the incubate scans. Based on inspection, amine **1** may be metabolized according to several possible pathways as shown in Scheme I. Oxidation of the aromatic methyl group to the

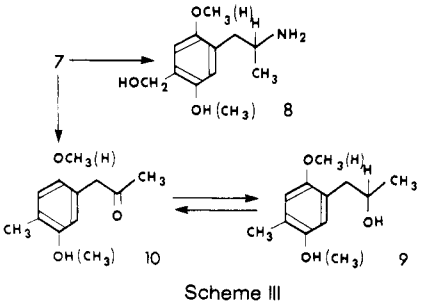


Scheme I

hydroxymethyl, **2**, and carboxyl functions has been shown by Ho and Tansey (*16*) to give rise to the major urinary metabolites in rat. By analogy with other 1-phenyl-2-aminopropanes additional pathways may be expected to proceed through oxidative deamination (*17*) to give **3** and **5**,



Scheme II



Scheme III

β-hydroxylation (*18*) and further side chain oxidation to the benzoic acid (*19*), and finally *O*-demethylation of the aromatic methoxy groups to give **7a** and **7b** (*20*). There are thus five feasible sites of metabolism, each having a differ-

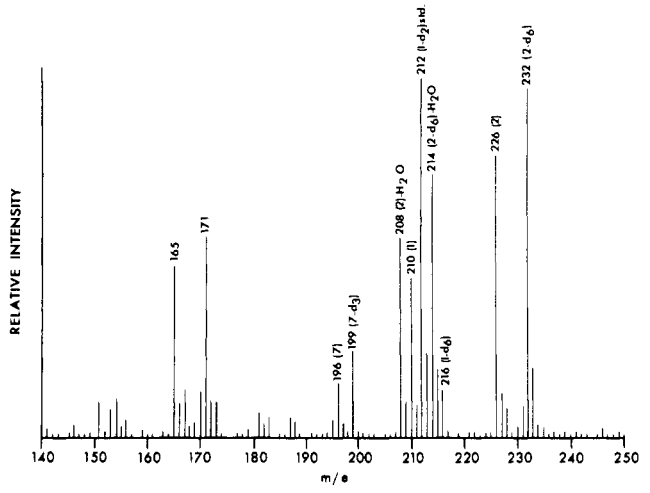


Figure 1. Isobutane CIMS of combined amine and aminophenol incubate extracts from the rabbit liver microsomal metabolism of (*R*)-**1** and (*S*)-**1**-d_6

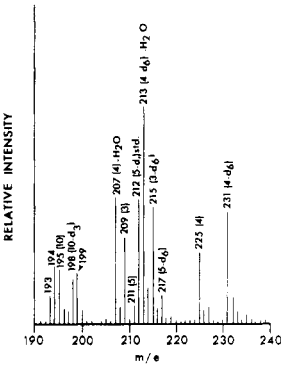


Figure 2. Isobutane CIMS of combined neutral and phenol incubate extracts from the rabbit liver microsomal metabolism of (*R*)-**1** and (*S*)-**1**-d_6

ent orientation with respect to the asymmetric center. Further metabolism of these primary metabolites is also to be expected. Possible secondary reactions are shown in Schemes II and III.

An inspection of the compounds listed in Table I reveals that all parent ions and significant fragment ions can be detected without ambiguity when liquid–liquid partitioning is used to separate amine, aminophenol, neutral, and acid metabolites from the incubate mixture. Examples of typical scans are given in Figures 1 and 2. The combined amine (pH 13) and aminophenol (pH 9.5) benzene extracts of Figure 1 show the presence of unreacted substrates (*R*)-**1** and (*S*)-**1**-d_6 and internal standard **1**-d_2 as well as metabolites of each substrate. The peak at *m/e* 196 corresponds to the MH^+ ion of the isomeric (*S*)-**1** *O*-demethylated phenols, **7**. The corresponding phenols **7**-d_3, *m/e* 199, of the (*R*)-**1**-d_6 substrate would show the loss of three deuterium atoms on *O*-demethylation. The peaks at *m/e* 226 and 232 correspond to the hydroxymethyl metabolites **2** and **2**-d_6. The observed fragment ion intensities agree with the spectrum of pure **2** taken under the same conditions. The presence of four other possible metabolites for each substrate can be detected in the combined neutral and phenol (pH 2) scans shown in Figure 2.

High resolution mass measurement of the protonated molecular ions for the metabolites indicated in Figures 1 and 2 were within 0.001 Dalton of those expected for the molecular structures shown in Table I. High resolution scans also confirm that these ions were the only significant peaks at their respective nominal masses. The absence of interfering ion current permitted quantification of minor constituents such as the propanol **5**. Limits of detection were approximately 0.2 nmol for a metabolite formed in 16 ml of microsomal enzyme preparation. It is significant to note that, with this sensitivity and simple workup procedure, these data indicate the absence of other possible metabolites such as the hydroxymethyl propanol **6** or the *O*-demethylated compounds **8** and **10**.

Two techniques have been used to estimate the amounts of metabolites formed and the consumption of the substrate **1**. A stable isotopically distinct analogue of each potential incubate constituent can be added to the incubate at the conclusion of the metabolic reaction. The amount of metabolites formed or substrate consumed can then be calculated from the relative ion currents observed for the internal standard and the unknown in the mass spectra (*21*). Another approach, which does not require isotopically labeled internal standards, involves summation of the ion current produced by an individual metabolite during the exhaustive evaporation of the incubate constituents into the ion source (*22*). The total ion current produced may then be compared to that of a known amount of internal standard, and the constituents quantified.

The first approach was used in the 10000 × *g* rabbit liver supernatant metabolism of racemic **1**-d_6 (*23*). The unlabeled substrate **1** and seven possible unlabeled metabolites, compounds **2, 3, 4, 5, 7, 11,** and **12** (Table II), were added as internal standards at the conclusion of the metabolic reaction. The incubation mixture was extracted successively at pH 2.0, 13.0, and 9.5 and a series of spectra were taken as the extract was evaporated into the ion source. The data obtained from the unlabeled standard peak intensities and those of the metabolites of labeled substrate as shown in the isobutane CI scans are summarized in Table II, column 1.

After 37% metabolism of the substrate, the seven compounds investigated accounted for 77% of the reaction products although the oxime **11** and acid **12** were not detected (<1 nmol). The isomeric phenols, **7**, are indicated as their sum. The nonphenolic hydroxyl compounds **2** and **5** showed the same d_6/d_0 ratios for their molecular (MH^+) and fragment ions, confirming the structural assignments. These data also indicate that the possible isomeric hydroxylated metabolite, 1-(2,5-dimethoxy-4-methylphenyl)-1-hydroxy-2-aminopropane, would be a minor metabolite, if present, as this compound would not be expected to give a *m/e* 165 ion in its isobutane CI spectrum (*15*). Another possible hydroxylated metabolite, the *N*-hydroxy derivative, has been studied using glc-electron bombardment MS (*24*); CIMS has been found unsuitable for the detection of this compound (*25*).

Isotope Effects. The deuterium isotope effects occurring in the liver homogenate metabolism of **1**-d_6 are indicated in Table II, column 2. In this experiment, equivalent amounts of racemic **1** and **1**-d_6 were incubated and quantitative analyses were performed using **1**-d_2 as internal standard. Variation of the d_6/d_0 peak ratios from unity provides a direct measure of the deuterium isotope effect. After 46% metabolism of the substrate only the *O*-demethylated phenols **7** showed an isotope effect in the formation of the labeled metabolites (*26*). An average apparent k_H/k_D isotope effect of 4.3 was observed. Peak height ratios of the internal standards and substrates at *m/e* 210, 212, and 216 remained within the ±1.5% experimental precision throughout sample analysis; therefore, no isotope effect could be detected in the process of evaporation of these compounds from the ceramic tip of the direct insertion probe at ambient temperature.

Table II. The in vitro Rabbit Liver 10000 × *g* Supernatant Metabolism of 1-(2,5-Dimethoxy-4-methylphenyl)-2-aminopropane (1)

	Column 1	Column 2	Column 3	Column 4
Substrate	(*R*,*S*)-1-d_6	(*R*,*S*)-1-d_6 (*R*,*S*)-1	(*S*)-1-d_6 (*R*)-1	(*S*)-1 (*R*)-1-d_6
Substrate added, nmol	474	d_6, 581 d_0, 598	*S*, 581 *R*, 598	*S*, 598 *R*, 581
Substrate metabolized, nmol	175 (37%)	d_6, 131 (23%) d_0, 134 (22%)	*S*, 180 (31%) *R*, 84 (14%)	*S*, 185 (31%) *R*, 99 (17%)
Constituents	% of metabolism	(*R*,*S*)-1-d_6/(*R*,*S*)-1	(*S*)-1-d_6/(*R*)-1	(*S*)-1/(*R*)-1-d_6
1 before incubation		1.02	1.05	1.05
1 after incubation		0.97	0.78	0.86
Hydroxymethyl, **2**	70	0.98	1.8	1.3
Propanone, **3**	1	0.90	4.3	3.2
Hydroxymethyl propanone, **4**	0.5	0.96	1.9	3.1
Propanol, **5**	3	1.03	2.1	2.6
Phenols, **7**,[b]	3	0.23	2.5	10.0
Oxime, **11**	not obsd			
Acid, **12**	not obsd			

[a] Substrate mixtures of cols 2, 3, and 4 were metabolized using aliquots corresponding to 2 g of same liver supernatant, after 1 hr. [b] Data for the phenols, **7** and **8**, were taken from a different incubation mixture after 42% metabolism; the supernatant was prepared by the same procedure but from a different liver.

Table III. The in vitro Rabbit Liver Microsomal Metabolism of 1-(2,5-Dimethoxy-4-methylphenyl)-2-aminopropane (1)

Substrate	(*R*)-1	642 nmol Found,	(*S*)-1-d_6	625 nmol Found,
Constituents	Ions monitored	nmol, (%)	Ions monitored	nmol, (%)
Recovered **1**	210	294 (45.8)	216	49 (7.8)
Hydroxymethyl, **2**	226, 208, 165	248 (38.6)	232, 214, 171	310 (49.6)
Propanone, **3**	209	16 (2.5)	215	19 (3.0)
Hydroxymethyl propanone, **4**	225, 207	33 (5.1)	231, 213	53 (8.5)
Propanol, **5**	211, 193	3 (0.5)	217,199	5 (0.8)
Phenols, **7**	196	13 (2.0)	199	25 (4.9)
Phenolpropanones, **9**	195	15 (2.3)	198	9 (1.4)
	Total	622 (97.8)		470 (75.1)

The presence of two equally intense peaks at *m/e* 225 and 231, facilitated the tentative identification of a new metabolite as the neutral hydroxymethyl ketone **4**. Peaks were also observed at *m/e* 207 and 213 corresponding to the formation of a fragment by the loss of H_2O as would be expected from **4**. The parent-fragment peak ratios remained constant during the slow evaporation of the incubate neutral-acid fraction.

Stereoselective Metabolism. Experiments designed to investigate the stereoselective consumption of substrate and metabolite formation by the 10000 × *g* rabbit liver supernatant fraction are summarized in columns 3 and 4 of Table II. In these experiments, pseudoracemic mixtures consisting of 50% (*R*)-(−)-**1** and 50% (*S*)-(+)-**1**-d_6 (column 3) and the alternate pair 50% (*S*)-(+)-**1** and (*R*)-(−)-**1**-d_6 (column 4) were metabolized using aliquots of the same liver homogenate used in the experiment summarized in column 2. Variance of the S/R ratio from unity indicates the stereoselective metabolism of the substrate or formation of metabolites which are labeled according to the configuration of their precursors. A change in the S/R ratio between the alternate labeled substrate pairs (column 3 vs. column 4) would be a measure of any operating isotope effects, although this approach is less precise than that described above.

These data show that the disappearance of the inactive (*S*)-(+)-**1** enantiomer was approximately two times that of (*R*)-(−)-**1**. Metabolites derived from the (*S*)-**1** substrate were present in greater abundance than those from (*R*)-**1**. The S/R ratio was greatest (3.2–4.3) for the propanone **3** which is formed through reaction at the chiral center and least (1.3–1.8) for the hydroxylmethyl metabolite **2** involving hydroxylation at the distant aromatic methyl position. These ratios do not necessarily measure relative stereoselective formation, however, as nonstereoselective formation of a metabolite followed by stereoselective disappearance would also affect the S/R ratios. The question can be satisfied through measurement of the initial rates of formation of the enantiomeric metabolites.

In a separate experiment, an example of a secondary metabolic reaction of **1** was shown by the 10000 × *g* rabbit liver supernatant metabolism of **5**-d_1 labeled at the 2 position of the 2-hydroxypropyl side chain. This demonstrated that the propanol, **5**, and propanone, **3**, are interconverted. Partial metabolism of **5**-d_1 gave an incubate mixture consisting of the propanone, **3**, and the propanol, **5**, as well as unreacted **5**-d_1.

Because of the presence of the isotope effect in the *O*-demethylation, the indicated stereoselective formation of the two resulting phenols can not be defined from these data. The stereoselective formation of these compounds has been studied using glc separation and CIMS/internal standard quantification techniques (*13*).

Quantification. Quantitative analysis of the rabbit liver microsomal metabolites of (*R*)-**1** and (*S*)-**1**-d_6 (Table III) employed the method of total ion current summation (*22*) during exhaustive incubate fraction evaporations. In this procedure, internal standards were added to the post-incubate mixture after cooling. The mixture was separated into amine, neutral, phenol, and aminophenol fractions through

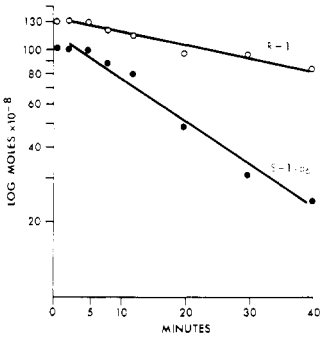


Figure 3. Plot showing the disappearance of (*R*)-**1** and (*S*)-**1**-d_6 vs. time in rabbit liver homogenate

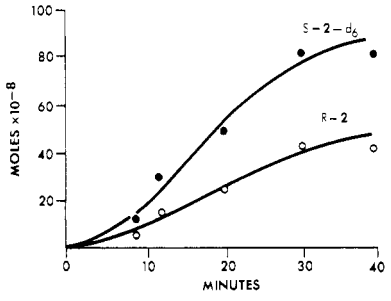


Figure 4. Plot showing formation of **2** and **2**-d_6 from (*R*)-**1** and (*S*)-**1**-d_6 vs. time in rabbit liver homogenate

complete benzene extractions at the appropriate pH values. An aliquot of each extract was introduced into the CI ion source and the sample evaporated by slowly increasing the temperature of the direct insertion probe. A series of isobutane CI scans were taken every 15 sec until the sample had completely evaporated, about 3 min. The recorded ion current for the parent and fragment ions of each metabolite were totaled and compared with the total ion current from an internal standard. The amount of each incubate constituent was calculated from these total ion currents.

This approach rapidly yields quantitative data on all of the extractable incubate constituents without necessitating the use of isotopically labeled internal standards although **1**-d_2 and **5**-d_1, were used in these experiments. There are a number of approximations inherent in this scheme, however, which modulate the accuracy of the quantitative data. The efficiency of protonation of a molecule is a function of the gas phase basicities of the sample molecules (*27*), so that discrimination may occur when a mixture is introduced into the ion source. Further, when a large amount of sample is introduced, ion quenching may occur between a sample or fragment ion and a neutral sample molecule of higher gas phase basicity.

The first factor was minimized in this scheme as the extract components have common functional groups (amine, hydroxyl, methoxy, carbonyl, etc.) and, therefore, have similar proton affinities. Both factors can be minimized by maintaining a low sample partial pressure. This, however, was not feasible in the total evaporation series. The MH^+/fragment ion ratios varied during the course of recording the sequential spectra. For this reason, the parent and fragment ions were totaled for all constituents. This technique was used to quantify standard mixtures of the available substrate and metabolic products. In standard solutions, observed values were within 15% of the known amounts with a standard deviation of ±12% although these values vary for different compounds. Quantification is more accurate and precise if the standard and sample being determined are chemically similar.

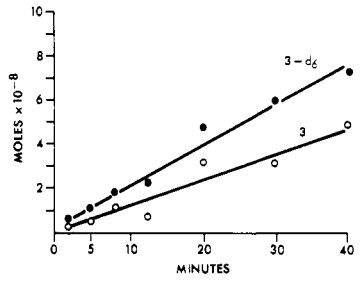


Figure 5. Plot showing formation of **3** and **3**-d_6 from (*R*)-**1** and (*S*)-**1**-d_6 vs. time in rabbit liver homogenate

The data presented in Table III account for 98% of added (*R*)-1 substrate after 1-hr incubation. The amount of recovered (*R*)-1 showed that 54% of this substrate had been consumed. The (*S*)-**1**-d_6 was more fully metabolized as 8% unreacted substrate remained; however, only 75% of the added substrate mass could be accounted for in the extracts. Several differences were observed between the rabbit liver microsomal (Table III) and 10000 × *g* supernatant (Table II) enzyme systems. The amount of propanol, **5**, was less relative to the ketone **3** in the microsomal system. The presence of two other metabolites was indicated in the microsomal phenol fraction by peaks at *m/e* 195 and 198. These ions correspond to the expected molecular ions of the isomeric phenol propanones **10**.

Metabolism Kinetics. The total ion current summation method was used to measure the initial stereoselective rates of disappearance for a pseudo-racemic mixture of the enantiomeric substrates (*S*)-**1**-d_6 and (*R*)-1 and the stereoselective formation of the two initially most abundant metabolites, the hydroxymethyl derivative, **2**, and the propanone, **3**. The data were obtained from analyses of timed aliquots of a single microsomal enzyme system prepared from 2 g of wet tissue and containing ~1 μM of each enantiomer.

Figure 3 shows the semilog plot of unreacted (*R*)-1 and (*S*)-**1**-d_6 vs. time during incubation in the rabbit liver microsomal enzyme system. The data suggest a nearly first-order rate of substrate disappearance with (*S*)-**1**-d_6 having a half life, $T_{1/2}$ = 20 min, 2.8 times shorter than the enantiomeric (*R*)-1, $T_{1/2}$ = 57 min. The apparent first-order disappearance suggests that the enzyme system was not saturated at the low substrate levels of less than 1.3 μmol.

Figures 4 and 5 show the amounts of hydroxymethyl and propanone metabolites formed vs. time. These data were not sufficiently precise to permit a detailed kinetic analysis of the rates of formation, but they do suffice to demonstrate that the initial rates of formation of these compounds were influenced by the stereochemistry of the aminopropyl side chain of **1**. A detailed investigation of the metabolism kinetics of this system is in progress (*28*).

CONCLUSION

This study has employed stable isotopes and direct sample insertion chemical ionization mass spectrometry to study the stereoselective in vitro metabolism of the psychotomimetic amine 1-(2,5-dimethoxy-4-methylphenyl)-2-aminopropane (1). The method of analysis has proved to be a rapid and effective approach toward the measurement of deuterium isotope effects, stereoselective metabolism, and product formation, and for the quantitative estimations of incubate constituents. Emphasis has been placed on techniques which provide detailed information on the metabolic system while, at the same time, require few isotopically labeled compounds. This methodology is thus well suited for use as an initial data gathering tool in the investigation of metabolic reactions. Because of the many features com-

mon to in vitro small molecule metabolic reactions, the techniques described may prove equally effective in the study of other substrate–enzyme systems.

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Graphite-Epoxy Mercury Thin Film Working Electrode for Anodic Stripping Voltammetry

Jeffrey E. Anderson and Dennis E. Tallman*

Department of Chemistry, North Dakota State University, Fargo, N.D. 58102

A graphite-epoxy electrode is described which appears to be a viable alternative to other working electrodes currently used in anodic stripping voltammetry (ASV). Below pH 4, the electrode is essentially free of intrinsic electrochemical interference over the potential range of interest in ASV. A comparison is made of results obtained by DPAS analysis of Cd, Pb, and Cu in EPA reference samples with published results and with results obtained by flameless atomic absorption in our laboratory. Precision and accuracy of DPAS analysis of these samples with the graphoxy electrode is typically 5–10%. The electrode is easy and inexpensive to make, is easily polished, and is both physically and electrochemically durable.

Anodic stripping voltammetry (ASV) has proved to be a powerful method for the trace analysis of certain metal ions of environmental concern (*1–3*). The sensitivity of this technique is inherently associated with the preconcentration step during which metal species are electrochemically deposited at a working electrode. The various problems and attributes which are associated with the different types of working electrodes have been treated elsewhere (*3, 4*). We direct our attention here to mercury thin film electrodes (MTFE) which have found widespread use in anodic stripping voltammetry. Of the substrates used for plating these films, carbon appears to best fulfill the modest requirements of 1) conductivity, 2) chemical inertness to solution and to mercury, and 3) electrochemical inertness throughout the potential region of interest.

Perhaps the two most widely used electrodes in stripping analysis are the glassy or vitreous carbon electrode and the wax impregnated graphite (WIG) electrode. There appears, however, to be some controversy over which of these electrodes performs best and, indeed, most workers soon find preference for one over the other. Both of these electrodes appear to fail upon extended use, particularly under the acidic conditions often required to ensure dissociation of the metals from naturally occurring ligands. Modes of electrode failure have been studied by Clem and co-workers (*5–7*) and two modes appear to exist. One is common to the WIG electrodes in which solution gains access to the interior of the electrode, such as in the event of crystallization and cracking of the wax impregnator (*5, 6*). Much effort has been devoted to finding appropriate waxes to circumvent this problem; however, wax being a rather complex substance, these attempts have suffered somewhat from irreproducible and inconsistent behavior. The other mode of failure appears to result from changes in the carbon itself involving formation of carboxyl compounds at the surface of the electrode (*7*). Thus, in acidic solutions, the hydrogen ion concentration at the surface is effectively increased yielding the first sign of electrode failure, an apparent decrease in hydrogen overpotential. Eventually the surface becomes unuseable and must be repolished, this problem being common to both WIG and glassy carbon electrodes.

Our work began as a result of limited success with wax impregnated graphite electrodes and our desire to find an electrode which would be inexpensive and easy to make, durable, yet not require constant attention. The first electrode which came to mind was the carbon paste electrode used in organic voltammetry, the surface of which is easily renewable in the event of film formation at the surface (*4*). If a similar electrode could be produced which would harden, yet still have an easily renewable surface, it may prove