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Identification of the *in vitro* *N*-oxidized metabolites of (+)- and (−)-*N*-benzylamphetamine

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This study has identified (+)- and (−)-*N*-benzyl-*N*-hydroxyamphetamine as metabolites after incubation of both (+)- and (−)-*N*-benzylamphetamine with fortified rabbit liver homogenates. The isomeric hydroxylamine metabolites were identified using the techniques of g.l.c., t.l.c. and combined g.l.c.-mass spectrometry (ms) and by comparison with results from reference samples. An additional novel metabolic product was identified after incubation of *N*-benzylamphetamine which had properties consistent with that of *N*-benzylamphetamine nitrone.

The *N*-dealkylation of basic arylalkylamines by liver homogenates has been well documented and includes the biotransformation of the *N*-substituted amphetamine and fenfluramine series of compounds (Hewick & Foutts, 1970; Chissick, 1973; Ogunbona, 1973). In contrast, the isolation of *N*-oxidized products after the incubation of *N*-substituted amphetamines with liver homogenates has proved to be more difficult, because of the labile nature of the products formed.

Preliminary evidence for the existence of *N*-oxidized metabolites arising from arylalkylamines was found using *N*-benzylamphetamine (norbenzphetamine) as substrate (Beckett, Van Dyk & others, 1971a, b). These workers postulated that certain oxidation products of *N*-benzylamphetamine could arise via the breakdown of an unstable intermediary hydroxylamine, *N*-benzyl-*N*-hydroxy-amphetamine, although no authentic compound was available at the time for comparison. The synthesis of the *N*-oxidized products of both (+)- and (−)-*N*-benzylamphetamine and their subsequent characterization from metabolic extracts are now reported.

MATERIALS AND METHODS

Synthesis of (+)- and (−)-N-benzyl-N-hydroxyamphetamine (IIa, b) and (+)- and (−)-N-(1-benzylethyl)-α-phenylnitrone (IIIa, b)

These compounds were synthesized by oxidation of the parent amine (Ia, b, Fig. 1) with *m*-chloroperbenzoic acid (Beckett, Coutts & Ogunbona, 1973a). The nitrone IIIb was recrystallized as the free base from an ether-light petroleum (50:50) (b.p. 40–60°) mixture to yield a white solid m.p. 109–110°; ultra-violet λ_{max} (borate buffer, pH 9·0): 290 nm; infrared (Nujol mull) 1140 cm^{-1} (N–O), 1575 cm^{-1} (C=N); nmr ($CDCl_3$) δ 1·50 (d, 3H, CH_3), 2·65–3·55 (m, 2H, CH_2), 3·90–4·35 (m, H, $CHCH_3$), 7·07 (s, H, CH=N), 7·15–8·30 (m, 10H, Ar); ms, *m/e* 239 (3%, molecular ion), 132 (13), 118 (100), 91 (32), 77 (13), 65 (11). Found: C, 80·0; H, 7·0; N, 5·3. Calculated for $C_{16}H_{17}NO$: C, 80·3; H, 7·1; N, 5·9%. The isomeric nitrone (IIIa) was prepared as above to yield a white crystalline solid which was recrystallized from ether–light petroleum (50:50) to give IIIa: m.p. 108–109·5°. Found: C, 80·5; H, 7·3; N, 5·7. Calculated for $C_{16}H_{17}NO$: C, 80·3; H, 7·1; N, 5·9%. The ultraviolet, nmr and ms characteristics of IIIa were identical to that of IIIb above.

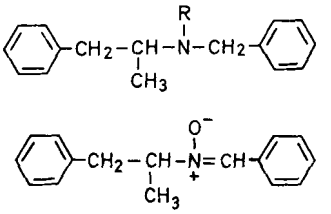

FIG. 1. Structure of (+)- and (−)-*N*-benzylamphetamine and their metabolic *N*-oxidized products.
R = H, (+)-*N*-Benzylamphetamine (Ia)
R = H, (−)-*N*-Benzylamphetamine (Ib)
R = OH, (+)-*N*-Benzyl-*N*-hydroxyamphetamine (IIa)
R = OH, (−)-*N*-Benzyl-*N*-hydroxyamphetamine (IIb)
(+)-*N*-(1-Benzylethyl)-α-phenylnitrone (IIIa)
(−)-*N*-(1-Benzylethyl)-α-phenylnitrone (IIIb)

The acid oxalate salts of the hydroxylamines IIa, b) were formed to yield the title compound IIb and recrystallized (96% ethanol) to yield white crystals: m.p. 166–167·5°; infrared (thin capillary film of free base) 3200–3600 cm^{-1} (OH); nmr ($CDCl_3$) δ 1·1 (d, 3H, CH_3), 2·4–3·4 (m, 3H, CH_2CH), 3·95 (d, 2H, NCH_2), 6·9 (broad, H, exchanges with D_2O, NO*H*), 7·1–7·4 (m, 10H, Ar); ms, *m/e* 150

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(30%), 134 (5), 91 (100), 65 (10). Found: C, 64·8, H, 6·6; N, 4·7%. Calculated for $C_{18}H_{21}O_5N$ (acid oxalate): C, 65·2, H, 6·3, N, 4·2%. The acid oxalate salt of IIa was recrystallized (acetonitrile) to yield white crystals: m.p. 167–168·5°. Found: C, 65·3; H, 6·4; N, 4·6%. Calculated for $C_{18}H_{21}O_5N$ (acid oxalate): C, 65·2; H, 6·3; N, 4·2%.

The molecular rotations of Ia, b; IIa, b; IIIa, b were determined either in aqueous or methanolic solutions on a Bendix-Ericsson Polarmatic 62 apparatus and the specific molecular rotations were equal in magnitude but opposite in direction for each pair of isomers.

Animals and preparation of liver homogenates

Male rabbits (New Zealand White, 2–3 kg) were killed after being fasted overnight and liver microsomal fractions were prepared by ultracentrifugation after a modification of the procedure of Gorrod, Temple & Beckett (1975). Liver homogenates were suspended in tris-KCl buffer, pH 7·4 to a volume equivalent to 0·5 g of original liver weight ml^{-1}. When not in use, liver homogenates were stored at 4° and not used in incubation experiments after the third day of preparation.

Standard incubation techniques

Incubations were carried out aerobically at 37° for 30 min, in duplicate, with shaking in open 25 ml Erlenmeyer flasks. 10 μmol of (+)- and (−)-*N*-benzylamphetamine (in 1 ml water) were incubated with 1 ml liver microsomal preparation (equivalent to 0·5 g original weight), 3 ml phosphate buffer, pH 7·4 and 1 ml cofactor solution as previously described (Beckett & Gibson, 1975), to a final volume of 6 ml. Incubations were terminated by immediate extraction with freshly-distilled, peroxide-free diethyl ether.

Protein assay

The protein content of rabbit liver homogenates was measured according to Lowry, Rosebrough & others (1951), using bovine serum albumin as the standard.

Mass spectrometry

The mass spectra of synthetic and metabolic *N*-oxidation products of Ia and Ib were obtained using an integrated Perkin Elmer model 270 gas chromatograph-mass spectrometer system at an electron energy of 70 eV. The instrument was fitted with a 1 m glass column, packed with 10% UCW98 on Chromosorb W, 80–100 mesh; helium pressure 15 lb in^{-2} (104 kNm^{-2}) and column temperatures 200 or 220°. In addition, solid inlet mass spectra of the synthetic *N*-oxidation products of Ia and Ib were obtained on a VG Micromass 12f mass spectrometer at an ionization energy of 70 eV and a source temperature of 240°.

Gas-liquid chromatography

All analyses of chemical and biological oxidation products of (+)- and (−)-*N*-benzylamphetamine were determined on a Perkin-Elmer F11 gas chromatograph fitted with a flame ionization detector and linked to a Hitachi Perkin-Elmer 159 recorder. The conditions used were as follows—System A: glass column, 1 m, 0·64 cm i.d., packed with 2·5% SE 30 on Chromosorb G, 100–120 mesh, oven temperature 180°, N_2 200, H_2 155, air 138 kNm^{-2}, flow rate 120 ml min^{-1}. System B: glass column, 1 m, 0·64 cm i.d., packed with 2% XE 60 on Chromosorb G, 80–100 mesh, oven temperature 185°, N_2 138, H_2 155, air 138 kNm^{-2}, flow rate 120 ml min^{-1}.

Thin-layer chromatography

Glass plates (20 × 20 cm) covered with silica gel G (0·25 mm; 1:2, silica–water), dried at room temperature, activated at 110° for 60 min were used. Authentic samples of hydroxylamine and nitrone were co-chromatographed with ethereal incubation extracts in the solvent system benzene–ethyl acetate–water (36:12:1) components on the plate were visualized with either Dragendorff's reagent or ammoniacal silver nitrate (Tollen's reagent).

Extraction and analysis of (+)- *and* (−)-N-*benzyl*-N-*hydroxy-amphetamine* (*IIa, b*)

The 6 ml incubation mixture was added to 200 μg diphenylmethyl-cyanide (in 0·1 ml methanol) as internal standard and extracted into freshly distilled, peroxide-free diethyl ether (10 ml, 2 × 20 min). The combined ether extracts were evaporated to dryness at 42° and care was taken to ensure that all the water was excluded. The extract was taken up in 50–100 μl of dry, redistilled acetonitrile and the trimethylsilyl (TMS) derivative formed by the addition of 20–50 μl of trimethylsilylimidazole, and analysed on g.l.c. system A. The amount of secondary hydroxylamine present was calculated from a standard curve using liver homogenates to which known amounts of IIa and IIb had been added.

Analysis of (+)- *and* (−)-N-(*1-benzylethyl*)-α-*phenylnitrone* (*IIIa, b*)

3 ml aliquots of the incubation medium were added

to 1 ml 10% $K_3Fe(CN)_6$ in water and shaken for 30 min. Preliminary experiments showed that the oxidation of the hydroxylamine to the corresponding nitrone was quantitative under these conditions (results not shown). 2 g of NaCl, and 100 μg benzoin in 0·1 ml methanol, as internal standard, were added and extracted into ether (10 ml, 2 × 30 min). The combined ether extracts were transferred to a finely tapered test-tube and concentrated to approximately 40 μl at 42°. The total amount of nitrone present was determined by reference to a standard curve and the amount of nitrone present in the incubation mixture was determined by subtracting the amount of hydroxylamine formed from the total amount of nitrone present after oxidation with $K_3Fe(CN)_6$, and subsequent analysis on g.l.c. system B.

RESULTS AND DISCUSSION

Initial experiments with synthetic (+)-*N*-benzyl-*N*-hydroxyamphetamine (IIa) suggested that the secondary hydroxylamine decomposed at elevated temperatures on various g.l.c. stationary phases, which precluded their use in quantitative analysis. Using g.l.c.-ms at 220° (as described under methods), IIa decomposed to form two additional peaks with Rt values of 3·5 and 7·9 min, corresponding to the secondary amine (Ia) and the nitrone (IIIa) respectively. When these peaks were scanned, they gave mass spectra which were virtually identical to those of authentic Ia and IIIa (Fig. 2). When the hydroxylamine IIa was silylated, the two breakdown peaks disappeared with the concomitant appearance of a new peak which when scanned, was shown to be the TMS derivative of IIa (Fig. 3a). The secondary amine (Ia) and the nitrone (IIIa) were stable to the g.l.c. conditions and single, sharp peaks appeared with no detectable decomposition.

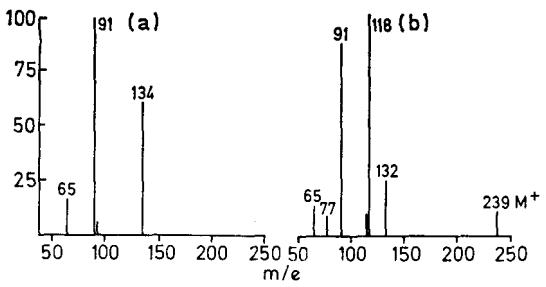

FIG. 2. Mass spectra of synthetic (a) *N*-benzylamphetamine and (b) *N*-(1-benzylethyl)-α-phenylnitrone.

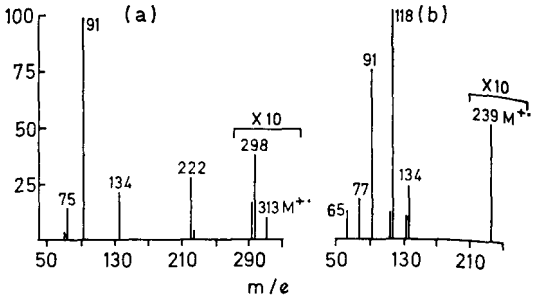

FIG. 3. Mass spectra of (a) metabolically produced *N*-benzyl-*N*-hydroxyamphetamine as its TMS derivative and (b) metabolically produced *N*-(1-benzylethyl)-α-phenylnitrone.

Identification of N-*benzyl*-N-*hydroxyamphetamine* (*IIa, b*) *after incubation of Ia and Ib with fortified rabbit liver microsomes*

After extraction of the incubation medium at neutral pH (7·4), derivatization with *N*-trimethylsilylimidazole and analysis on g.l.c. system A, a peak was observed which had an identical Rt (5·0 min) to that of the synthetic *N*-benzyl-*N*-hydroxyamphetamine TMS derivative after incubation of both isomers of benzylamphetamine with microsomal preparations. The peak was sharp and symmetrical and suitable for quantitative analysis and the isomers had identical retention times on g.l.c. system A.

In addition, after incubation of Ia or b, two additional spots appeared on t.l.c. One of these spots gave a positive reaction with Tollen's reagent producing an immediate black spot, indicative of hydroxylamine formation. This area of the chromatogram produced an orange-brown colouration with Dragendorff's reagent. The R_F value of this first spot was identical to that of authentic IIa or b (0·75). Furthermore, after oxidation of the incubation extract with $K_3Fe(CN)_6$, no hydroxylamine could be detected on g.l.c. system A. This was substantiated by t.l.c., where no hydroxylamine could be visualized with either Tollen's or Dragendorff's reagents.

After extraction of the incubation medium at neutral pH and analysis on the integrated g.l.c.–ms system, the ms of IIa (as the TMS derivative) produced a base peak at *m/e* 91 and a molecular ion at *m/e* 313. The diagnostic ions in the spectrum of the TMS derivative of IIa include fragments at *m/e* 313 (1% relative abundance, molecular ion), 312 (1), 298 (3), 297 (1), 222 (20, $M^{+\cdot} - 91$), 134 (20), 91 (100), 75 (15) and 73 (8).

Identification of (+)- *and* (−)-N-(*1-benzylethyl*)-α-*phenylnitrone* (*IIIa, b*) *after incubation of Ia and b with rabbit liver homogenates*

T.l.c. analysis of neutral incubation extracts (salted out with NaCl) and subsequent visualization with either Tollen's or Dragendorff's reagents, yielded another spot in addition to the hydroxylamine identified above. This spot produced only a slowly developing grey colouration with Tollen's reagent and an immediate orange-brown spot when sprayed with Dragendorff's reagent, and had the same R_F value (0·40) as the synthetic nitrone. Analysis of the neutral incubation extract on g.l.c. system B, after salting out with NaCl, showed the existence of a sharp, symmetrical peak which had the same Rt (12·0 min) to that of synthetic nitrone IIIa and b, which was not present in extracts of control incubations. Furthermore, the nitrone was present both before and after oxidation of the incubation medium. Unlike the hydroxylamine, the nitrone was stable on g.l.c. and could be quantitatively analysed as such without detectable decomposition.

After incubation of Ib with fortified rabbit liver 9000*g* supernatant, oxidation with $K_3Fe(CN)_6$ and analysis on the combined g.l.c.–ms system (200°), an additional peak appeared (Rt 15·2 min) which was distinct from both the hydroxylamine-TMS derivative (12 min) and the parent amine (6·2 min). The mass spectrum of this peak exhibited a base peak at *m/e* 118 and ion fragments at *m/e* 239 (5% relative abundance, molecular ion), 134 (23), 132 (11), 117 (26), 91 (74), 77 (9), 65 (10) and 51 (5). This was consistent with the structure of the nitrone and the spectra of synthetic and metabolic nitrones were essentially the same (Fig. 3b).

Evidence for the enzymatic formation of N-*benzyl*-N-*hydroxyamphetamine and* N-(*1-benzylethyl*)-α-*phenylnitrone*

Both isomers of *N*-benzylamphetamine were incubated with rabbit liver 9000*g* supernatant in the presence and absence of the cofactor solution, as described under methods. When the cofactor was omitted, only trace amounts of the secondary hydroxylamine and total *N*-oxidized products (hydroxylamine plus nitrone) could be detected on g.l.c. systems A and B respectively. The requirement for reduced nucleotide in the *in vitro N*-oxidation process has been reported both for aliphatic and aromatic amines (Uehleke & Tabarelli, 1973; Ziegler, McKee & Poulsen, 1973; Cho, Lindeke & Sum, 1974), and is an essential requirement for the conversion of *N*-benzylamphetamine to the hydroxylamine and nitrone.

In addition, when (+)- and (−)-*N*-benzylamphetamine were incubated with increasing amounts of liver microsomal protein and an NADPH regenerating system, the amounts of hydroxylamine and total *N*-oxidized products formed (measured as nitrone), were found to be proportional to the concentration of protein, at least up to 1 mg ml^{-1} of incubation medium (Fig. 4). The formation of the secondary hydroxylamine (IIb) and the total *N*-oxidized products (IIb + IIIb) were linear with time, at least up to 60 min, after incubation of Ib with fortified rabbit liver microsomal preparations (Fig. 5). This remarkable linearity range appears to be peculiar to the rabbit where linearity of metabolism with time has been noted for periods up to 90 min (Gram & Fouts, 1966; Cho & others, 1974). Linear regression analysis of the metabolism *vs* time curves yielded rates of formation of 0·30 and 0·83 nmol product formed min^{-1} mg^{-1} protein for the hydroxylamine (IIb) and the total *N*-oxidized products (IIb + IIIb) respectively; thus the nitrone (IIIb) was the major *N*-oxidation product after incubation of Ib with rabbit liver microsomes.

Thus, the physicochemical properties of *N*-benzyl-*N*-hydroxyamphetamine and *N*-(1-benzylethyl)-α-phenylnitrone derived from both (+)- and (−)-*N*-benzylamphetamine have been described and shown to be metabolites of the parent amine after incubation with fortified rabbit liver homogenates and their

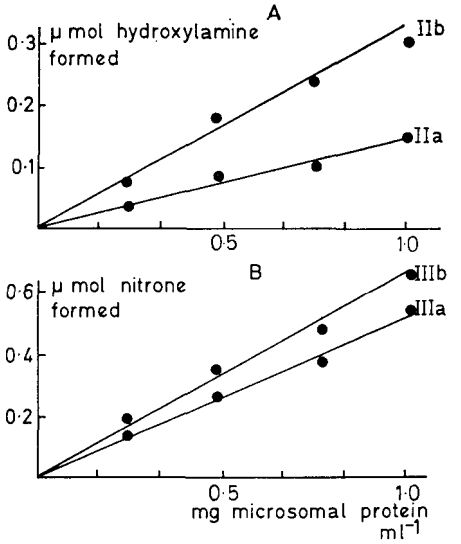

FIG. 4. Effect of liver microsomal protein concentration on the metabolic production of (A) (+)- and (—)-*N*-benzyl-*N*-hydroxyamphetamine (IIa, b) and (B) (+)- and (—)-*N*-(1-benzylethyl)-α-phenylnitrone (IIIa, b).

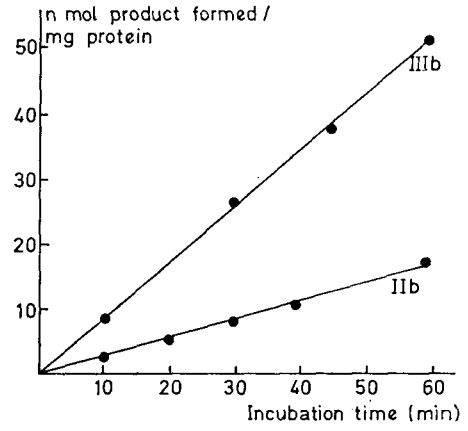

FIG. 5. Rate of formation of (—)-*N*-benzyl-*N*-hydroxyamphetamine (IIb) and (—)-*N*-(1-benzylethyl)-α-phenylnitrone (IIIb) after incubation of the parent amine (Ib) with rabbit liver microsomes in the presence of an NADPH generating system.

structures determined by g.l.c., t.l.c. and g.l.c.–ms. Although hydroxylamines have previously been shown to be metabolic products of secondary aliphatic amines (Beckett & Al-Sarraj, 1973; Midha, Beckett & Saunders, 1974; Beckett, Al-Sarraj & Essien, 1975), the existence of nitrones in drug metabolism is comparatively novel (Beckett, Coutts & Ogunbona, 1973b). Previous workers have shown that *N*-alkyl nitrones may undergo solvolysis and generally hydrolyse readily (Grammaticakas, 1937; Exner, 1951), although aryl nitrones have been reported as being more resistant to hydrolysis (Hamer & Macaluso, 1964). The stability of *N*-benzylamphetamine nitrone (IIIa, b) can be accounted for by the presence of a double bond in conjugation with the aromatic system and consequently, the nitrone is identified and quantified both chemically and metabolically.

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