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Microsomal formation of *N*-benzyl-4-hydroxymethylaniline from *N*-benzyl-4-methylaniline

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Abstract—The in-vitro metabolism of *N*-benzyl-4-methylaniline was re-examined using male hamster and rabbit hepatic microsomes; both species generated *N*-benzyl-4-hydroxymethylaniline, confirmed by TLC and HPLC, comparison with authentic compound. Further confirmation of the formation of this metabolite was achieved by use of a rapid scan UV detector.

Secondary aromatic amines are metabolized by both *C*- and *N*-oxidation (Uehleke 1971). Some *N*-substituted anilines have been shown to undergo metabolic *N*-dealkylation (Gorrod et al 1975) and ring hydroxylation (Gorrod & Gooderham 1981).

A methyl group attached to a ring system is a common structure in drugs and other xenobiotics, and often undergoes hydroxylation to produce the corresponding hydroxymethyl compounds. The role of hydroxymethyl compounds in toxicity has been reviewed by Gorrod (1979).

The metabolism of *N*-benzyl-4-methylaniline (NB4MA) in-vitro has been reported previously (Gorrod & Gooderham 1985) who showed the formation of a number of metabolites including benzaldehyde, the corresponding nitrone, amide and 4-toluidine together with an unknown metabolite which was tentatively proposed as *N*-benzyl-4-hydroxymethylaniline (NB4HMA).

The aim of this study was to synthesize the suggested compound, develop sensitive and specific analytical methods, and to establish whether it is metabolically produced during incubation of NB4MA and microsomal preparations.

Materials and methods

The synthesis and characteristics of N-*benzyl-4-hydroxymethylaniline.* 4-Aminobenzylalcohol (Lancaster Synthesis, Lancaster, UK) (0·008 mol) was refluxed with sodium carbonate (0·43 mg) and acetone (25 mL) at 90°C. Benzyl chloride (0·020 mol) in acetone (15 mL) was added dropwise over 30 min. The reaction was monitored by TLC and the reaction stopped when the level of a third component was maximum. The reaction mixture was cooled and excess solvent removed using a rotary film evaporator to yield an oil. The required compound was obtained by column chromatography (35 × 2 cm) using silica gel as the stationary phase in conjunction with petroleum ether (bp 40–60°C): acetone 7:3 v/v). The eluates were monitored by TLC and fractions containing the product were bulked and evaporated to dryness to yield an oil. The structure of the new compound was established as *N*-benzyl-4-hydroxymethylaniline, by UV, IR, NMR, MS and elemental analysis methods.

IR: 3260 cm^{-1} (N–H stretch of secondary aryl amine), 1000 cm^{-1} (C–O stretch of hydroxyl group). UV: (methanol) max = 208 and 253 nm. MS: m/e = 213 (69%M^+), 196 (13%), 91 (100%). ^1H NMR: ($CDCl_3$): (ppm) = 4·3 (s, 2H, C_{10} protons), 4·7 (s, 2H, C_7 protons), 6·6 (d, 2H, C_3, C_5 protons), 7·2 (d, 2H, C_2, C_6 protons), 7·35 (s, 5H, C_{12}, C_{13}, C_{14}, C_{15}, C_{16} protons) (Fig. 1).

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$C_{14}H_{15}NO$ (213·16). Calcd: C: 78·85, H: 7·09, N: 6·57%; found: C: 78·99, H: 7·15, N: 6·27%.

HPLC separations of NB4HMA from NB4MA and its previously identified metabolites. The isocratic HPLC chromatograph (Gooderham & Gorrod 1984) comprised an ACB pump, a model 7125 syringe loading sample injector valve fitted with a 20 μL sample loop, a Milton Roy variable wavelength UV detector and a CI 4000 computing integrator. A rapid scan SA6508 UV detector was connected to obtain UV spectra of metabolites on the HPLC. The analytical column contained Spherisorb 5 μm ODS (250 × 4·6 mm i.d.) and the pre-column material was co-pellicular ODS. The mobile phase composition was acetonitrile: 0·02 M phosphate buffer (pH 7, 45:55 v/v) at a flow rate of 1 mL min^{-1}. The column eluate was monitored at 254 nm.

The HPLC separation of substrate and potential metabolites is shown in Fig. 2.

TLC separation. TLC systems and chromogenic agents used to separate and identify the substrate and potential metabolites are described in Table 1.

Incubation and extraction procedures. Glucose-6-phosphate dehydrogenase was purchased from Boehringer Mannheim Corporation (London) Ltd (UK). Nicotinamide adenine dinucleotide phosphate (NADP) was obtained from Sigma Ltd (Poole, Dorset, UK). Methylaniline was purchased from Aldrich Chemical Company (Gillingham, Dorset, UK). NB4MA, *N*-benzoyl-4-methylaniline and the corresponding nitrone were previously prepared in this laboratory. Glucose-6-phosphate was purchased from British Drug Houses, Dorset (UK). All other chemicals used in the experiments were supplied as mentioned earlier. The animals used were hamsters and rabbits. Hepatic microsomes used in experiments were prepared at 0–4°C using the calcium chloride precipitation method (Schenkman & Cinti 1978). Incubations were carried out in a shaking water bath at 37°C. Typical incubations were carried out using a standard cofactor solution at pH 7·4. Cofactors were preincubated for 5 min before the addition of microsomes and substrate (5 μmol). The incubation time was 30 min, the reaction was terminated and the reaction mixture extracted with dichloromethane (2 × 5 mL). Organic extracts were evaporated to dryness under a stream of nitrogen in a water bath at 45°C. The residues were reconstituted in 200 μL of methanol for HPLC and 50 μL of methanol for TLC studies.

Result and discussion

The in-vitro metabolism of NB4MA by both rabbit and hamster

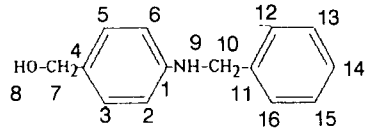

FIG. 1. The structure of NB4HMA.

Table 1. TLC systems, Rf (× 100) values and chromogenic agents used to separate and identify *N*-benzyl-4-methylaniline and its metabolites.

	Rf value (× 100) in solvent systems				Chromatographic response with detection systems				
Compound	S_1	S_2	S_3	S_4	D_1	D_2	D_3	D_4	D_5
N-Benzyl-4-methylaniline	61	51	67	80	Dark blue	Yellow	–	Red	Yellow
4-Methylaniline	25	18	33	74	Dark blue	Yellow	Orange	Red	Red
N-Benzoyl-4-methylaniline	38	23	52	69	Light blue	–	–	–	–
N-Benzyl-4-hydroxymethylaniline	14	9	27	37	Dark blue	Yellow	Grey	Red	Yellow
N-(4-methylphenyl)-α-phenylnitrone	9	6	35	61	Dark blue	Yellow	Black	–	–

S_1 = Benzene: ethylacetate (9:1). S_2 = Petroleum spirit (bp 40–60°C): ethyl acetate (8:2). S_3 = Benzene: ethyl acetate: acetone (8:1:1). S_4 = Petroleum spirit (bp 40–60°C): acetone (7:3). D_1 = UV light 254 nm. D_2 = Ehrlichs reagent (*p*-dimethylaminobenzaldehyde 0·1% in HCl). D_3 = Tollens reagent (Ammoniacal silver nitrate). D_4 = Diazotised *p*-nitroaniline followed by Na_2CO_3 10%. D_5 = Sodium nitrite (0·5%) (in 1 M NaOH in 50% aqueous ethanol).

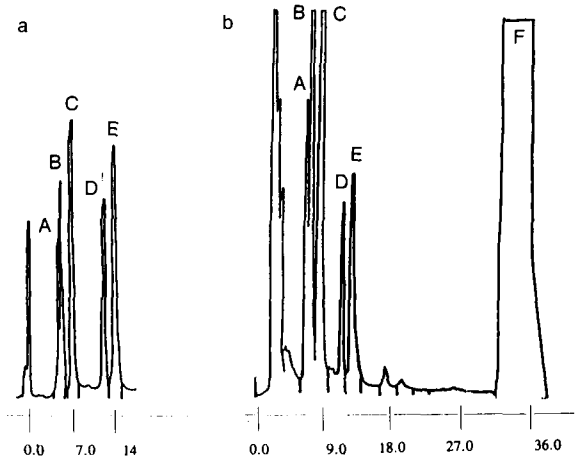

FIG. 2.a. HPLC separation of putative *N*-benzyl-4-methylaniline metabolites. b. HPLC chromatogram of in-vitro *N*-benzyl-4-methylaniline metabolites formed by hepatic microsomes of hamster. A, aniline; B, benzaldehyde; C, *N*-benzyl-4-hydroxymethylaniline; D, *N*-benzoyl-4-methylaniline; E, *N*-(4-methylphenyl)-α-phenylnitrone; F, *N*-benzyl-4-methylaniline.

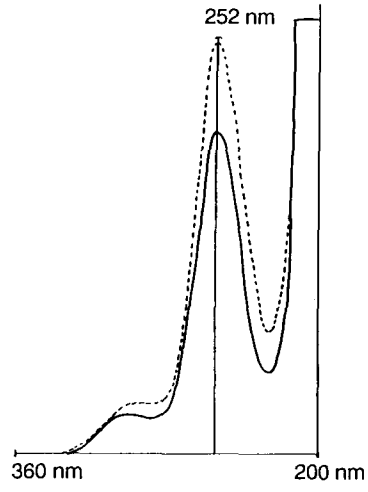

FIG. 3. UV spectra of authentic (——) and metabolically formed (– – –) NB4HMA, obtained with the rapid scan detector (peak C, Fig. 2a, b).

microsomes showed the formation of the expected uncharacterized metabolite together with the previously identified metabolites (Gorrod & Gooderham 1985). A typical HPLC chromatogram of an extract from hepatic hamster microsomes is shown in Fig. 2. The unidentifiable metabolite had a retention time of 8·6 min, co-chromatographed with authentic *N*-benzyl-4-hydroxymethylaniline (NB4HMA) and had an identical UV spectrum (Fig. 3). TLC characteristics of the metabolite were identical to the synthetic compound (Table 1). No metabolites were observed in control experiments. Hamster microsomes were more efficient compared with rabbit microsomes in producing the compound. Although hydroxymethyl compounds are usually intermediates in the conversion of methyl groups to carboxylic acids and therefore may only be present at low concentrations (Gorrod 1979), the metabolism of *N*-benzyl-4-methylaniline yielded the corresponding hydroxymethyl metabolite as a major product.

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