J. Pharm. Pharmac., 1977, **29**, 89–95 *Received June* 3, 1976

The identification and quantitation of the major metabolites of ethylamphetamine, produced by rabbit liver microsomal preparations

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Amphetamine (I), 1-phenylpropan-2-ol (III), phenylacetone (V), *N*-hydroxyethylamphetamine (VII), and α-methyl-*N*-(1′-phenylprop-2′-yl)nitrone (VIII) were obtained as metabolic products from the incubation of ethylamphetamine (II) with fortified male rabbit liver 9000 *g* supernatant fractions. These metabolites were identified by comparing their t.l.c., g.l.c. and g.l.c.-m.s. behaviour to that of reference compounds. A method for the quantitative analysis of the metabolites in mixtures using g.l.c. techniques is described.

Metabolic *N*-oxidation of aminated xenobiotics has received much attention due to the potential toxicological properties of the chemically and biologically reactive *N*-oxygenated products that are formed (Bickel, 1969, Bridges, Gorrod & Parke, 1972). The toxic effects of arylhydroxylamines and their conjugates is well established (Miller & Miller, 1966; Weisburger & Weisburger, 1973; Gutmann, Barry & Malejka-Giganti, 1969; Gillette, Mitchell & Brodie, 1974); alkylhydroxylamines may have important pharmacological actions (Beckett, 1973; Gorrod & Jenner, 1975).

It is now becoming evident that *N*-oxidation as well as *C*-oxidation is a general route of metabolism of primary and secondary amines. The *N*-oxygenated metabolites of aralkylamines may have previously gone undetected because of their labile nature (Beckett, 1974). A prior communication indicated that secondary amines, such as ethylamphetamine (II), are metabolized to nitrones and hydroxylamines *in vitro* (Beckett, Coutts & Ogunbona, 1973a). In the present report evidence for the identification of five *in vitro* metabolites of ethylamphetamine (II) is presented.

The quantitative analysis of *N*-oxygenated metabolites has often required time-consuming procedures (Beckett & Al-Sarraj, 1973; Beckett & Midha, 1974), to prevent chemical conversions of the metabolites during the analytical procedures. Recently g.l.c. techniques employing the formation of stable derivatives have been used (Cho, Lindeke & Sum, 1974; Beckett & Bélanger, 1975b; Beckett, Coutts & Gibson, 1975). A method involving g.l.c. for the quantitative analysis of the five major metabolites of ethylamphetamine (II) is developed in the present study.

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MATERIALS AND METHODS

Materials

(±)-Ethylamphetamine HCl was obtained from Oril Labs (Paris, France), (+)- and (−)-ethylamphetamine and (±)-n-propylamphetamine HCl were prepared from the respective enantiomers of amphetamine (Hilgetag & Martini, 1972), which were generous gifts from Smith Kline and French Laboratories. Published procedures were utilized for the synthesis of: (+)-, (−)-, and (±)-*N*-hydroxyamphetamine and *N*-hydroxyethylamphetamine (Beckett, Haya & others, 1975); α-methyl-*N*-(1′-phenylprop-2′-yl)nitrone (Beckett, Coutts & Ogunbona, 1973b; Morgan & Beckett, 1975); phenylacetone ketoxime (Hey, 1930). All other chemicals were purchased from commercial sources.

Thin layer chromatography

Glass plates (20 × 20 cm) were coated with an aqueous slurry of Silica Gel G (Merck) to a 0·25 mm thickness, dried at room temperature and activated at 110° for 60 min. The solvent systems employed were: (A) ether–benzene (4:1); (B) methanol–acetone (1:1). Spots were visualized with iodine vapour, or by spraying with ammonical silver nitrate (0·1 N $AgNO_3$–5 N ammonia 1:5), 2,3,5-triphenyltetrazolium chloride (TTC 4% methanolic TTC–1 N NaOH 1:1), 2% aqueous $CuCl_2$ or *NN*-dimethyl-*p*-aminobenzeneazobenzoyl chloride (DABBC, 0·1% methanolic). Metabolic samples were co-chromatographed with the appropriate reference compounds.

Gas liquid chromatography

A Hewlett-Packard model 5700A gas chromatograph equipped with a flame ionization detector and a Hewlett-Packard Strip Chart Recorder 7123B was used; glass columns (i.d. 4 mm) were used, the

detector temperature was 350° and the columns were conditioned for 24 h at 50° higher than the operating temperatures. The systems used for analysis were: stationary phase (length); solid support; flow rates in ml min^{-1}; oven temperature: (A) (2.0m) 3% w/w OV-17; Gas-Chrom Q 100–120 mesh; N_2 55, H_2 40, Air 165; 130°; (B) (2.0m) 5% w/w Carbowax 20M; Chromosorb G AW-DMCS 100–120 mesh; $N_2$55, H_2 40, Air 165; 140°; (C) (1.0m) w/w Apiezon L; Chromosorb G (HP) 80–100 mesh; N_2 60, H_2 60, Air 200; 190°; (D) as system C but oven temperature at 145°.

Combined gas-liquid chromatography-mass spectrometry

A Perkin-Elmer Model 270 instrument fitted with 1 m × 4 mm i.d. glass columns packed with similar materials as above was utilized. Helium (70 kN m^{-2}) was employed as the carrier gas and the ionization potential was 70 eV. The relevant temperatures were as follows: oven 140–190°; manifold temperature 200°.

In vitro *metabolic experiments*

Livers from male rabbits (New Zealand White) were used to prepare the subcellular fractions (9000 *g* or washed microsomes) by differential centrifugation (Gorrod, Temple & Beckett, 1975). The liver subcellular fractions were resuspended in pH 7·4 tris-KCl buffer to a volume equivalent to 0·5 g ml^{-1} of original liver.

Incubations were performed aerobically at 37° for 30 min with shaking in open 25 ml Erlenmyer flasks. Each incubation flask contained 1 ml of the liver preparation plus 1 ml of co-factor solution containing glucose-6-phosphate (6 mg), $NADPNa_2$ (3·4 g), $MgCl_2$ (0·2 ml, 0·1 M), distilled H_2O (to give 1 ml) and 2 units of glucose-6-phosphate dehydrogenase when appropriate; 0·2 M phosphate-buffer (pH 7·4, 3 ml); 10 μmol of the HCl salt of the substrate in 1 ml of H_2O. The reactions were initiated by the addition of the substrate after a preincubation period of 10 min to allow temperature equilibration and the reduction of NADP. The reactions were terminated by cooling the incubation mixture in an ice bath. The metabolic samples were extracted and analysed as described below.

Analysis for N-*hydroxyethylamphetamine* (*VII*)

The incubation mixture (pH 7·4) was transferred to a 20 ml centrifuge tube containing NaCl (2 g) and *p*-chlorophenyl cyclopropyl ketone (1 ml, 100 μg ml^{-1} in water containing 10% methanol) as the internal standard. Freshly distilled pentane (12 ml) was added and the mixture was mechanically shaken for 20 min, centrifuged and the pentane transferred to a tapered evaporating tube. The extraction procedure was repeated. The combined pentane extracts were concentrated to a small volume in a water bath (43°), the sides of the tube washed with 0·5 ml of pentane and evaporated to dryness under a stream of nitrogen at room temperature. The samples were stored at 0–5° until analysis by t.l.c. system (A) or by g.l.c. system (A). For the g.l.c. analysis, acetonitrile (30 μl) and BSTFA (*N*,*O*-bis-(trimethylsilyl)-trifluoroacetamide) (20 μl) were added, the solution mixed and allowed to stand for 5 min; a 4 μl sample was analysed for the trimethylsilyl (TMS) derivative of VII.

*Analysis for α-methyl-*N-*(1′-phenylprop-2′-yl)nitrone* (*VIII*).

A 5 ml aliquot of the incubation mixture (pH 7·4) was oxidized with a 10% aqueous solution of $K_3Fe(CN)_6$ (1 ml) in a centrifuge tube at room temperature for 30 min. The internal standard (benzophenone, 1 ml, 100 μg ml^{-1} in 10% aqueous methanol), NaCl (2 g) and freshly distilled peroxide free ether (Analar, 12 ml) were added. The mixture was extracted and concentrated to approximately 50 μl as above and immediately analysed on t.l.c. system (A) and g.l.c. system (C).

Analysis for phenylacetone(*V*) *and 1-phenylpropan-2-ol* (*III*)

The incubation sample was transferred to a 20 ml centrifuge tube containing dilute HCl (0·5 ml, 13% v/v, pH = 1 of final solution), and the internal standards (*p*-chloropropiophenone, 1 ml, 100 μg ml^{-1} in 10% aqueous methanol and propylamphetamine HCl, 1 ml, 500 μg ml^{-1} in H_2O). The mixture was extracted with ether and evaporated to dryness and stored as described above, except that 5 μl of n-butanol was added to the ether extracts before concentration. The samples were analysed by t.l.c. system (A) or redissolved in 50 μl of acetonitrile and analysed on g.l.c. system (B).

Analysis for amphetamine (*I*) *and ethylamphtamine* (*II*)

The acidic aqueous portion from above was basified (pH 12) with NaOH (0·5 ml, 30% in H_2O), extracted with ether, evaporated to dryness and stored as previously described. The samples were analysed by t.l.c. system (B) or redissolved in 50 μl of acetonitrile and analysed on g.l.c. system (D).

Quantitative analysis

The amount of substrate and metabolites in the incubation samples were quantitated by g.l.c. analysis (see Fig. 3) with reference to a calibration curve constructed using the internal standard technique (Beckett & Rowland, 1965). Freshly prepared solutions of (+)-, (−)-, (±)-I (0·1–4 μmol per assay), III (0·01–0·8 μmol per assay), V (0·02–0·4 μmol per assay), (+)-, (−)-, (±)-VII (0·02–0·8 μmol per assay) and (+)-, (−)-, (±)- VIII (0·04–1·0 μmol per assay) and (+)-, (−)-, (±)-II (5–10 μmol per assay) in concentration ranges approximating those found in metabolic samples were used for the calibrations. All samples were carried through the complete incubation and analytical procedures described above except that the co-factor solution was omitted or inactivated tissue preparations were used. Regression analysis of these lines gave calibration coefficients of not less than 0·995. The calibrations were routinely checked during the course of the study.

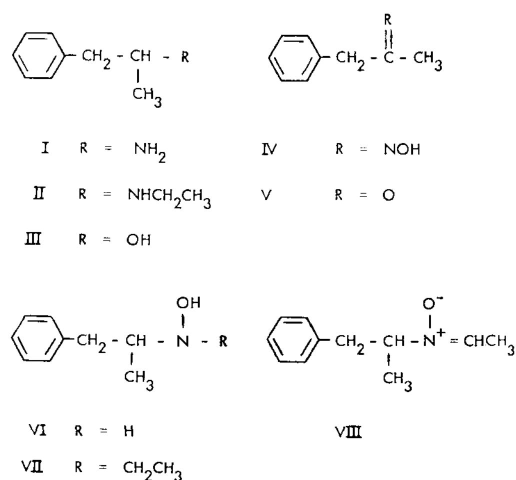

FIG. 1. Structure of ethylamphetamine (II) and its possible products following metabolic incorporation of oxygen on the nitrogen or the α-carbon atom.

RESULTS AND DISCUSSION

The metabolic oxidation of primary and secondary aralkylamines can occur by several routes (Beckett & Bélanger, 1975a). The incorporation of oxygen on the carbons adjacent to the nitrogen gives dealkylated and deaminated products, probably through unstable carbanolamines. These products, as well as a number of *N*-oxygenated compounds can also arise from a metabolic oxidative attack on the nitrogen. The possible metabolites or metabonates of ethylamphetamine (II) are given in Fig. 1; the dealkylated product, amphetamine (I), the deaminated products, 1-phenylpropan-2-ol (III) and phenylacetone (V) and the *N*-oxygenated products, *N*-hydroxyethylamphetamine (VII) and α-methyl-*N*-(1′-phenylprop-2′-yl) nitrone (VIII) were identified as products from the *in vitro* metabolism of II with male rabbit (New Zealand White) liver 9000 *g* supernatant fractions. The evidence is as follows.

Analysis of extracts from neutral solutions

A pentane extract of the incubation mixture at neutral pH was developed on t.l.c. system A. Spraying with ammoniacal silver nitrate or a basic solution of T.T.C. gave an immediate black or pink spot respectively, R_F = 0·51, which was identical to the behaviour exhibited by a co-chromatographed sample of *N*-hydroxyethylamphetamine (VII). A brownish-black spot, which developed slowly near the origin after spraying with ammoniacal silver nitrate corresponded to the characteristics of a reference sample of α-methyl-*N*-(1′-phenylprop-2′-yl)-nitrone (VIII).

A similar pentane extract when dissolved in acetonitrile and treated with BSTFA and analysed on g.l.c. system A gave a peak of retention time identical to that of the trimethylsilyl (TMS) derivative of a synthetic sample of the hydroxylamine VII; the mass spectrum obtained by scanning the peak was identical to that obtained by a scan of the reference peak. The mass spectrum gave a fragmentation pattern characteristic of TMS derivatives of *N*-hydroxyaralkylamines (Lindeke, Cho & others, 1973; Beckett & Achari, 1977) i.e. a weak molecular ion (0·5%), with diagnostic peaks at *m/e* 160 (100), 75 (23), 73 (36) and 70 (23) attributable to fragments XIX, XV, XIV and XII, respectively (Fig. 2).

When the incubation mixture was oxidized with 10% aqueous $K_3Fe(CN)_6$ at neutral pH and extracted with ether, development of the ether fraction on t.l.c. system A gave a brownish-black spot near the origin upon spraying the plate with ammoniacal silver nitrate. Analysis by g.l.c. system C of the ether extract gave a peak at Rt = 5·3 min. A synthetic sample of the nitrone VIII or that prepared by the $K_3Fe(CN)_6$ oxidation of the hydroxylamine VII as above gave identical behaviour on t.l.c. or g.l.c. and identical mass spectra. The mass spectral characteristics of the nitrone VII were similar to other arylisopropylnitrones (Beckett & others, 1973b) i.e. a weak molecular ion (1·5%), peaks at *m/e* 119 (100), 91 (75) and 70 (78) due to fragments XVI, tropylium and

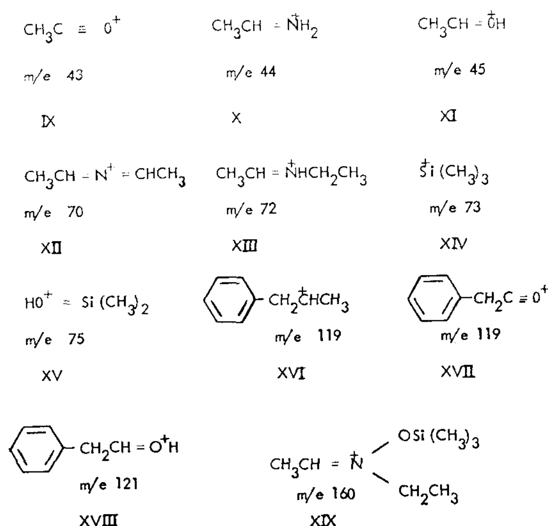

FIG. 2. Characteristic mass fragment ions of ethylamphetamine (II) and its metabolic products (I, III, V, VIII and TMS derivative of VII).

XII respectively (Fig. 2) and a small (3·5%) but important diagnostic ion at *m/e* M-15 due to the loss of a methyl radical.

Analysis of extracts from pH 12 solutions

Analysis of concentrated ethereal extracts of incubation mixtures, made alkaline before extraction, by t.l.c. system B gave two yellow spots, R_F = 0·26 and 0·41 when the chromatograms were sprayed with an ethanolic solution of DABBC. The R_F values were identical to co-chromatographed samples of ethylamphetamine (II) and amphetamine (I), respectively. Analysis of the ether extract on g.l.c. system D gave two peaks of the same retention times as reference samples of I (3·7 min) and II (6·5 min). These peaks also gave identical mass spectra to samples of I and II; a small molecular ion (0·4%), a base peak at *m/e* 44 due to fragment X (Fig. 2) with I and a small molecular ion (0·4%), base peak at *m/e* 72 and a strong peak at *m/e* 44 (45%) due to fragments XIII and X, respectively with II (Fig. 2).

Analysis of extracts from pH 1 solutions

Thin layer chromatograms (t.l.c. system A) of the ethereal extracts from acidified incubation mixtures, upon exposure to iodine vapour, gave two spots at R_F = 0·61, 0·53, i.e. R_F values identical to those of co-chromatographed samples of phenylacetone (V) and 1 phenylpropan-2 ol (VII), respectively. The R_F value of phenylacetone ketoxime (IV) using t.l.c. system A is identical to that of the ketone V which is a potential metabolite of II; however spraying a separate t.l.c. plate with 2% $CuCl_2$ (green coloration with oximes) indicated that IV was absent after incubation of II; also no peak attributable to the oxime was observed by g.l.c. analysis (system A). Thus the spot at R_F = 0·61 was attributed to the presence of V.

Analysis of the ether extract on g.l.c. system B gave two peaks, R_F = 4·6 and 6·3 min which are identical to the retention times of the ketone V and the alcohol III, respectively. The structure of these metabolites were further verified by comparison of mass spectral data to their appropriate reference compounds; a molecular ion (20%), diagnostic ions at *m/e* 119 (1%) and 43 (100%) attributable to fragments XVII and IX, respectively, as well as a strong tropylium ion peak (46%) for V; a weak molecular ion (2%), diagnostic peaks at *m/e* 121 (2%) and 45 (27%) due to fragments XVIII and XI, respectively, and a base peak at *m/e* 92 for III were obtained.

The amphetamine (I), which is formed from the metabolic dealkylation of ethylamphetamine (II), could undergo further metabolism on the nitrogen to give phenylacetone ketoxime (IV) and *N*-hydroxyamphetamine (VI). Also the nitrone VIII can chemically convert to IV or VI (Beckett & Bélanger, 1975a). However, neither of these potential products from the metabolism of ethylamphetamine (II) were detected and possible explanations are as follows. Under the neutral conditions employed for the analysis of the *N*-oxygenated products (VII and VIII), the nitrone VIII is stable and does not hydrolyse to VI. The amount of amphetamine (I) that is produced during a 30 min incubation period (maximum levels approximately $\frac{1}{3}$ that of II, Beckett & Haya, unpublished observations) is not sufficient to compete with II for the *N*-oxidase system to produce detectable levels of IV or VI. It is also possible that II is a better substrate than amphetamine (I) and thus II effectively inhibits the incorporation of oxygen to the nitrogen atom of I.

Quantitative analysis

The *N*-oxygenated metabolites of aralkylamines are unstable and may be converted to other products during the isolation and analytical procedures (Beckett, 1974); these reactions must then be prevented during the quantitative analysis of metabolites. Techniques for the quantitation of *N*-oxygenated metabolites of some aralkylamines have been reported (Cho & others, 1974, Beckett & Bélanger, 1975b; Beckett & others, 1975); however in these drugs the routes to *C*-oxygenated products

were limited or non-existant. On the other hand, ethylamphetamine (II) is susceptible to the metabolic incorporation of oxygen on the nitrogen or the α-carbons to give a complex mixture of products; however the analytical procedure outlined in Fig. 3 permits the quantitative analysis of the major *in vitro* metabolites.

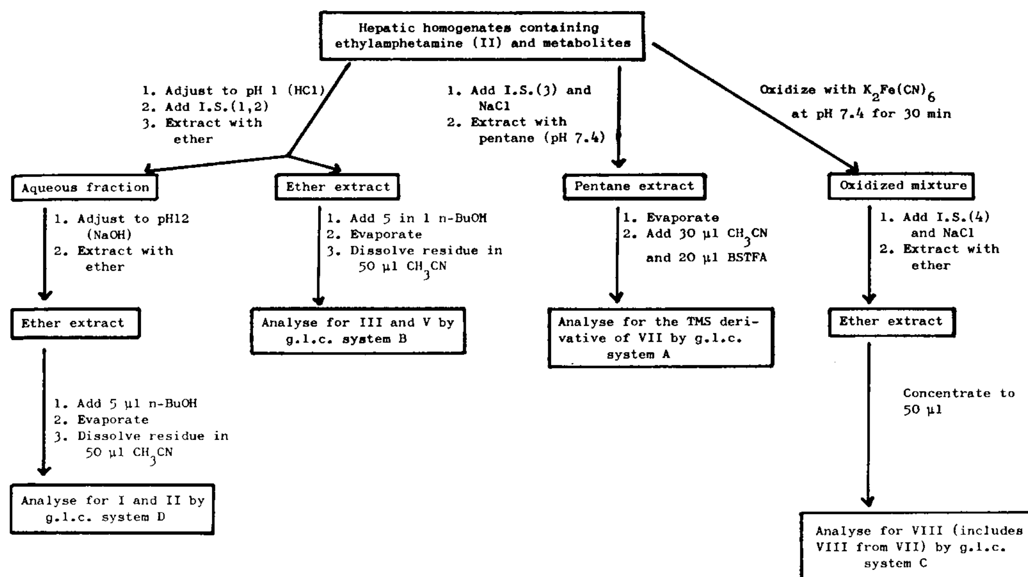

FIG. 3. Scheme for the quantitative analysis of ethylamphetamine (II) and its *in vitro* metabolic products. Internal standards (I.S): 1. *p*-chloropropiophenone; 2. *N*-propylamphetamine; 3. *p*-chlorophenyl cyclopropyl ketone; 4. Benzophenone.

N-Hydroxyethylamphetamine (VII) was unstable under the conditions of g.l.c. analysis, breaking down to the amine II and the nitrone VIII. Silylation with BSTFA in dry acetonitrile gave the immediate and quantitative formation of the trimethylsilyl (TMS) derivative of VII; TMS derivatives have been used in the quantitative analysis of other hydroxylamines (Lindeke, Cho & others, 1973; Beckett & Bélanger, 1975b; Beckett & others, 1975) and silylation appears to be a generally applicable procedure for hydroxylamines of aralkylamines (Beckett & Achari, 1977). The TMS derivative of VII was quantitated utilising g.l.c. system A. An impurity contained in ether interfered with the analysis and precluded the use of this solvent for VII; pentane was sufficiently 'clean' and complete extraction could be accomplished by saturating the aqueous media with sodium chloride.

α-Methyl-*N*-(1′-phenylprop-2′-yl)nitrone (VIII) is stable under the conditions of analysis by g.l.c. system C; however, under these conditions, the hydroxylamine VII is partially oxidized to VIII and thus direct quantitative measurement of the metabolic mixture for the nitrone VIII was not possible. The hydroxylamine VII can be stabilized as the TMS derivative, but under the conditions of the silylation procedure, partial hydrolysis of the nitrone VIII

Table 1. *T.l.c. and g.l.c. characteristics of ethylamphetamine (II) and its potential metabolites—amphetamine (I), 1-phenylpropan-2-ol (III), phenylacetone ketoxime (IV), phenylacetone (V),* N-*hydroxyamphetamine (VI),* N-*hydroxyethylamphetamine (VII), α-methyl-*N-*(1′-phenylprop-2′-yl) nitrone (VIII).*

Compound	T.l.c. *RF* values System A	T.l.c. *RF* values System B	Spray‡	G.l.c. Rt values (min) System A	G.l.c. Rt values (min) System B	G.l.c. Rt values (min) System C	G.l.c. Rt values (min) System D
I	0·03	0·41	1	1·4	3·3	0·9	3·7
II	0·05	0·26	1	2·0	3·3	1·8	6·5
VI	0·30	0·54	2, 3	4·0*	39·8†	1·3†	4·0†
VII	0·51	0·59	2, 3	6·5*	40·3†	2·0†	7·8†
VIII	0·05	0·44	2	15·5	57·5	5·3	22·4
V	0·61	0·61	4	1·6	4·6	0·9	3·0
IV	0·61	0·57	5	3·4*	39·8	1·9	7·0
III	0·53	0·58	4	1·5	6·3	1·1	3·4

* As the TMS Derivative.
† Accompanied by breakdown peaks corresponding to amine (I or II), nitrone (VIII) and/or oxime (IV).
‡ Spray reagents: 1. *NN*-dimethyl-*p*-amino benzeneazobenzoyl chloride. 2. Ammonical silver nitrate. 3. 2,3,5-triphenyltetrazolium chloride. 4. Iodine vapour. 5. Cuprous chloride (2% aqueous).

occurred. The levels of metabolically formed VIII were therefore determined by measuring the 'total amount of *N*-oxidized products' by oxidizing an aliquot of the incubation mixture with potassium ferricyanide, which quantitatively oxidizes VII to VIII (see below) and analysing by g.l.c. system C; substraction of the amount of VII, as determined above, gave the levels of metabolically formed nitrone VIII.

Complete extraction of the nitrone VIII was obtained with ether if the aqueous fraction was saturated with sodium chloride. The nitrone VIII was unstable even to storage in ether at 0° and thus samples were analysed immediately after the extraction process.

The quantitative nature of the oxidation procedure was supported by g.l.c. and t.l.c. data, i.e. complete disappearance of VII (g.l.c. system A and C, t.l.c. system A) was accompanied by the appearance of VIII. Furthermore, identical slopes were obtained for the nitrone VIII calibration curves using solutions of synthetic VIII or by oxidizing solutions of the hydroxylamine VII. Subsequent calibration curves for VIII were constructed by using freshly prepared solutions of VII followed by oxidation.

During isolation from acidic and basic solutions, the *N*-oxygenated metabolites may be partially converted to amphetamine (I) or phenylacetone (V), thus interfering with the quantitative analysis of I and V. However, when freshly prepared solutions of the hydroxylamine VII and the nitrone VIII at concentrations found in metabolic samples were subjected to the extraction procedure, the decomposition products did not significantly affect the analysis of I and V.

Straight line calibration curves were obtained for all the compounds analysed in concentration ranges (see experimental) comparable to those obtained in metabolic studies. The extractability of enantiomers from biological media may differ (e.g. stereospecific protein binding), but no significant differences were observed for the calibration curves of the enantiomers of I, II, VII and VIII in the present studies. With g.l.c. system D, the calibration curves slowly changed. When the column was used over several months a progressively increased tailing of the peaks from the amines occurred; replacing the first few cm of packing material of the column with fresh material restored the efficiency.

To ensure that the substrate II or its metabolic products, I, III, V, VII and VIII do not mutually interfere during the isolation and quantitation procedures as outlined in Fig. 3, solutions simulating *in vitro* metabolic mixtures in concentration and constituents (inactive rabbit liver washed microsomal fraction) were subjected to the incubation and analytical procedures. Quantitative recovery of all the compounds (Table 2) was obtained. Incubation of (±)-ethylamphetamine (II) with fortified 9000 *g* rabbit liver supernatant fractions gave (in μmol per 30 min) 1·68 of I, 0·17 of III, 0·14 of V, 0·28 of VII, 0·05 of VIII and 7·65 of unchanged drug; incubation with fortified washed microsomal fraction gave (in μmol per 30 min) 0·54 of I, 0·01 of V, 0·03 of VII, 0·11 of VIII and 9·29 of unchanged drug. The amounts represent total recovered of 99·7 and 100·4% respectively. Thus, the analytical scheme developed in this study permits the quantitation of ethylamphetamine II and its five major metabolites from rabbit liver homogenates. Also the results indicate that under the condition of these experiments, aromatic hydroxylation or β-carbon oxidation are not significant routes of metabolism for ethylamphetamine (II).

Table 2. *The analysis and recovery of a simulated metabolic mixture of ethylamphetamine (II) and its metabolic products (I, III, V, VII and VIII) in phosphate buffer containing rabbit liver washed microsomal fraction.*

Compound	Amount in mixture (μmol)	G.l.c. system	% recovery ±s.e.m.[a]
I	2·0	D	104 ± 3
II	8·0	D	101·8 ± 0·2
VII	0·5	A[b]	99·2 ± 1·8
VIII	1·0[c]	C	99·6 ± 0·8
V	0·2	B	100·6 ± 7·5
III	0·1	B	103·2 ± 1·5

(a) S.e. m. from 3 determinations.
(b) Analysed as the TMS derivative.
(c) 0·5 μmol of synthetic VIII + 0·5 μmol of VIII from the oxidation of VII.

Acknowledgement
One of us (K.H.) thanks the Medical Research Council of Canada for a post-doctoral fellowship.

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