Journal of Chromatography, 202 (1980) 287–293
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 13,221

Note

Identification of a new metabolite after incubation of N-benzylaniline with rabbit liver microsomes

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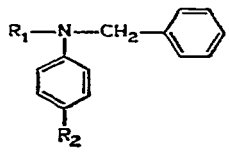
(Received August 6th, 1980)

Previous workers reported the *in vitro* metabolism of N-benzylaniline in an investigation of the toxicity of alkyl- and aryl-substituted anilines[1]. Aniline and nitrobenzene were obtained as metabolites after intravenous injection of N-benzylaniline (1) into cats.

Primarily, we were studying the metabolism of the antihistaminic drug antazoline (3). We have shown that N-benzylaniline was one of the major metabolites of antazoline[2]. We therefore found it necessary to examine the metabolic fate of N-benzylaniline (1) using rabbit liver microsomal fraction. The stability and extractability of the metabolic product under the conditions of incubation and analysis were also examined. Structures of the compounds concerned are shown in Fig. 1.



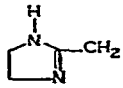
R_1	R_2	Compound
	H	Antazoline, 2-(N-benzylanilinomethylimidazoline)
H	H	N-Benzylaniline
H	OH	p-Benzylaminophenol

Fig. 1. Structures of antazoline (3), N-benzylaniline (1) and *p*-benzylaminophenol (2).

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EXPERIMENTAL

Compounds and reagents

Antazoline·HCl (3) was supplied by Ciba-Geigy (Basle, Switzerland). N-Benzylaniline (1) was obtained from BDH (Poole, Great Britain), *p*-benzylaminophenol (2) from Eastman (Rochester, NY, U.S.A.), $NADPNa_2$, glucose-6-phosphate, disodium salt, and glucose-6-phosphate dehydrogenase from Boehringer (Mannheim, G.F.R.), NO-bis(trimethylsilyl)trifluoroacetamide (BSTFA) from Pierce (Rockford, IL, U.S.A.), acetonitrile from Fisons (Loughborough, Great Britain) (redistilled and kept over calcium chloride at room temperature), AnalaR diethyl ether from BDH (freshly distilled), 2,3,5-triphenyl-2*H*-tetrazolium chloride monohydrate (98%) from Aldrich (Milwaukee, WI, U.S.A.), *n*-pentane from Fisons, iron(III) chloride from May and Baker (Dagenham, Great Britain) and sodium metabisulphite ($Na_2S_2O_5$) from BDH.

Thin-layer chromatography (TLC)

Glass plates (20 × 20 cm) were sprayed to a thickness of 0.25 mm with a mixture of silica gel G (Merck, Darmstadt, G.F.R.) and water (1:2). The plates were allowed to dry at room temperature for 1 h at 110°C before use. The solvent system was chloroform–acetone (9:2). The various spots were revealed by spraying with (a) ammoniacal silver nitrate solution; (b) triphenyltetrazolium chloride (TTC); (c) iron (III) chloride solution (5% in 0.5 *N* hydrochloric acid) and (d) Dragendorff's reagent. These spray reagents were prepared and used according to the methods described by Merck[3].

Gas–liquid chromatography (GLC)

A Perkin-Elmer Model F33 gas chromatograph equipped with a flame-ionization detector and a 1.0-mV Perkin-Elmer recorder was used. All columns were acid-washed and treated with dimethyldichlorosilane. The conditions were as follows. System A: 2-m glass column packed with Chromosorb Q (100–120 mesh) coated with OV-17 (3%, w/w) and operated with a hydrogen pressure of 1.12 kg/cm², air 1.68 kg/cm² and nitrogen 2.1 kg/cm². System B: 2-m glass column packed with Chromosorb W (80–100 mesh) coated with OV-17 (5%, w/w) and operated under the conditions described under system A. System C: 1-m glass column packed with Chromosorb W (80–100 mesh) coated with UCW-98 (10%, w/w) and operated with a hydrogen pressure of 2.1 kg/cm², air 0.7 kg/cm² and nitrogen 1.4 kg/cm². All of the columns were conditioned at 250°C for 24 h before use and the injection port temperature was 250°C. Each of these columns was silanized with 2 × 5 ml of hexamethyldisilazane (HMDS) before use.

Combined gas–liquid chromatography and mass spectrometry

All mass spectra were obtained using a Perkin-Elmer Model 270 gas chromatograph–mass spectrometer at an electron energy of 70 eV. A 1.0-m × 0.64 cm O.D. glass column packed with UCW-98 (10%, w/w) on Chromosorb W (80–100 mesh) was used at 190°C (oven temperature); helium (1.4 kg/cm²) was used as the carrier gas.

Metabolism

Incubation procedure. N-Benzylaniline·HCl (1) (10 μmol/ml; 1 ml) was in-

cubated at 37°C for 40 min with the microsomal fraction from a liver homogenate of a New Zealand white rabbit. Each incubation mixture contained 1 ml of substrate, 1 ml of cofactor solution made of glucose-6-phosphate, disodium salt (6 mg, 20 μmol), 0.6 *M* nicotinamide (0.1 ml, 60 μmol), $NADPNa_2$ (3.4 mg, 4 μmol), distilled water (0.7 ml); phosphate buffer, pH 7.4 (3 ml) and liver microsomal fraction (1 ml). Glucose-6-phosphate dehydrogenase (2 units) was added to the microsomal fraction preparation. Control experiments were carried out at the same time. In all instances, the incubation mixtures were incubated for 5 min at 37°C with shaking before the addition of the substrate. The incubation reactions were stopped by putting the flasks in ice and extracted as described.

p-Benzylaminophenol (2). To the incubation mixture (6 ml) was added $Na_2S_2O_5$ solution (10%, 1 ml) and sodium chloride (2 g). The pH was adjusted to 8.0–8.2 with ammonia solution (10%) and the mixture was extracted with freshly distilled diethyl ether. The concentrated ethereal extracts were examined by GLC systems A, B and C and by TLC. A separate portion of the concentrated ethereal extracts was allowed to dry under nitrogen gas; dry acetonitrile (10–15 μl) and BSTFA (10 μl) were added and the mixture was allowed to stand at room temperature for 5 min. The trimethylsilyl derivative of *p*-benzylaminophenol (4) was then examined by GLC system A.

Extractability of p-benzylaminophenol (2) from buffer and microsomal preparations. Two solutions (a, b) of *p*-benzylaminophenol (2, 20 μg/ml, base) were freshly prepared in phosphate buffer (pH 7.4)–methanol (9:1, v/v) (a) and in freshly distilled diethyl ether (b). To two tubes each containing 5 ml of solution (a) were added rabbit liver microsomes (0.25 g/ml, 1 ml), sodium chloride (2 g) and $Na_2S_2O_5$ solution (10%, 1 ml). To another set of tubes [2 × 5 ml, (a)] was added phosphate buffer (pH 7.4, 1 ml) instead of the liver microsomes. All of the samples were adjusted to pH 8.0 with ammonia solution (10%) and extracted with freshly distilled diethyl ether (4 × 5 ml). N-Benzoylaniline (5) as an external standard (50 μg/ml, base in *n*-pentane, 1 ml) was added to each of the ethereal extracts and also to each of two samples (2 × 5 ml) of solution (b). The ethereal solutions were concentrated and analysed by GLC system B. The average peak-height ratios (*p*-benzylaminophenol/N-benzoylaniline) were compared and the value obtained for solution (b) was arbitrarily taken as 100%.

Stability of p-benzylaminophenol (2) in phosphate buffer (pH 7.6). A freshly prepared solution of *p*-benzylaminophenol (2, 20 μg/ml, 5 ml) in phosphate buffer (pH 7.4)–methanol (4:1, v/v) was placed in each of two 20-ml centrifuge tubes (a and b). Phosphate buffer (1 ml) was added to tube (a) and $Na_2S_2O_5$ solution (10%, 1 ml) to tube (b). Duplicate samples were taken in each instance. Both tubes were stored at room temperature for 40 h and the samples were then extracted and analysed by GLC system B as described above. Two control samples (1 and 2) of *p*-benzylaminophenol (2, 20 μg/ml, in diethyl ether, 5 ml each) were also prepared. The average peak-height ratios (*p*-benzylaminophenol/N-benzoylaniline) were compared and the value obtained for the control samples (1 and 2) was arbitrarily taken as 100%.

Quantitative analysis of p-benzylaminophenol (2). Standard solutions were always freshly prepared. Calibration graphs (in the appropriate biological fluid or buffer) based on the ratios of the peak heights of the compounds to be quantitated to

those of their GLC reference standards using the methods described were obtained representing six different concentrations (50–5 μg/ml, base). The results were subjected to linear regression analysis to give the appropriate calibration factors. In all metabolic studies *p*-benzylaminophenol (2) was analysed as its trimethylsilyl (TMS) derivative with methadone as the external standard using GLC system A.

RESULTS AND DISCUSSION

Stability and extractability of p-benzylaminophenol (2)

Complete decomposition of *p*-benzylaminophenol (2) occurred when it was kept in phosphate buffer (pH 7.4) for 40 h. However, 62% of *p*-benzylaminophenol was recovered from a similar buffer solution containing sodium metabisulphite (10%, Table I). Sodium metabisulphite, a known antioxidant, has been used to stabilize adrenaline injectable solutions (BP 1973)[4]. Sodium metabisulphite was therefore used to stabilize *p*-benzylaminophenol (2) aqueous solution throughout these studies.

TABLE I

STABILITY OF *p*-BENZYLAMINOPHENOL (2) STORED (a) IN PHOSPHATE BUFFER (pH 7.4) AND (b) IN PHOSPHATE BUFFER CONTAINING $Na_2S_2O_5$ (10%, 1 ml) FOR 40 h AT ROOM TEMPERATURE

Results are expressed as a percentage of the quantitative recovery of a control (samples 1 and 2, as described under Experimental). Duplicate samples were analysed in each instance.

Sample	*Average peak-height ratio*	*Recovery (%)*
Control	2.4	100
a	0	0
b	1.5	62

When *p*-benzylaminophenol (2) was extracted from phosphate buffer (pH 7.4) and phosphate buffer containing liver microsomes (the final microsome concentration was the same as that used in incubation experiments) at pH 8.0 with diethyl ether, the recoveries were 98 and 93%, respectively (Table II). The extraction method was thus demonstrated to be satisfactory. Extraction at pH values above 8.5 or the use of strong alkalis for pH adjustment is to be avoided as such conditions may lead to

TABLE II

RECOVERY OF *p*-BENZYLAMINOPHENOL (2) (a_1) IN PHOSPHATE BUFFER pH 7.4 AND (a_2) IN PHOSPHATE BUFFER CONTAINING RABBIT LIVER MICROSOMAL FRACTION AT THE SAME DILUTION AS USED IN INCUBATION EXPERIMENTS

Results are expressed as a percentage of quantitative recovery of a control (sample b, as described under Experimental). Duplicate samples were analysed in each instance.

Sample	*Average peak-height ratio*	*Recovery (%)*
Control	2.3	100
a_1	2.2	98
a_2	2.1	93

chemical breakdown of *p*-benzylaminophenol (2). It is important to check the recovery of compounds from liver homogenate preparations, as binding to such homogenates may lead to large errors in analytical procedures[5,6].

Quantitative analysis of p-benzylaminophenol (2)

Column A (Table III) was chosen for the quantitative analysis of *p*-benzylaminophenol (2) produced metabolically because of the shorter retention time obtained for *p*-benzylaminophenol (2). Both *p*-benzylaminophenol (2) and N-benzoylaniline (5) (external standard) gave tailing peaks and were not well separated on column A. A sharp and symmetrical peak was obtained for *p*-benzylaminophenol (2) after trimethylsilylation with BSTFA. Methadone (6) was found to be a suitable reference compound on the same column (A), giving sharp, symmetrical and well separated peaks. However, it was not possible to extract methadone (6) completely in the pH range (8.0–8.2) used for the *p*-benzylaminophenol (2) extraction. Therefore, methadone (6) was used as an external standard, to be added to the ethereal extract containing *p*-benzylaminophenol (2). Straight-line calibration graphs were obtained for *p*-benzylaminophenol (2) using the above method. In all instances the calibration graphs were constructed using the appropriate liver homogenate preparation.

Identification of the metabolic product

p-Benzylaminophenol (2) was identified as a metabolic product of N-benzoylaniline (1) as follows: (i) TLC of the ethereal extract (pH 8.0) gave a spot with an R_F value of 0.39. Black, violet and yellow spots were obtained upon spraying with ammoniacal silver nitrate, iron(III) chloride and triphenyltetrazolium chloride (TTC) reagents, respectively (Table III). (ii) GLC of the ethereal extract (pH 8.0) gave peaks with retention times of 6.0, 17.0 and 15.0 min on columns A, B and C, respectively (Table III). Derivatization with BSTFA gave the trimethylsilyl derivative of *p*-benzylaminophenol (4), with a retention time of 7.0 min (column A, Table III). (iii) The GLC–mass spectrum of the ethereal extract (pH 8.0) showed the presence of fragment ions at *m/e* 91 (100%) and 199 (50%), corresponding to the base peak and the molecular ion, respectively (Fig. 2a and b). The mass spectrum of the trimethyl-

TABLE III

GLC AND TLC CHARACTERISTICS OF ANTAZOLINE (3) N-BENZYLANILINE (1), *p*-BENZYLAMINOPHENOL (2) AND RELATED COMPOUNDS

*Compound**	*GLC retention times (min)***			*TLC R_F values*
	Column A	*Column B*	*Column C*	
Antazoline	11.0(240°C)	—	—	On the baseline
N-Benzylaniline	1.5(220°C)	5.0(230°C)	4.0(190°C)	0.96
AC N-benzylaniline	—	—	8.0(190°C)	—
p-Benzylaminophenol	6.0(220°C)	17.0(230°C)	15.0(190°C)	0.66
TMS *p*-benzylaminophenol	7.0(215°C)	—	—	—

* TMS = trimethylsilyl derivative; AC = acetyl derivative.

** The appropriate GLC oven temperatures are given in parentheses.

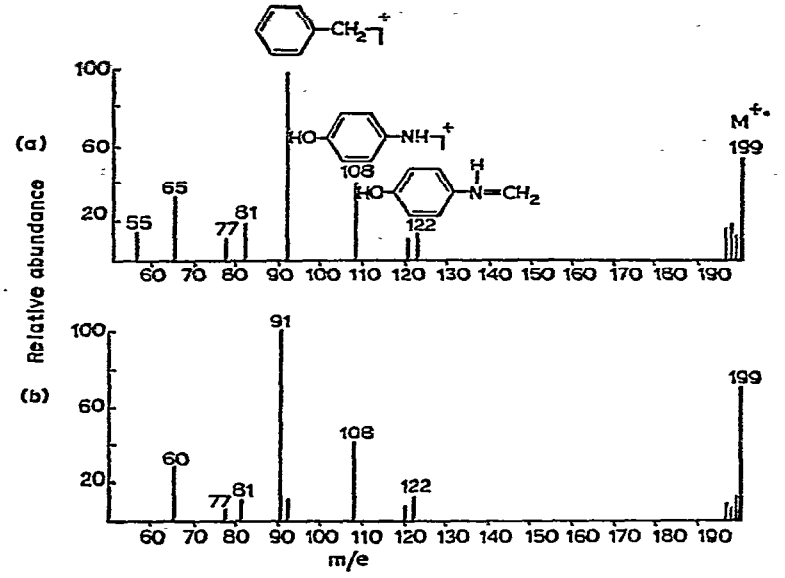

Fig. 2. Mass spectra of (a) synthetic *p*-benzylaminophenol (2) and (b) metabolically produced *p*-benzylaminophenol from N-benzylaniline (1).

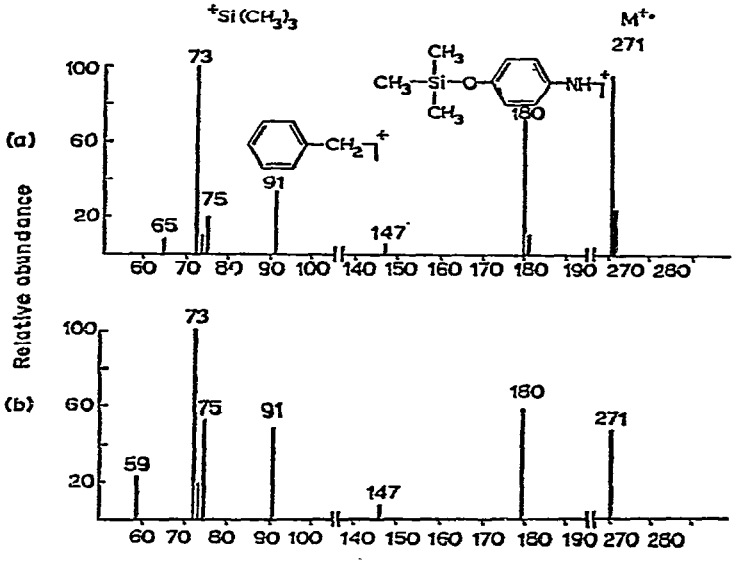

Fig. 3. Mass spectra of (a) synthetic *p*-benzylaminophenol and (b) metabolically produced *p*-benzylaminophenol from N-benzylaniline (1) as their trimethylsilyl derivatives.

silyl derivative (4) gave fragment ions at *m/e* 73 (base peak), 271 (molecular ion), 180, 91 and 75 (Fig. 3a and b). The fragment ions at *m/e* 73 and 75 are indicative of the trimethylsilyl derivatives[7,8].

The characteristics of the metabolically produced *p*-benzylaminophenol described above are identical with those of the authentic reference compound (2).

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