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Note

Identification of two *in vitro* metabolic products after liver microsomal incubation of antazoline

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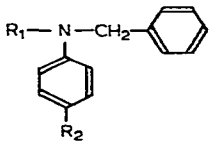
Antazole (Fig. 1) is an antihistaminic drug, with local anaesthetic and some anticholinergic properties. It is claimed to be less irritating to the tissues than most other antihistamines, and only two cases of lethal antazoline intoxication have been reported[1]. Blomquist *et al.*[1] reported two hydrolysis products of antazoline after autopsy in a case of lethal intoxication; (phenylbenzylaminoacetyl)ethylenediamine was the major hydrolysis product, together with N-benzylaniline in trace amounts.



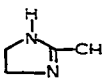
R_1	R_2	Compound
	H	Antazoline [2-(N-benzyl-anilinomenyl)imidazoline]
H	H	N-benzylaniline
H	OH	*p*-benzylaminophenol

Fig. 1. Structures of antazoline, N-benzylaniline and *p*-benzylaminophenol.

No reports on antazoline metabolism, to our knowledge, have been published previously. Therefore, we decided to study the metabolism of this drug to establish whether the two products reported[1] are metabolites or chemical decomposition products.

This paper describes investigations into the *in vitro* metabolism of antazoline using rabbit liver microsomal fraction.

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EXPERIMENTAL

Compounds and reagents

Antazoline hydrochloride was kindly supplied by Ciba-Geigy (Basle, Switzerland). N-Benzylaniline was obtained from BDH (Poole, Great Britain) and *p*-benzylaminophenol from Eastman (Rochester, NY, U.S.A.). $NADPNa_2$, glucose-6-phosphate Na_2 and glucose-6-phosphate dehydrogenase were purchased from Boehringer (Mannheim, G.F.R.), N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) from Pierce (Rockford, IL, U.S.A.), acetic anhydride from BDH, 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 & Baker (Dagenham, Great Britain) and sodium metabisulphite from BDH.

Thin-layer chromatography (TLC)

Glass plates (20 × 20 cm) were spread 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 first allowed to dry at room temperature and were heated for 1 h at 110°C before use. The solvent system was chloroform–acetone (90:20, v/v). The various spots were revealed by spraying with (a) ammoniacal silver nitrate solution, (b) triphenyltetrazolium chloride (TTC), (c) iron(III) chloride (5% in 0.5 *N* hydrochloric acid) and (d) Dragendorff's reagent. These spray reagents were prepared and used according to the methods described in ref. 2.

Gas–liquid chromatography (GLC)

A Perkin-Elmer Model F33 gas chromatograph equipped with a flame-ionization detector and a 1.0-mV Perkin-Elmer 56 recorder were used. All columns were made of glass, 0.64 cm O.D., and the solid supports were acid washed and treated with dimethyldichlorosilane.

System A was a glass column, 2 m long, packed with Chromosorb Q (100–120 mesh) coated with OV-17 (3%, w/w) and operated under the following conditions: hydrogen, 1.12 kg/cm^2; air, 1.68 kg/cm^2; nitrogen, 2.1 kg/cm^2.

System B was a glass column, 2 m long, packed with Chromosorb G (80–100 mesh) coated with OV-17 (5%, w/w) and operated under the same conditions as for system A.

System C was a glass column, 1 m long, packed with Chromosorb W (80–100 mesh) coated with UCW-98 (10%, w/w) and operated under the following conditions: hydrogen, 2.1 kg/cm^2; air, 0.7 kg/cm^2; nitrogen, 1.4 kg/cm^2.

All the columns were conditioned at 250°C for 24 h before use and the injection port temperature was 250°C. Each column was silanized with two 5-μl portions of hexamethyldisilizane (HMDS) before use.

Combined gas–liquid chromatography–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/cm^2) was used as the carrier gas.

Incubation procedure

Antazoline hydrochloride (10 μmol/ml as the base; 1 ml) was incubated at 37°C for 40 min with the microsomal fraction from a liver homogenate of a New Zealand white rabbit[3]. Each incubation mixture contained 1 ml of substrate; 1 ml of cofactor solution consisted of glucose-6-phosphate Na_2 (6 mg, 20 μmol), nicotinamide (0.6 *M*) (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) were also used. 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.

Extraction procedure

(a) Antazoline and N-benzylaniline. To the incubation mixture (6 ml) was added sodium chloride (2 g); the pH was adjusted to 9–10 with ammonia solution (10%) and the mixture was extracted with freshly distilled diethyl ether. The concentrated ethereal extracts were analysed by GLC systems A and B and by TLC. Also, acetic anhydride was added to ethereal solutions of authentic N-benzylaniline and to the concentrated ethereal extracts of antazoline incubation mixture prior to GLC.

(b) p-Benzylaminophenol. To the incubation mixture (6 ml) was added $Na_2S_2O_5$ (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 using GLC with systems A, B and C and by TLC. A separate portion of the concentrated ethereal extracts was allowed to dry under nitrogen; dry acetonitrile (10–15 μl) and BSTFA (10 μl) were added and the mixture allowed to stand at room temperature for 5 min. The trimethylsilyl derivative of *p*-benzylaminophenol was then examined by GLC using system A.

Stability of antazoline during incubation and extraction

Antazoline hydrochloride (10 μmol as the base) was incubated at 37°C for 40 min as described earlier, but omitting the cofactor solution. The incubation mixture was extracted as described above. The concentrated ethereal extracts were examined by GLC using system A and by TLC.

RESULTS AND DISCUSSION

Stability of antazoline during incubation and extraction

When antazoline was incubated under conditions similar to those used in metabolic studies but without the cofactor solutions and the incubation mixture was extracted at pH 9.0 with diethyl ether, the ethereal extract gave only one peak on GLC (system A) and one spot on TLC corresponding to antazoline itself. It is therefore concluded that antazoline is stable under the above conditions.

Identification of metabolic products

N-Benzylaniline. When antazoline was incubated with rabbit liver microsomal fraction plus the necessary cofactor requirements and the incubation mixture was extracted at pH 9.0 with diethyl ether, N-benzylaniline was identified as a metabolic product. TLC of the ethereal extract gave a spot with an R_F value of 0.96 (Table I). GLC of the ethereal extract gave a peak at a retention time of 1.5, 5.0 and 4.0 min on columns A, B and C, respectively (Table I).

TABLE I

GLC AND TLC CHARACTERISTICS OF ANTAZOLINE, N-BENZYLANILINE, *p*-BENZYLAMINOPHENOL AND THEIR TRIMETHYLSILYL AND ACETYL DERIVATIVES

GLC oven temperatures are given in parentheses.

Compound	*GLC retention time (min)*			*TLC R_F values*
	Column A	*Column B*	*Column C*	
Antazoline	11.0 (240°C)	–	–	0.0
N-Benzylaniline	1.5 (220°C)	5.0 (230°C)	4.0 (190°C)	0.96
Acetyl derivative of N-benzylaniline	–	–	8.0 (190°C)	
p-Benzylaminophenol	6.0 (220°C)	17.0 (230°C)	15.0 (190°C)	0.39
Trimethylsilyl derivative *p*-benzylaminophenol	7.0 (215°C)	–	–	

Acetylation gave mainly N-acetylbenzylaniline together with traces of unreacted N-benzylaniline (Table I). GC–MS of the ethereal extract gave fragment ions at *m/e* 91 (100%) and 183 (40%), with the latter fragment ion corresponding to the molecular ion, in agreement with published data[1] (Fig. 2a and b). The above characteristics of the metabolically produced N-benzylaniline are identical with those of the authentic compound.

p-Benzylaminophenol. p-Benzylaminophenol was identified as a metabolic product of antazoline. TLC of the ethereal extract of the incubation mixture (pH 8.0) gave a spot with an R_F value of 0.39 (Table I). Black, violet and yellow spots were obtained upon spraying with ammoniacal silver nitrate, iron(III) chloride and TTC reagents, respectively. GLC of the ethereal extract (pH 8.0) gave a peak with a retention time of 6.0, 17.0 and 15.0 min on columns A, B and C, respectively (Table I). Derivatization with BSTFA gave the trimethylsilyl derivative of *p*-benzylaminophenol (retention time 7.0 min on column A, Table I). GC–MS of the ethereal extract (pH 8.0) showed the presence of the fragment ions *m/e* 91 (100%) and 199 (50%), corresponding to the base peak and the molecular ion, respectively (Fig. 3a and b). GC–MS of the trimethylsilyl derivative gave fragment ions at *m/e* 73 (base peak), 271 (molecular ion), 180, 91 and 75 (Fig. 4a and b). The fragment ions at *m/e* 73 and 75 correspond to the trimethylsilyl derivatives[4,5]. The characteristics of the metabolically produced *p*-benzylaminophenol, described above, are identical with those of the authentic reference compound.

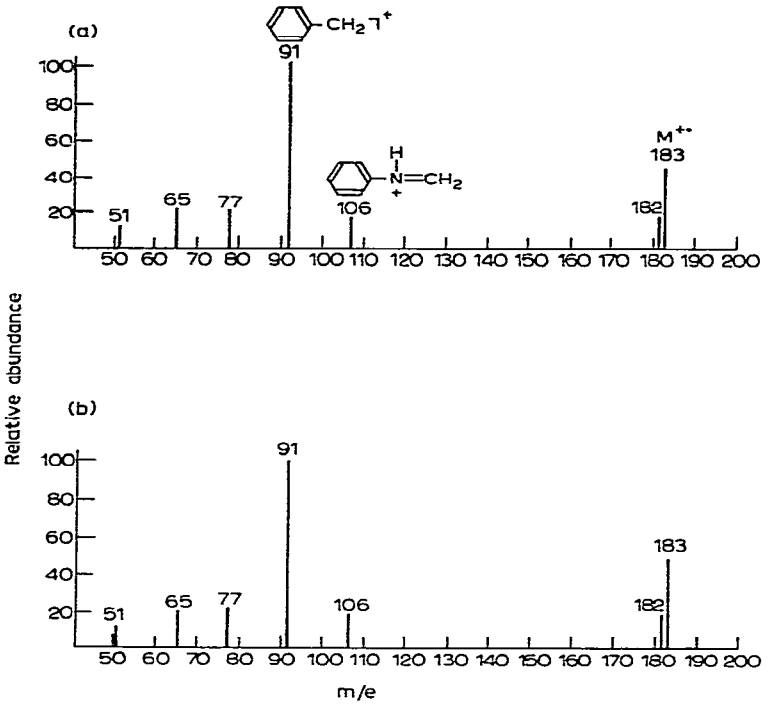

Fig. 2. Mass spectra of (a) synthetic N-benzylaniline and (b) metabolically produced N-benzylaniline.

Fig. 3. Mass spectra of (a) synthetic *p*-benzylaminophenol and (b) metabolically produced *p*-benzylaminophenol.

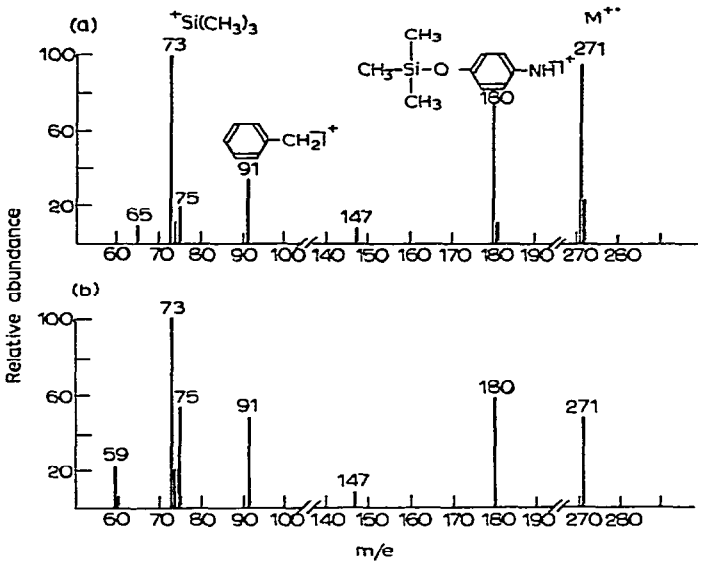

Fig. 4. Mass spectra of (a) synthetic *p*-benzylaminophenol and (b) metabolically produced *p*-benzylaminophenol as their trimethylsilyl derivatives.

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