THE *IN VITRO* HEPATIC MICROSOMAL METABOLISM OF BENZOIC ACID BENZYLIDENEHYDRAZIDE

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SUMMARY

The *in vitro* hepatic microsomal metabolism of benzoic acid benzylidenehydrazide was studied using rat microsomal preparations fortified with NADPH. The substrate and its potential metabolites were synthesised and their structures were elucidated by use of their UV, IR, ^{1}H-NMR and mass spectral characteristics. The results showed this compound produced hydrolytic metabolites, i.e. benzoic hydrazide and benzaldehyde, together with a phenolic metabolite, benzoic acid p-hydroxybenzylidene hydrazide.

KEY WORDS

aryl hydrazones, metabolism *in vitro*, aromatic hydroxylation

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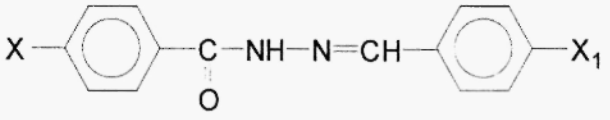
INTRODUCTION

Although a number of synthetic and microbiological studies on hydrazide hydrazones have been previously reported /1-6/, studies on their *in vitro* metabolism are very rare. We recently studied the *in vitro* microsomal metabolism of benzoic acid p-amino-[(4-fluorophenyl)-methylene]hydrazide, a substrate in which the para positions of both aromatic rings are substituted, and found hydrolytic metabolites, i.e. p-fluorobenzaldehyde and p-aminobenzoic acid hydrazide /5/. In the present study we used a similar hydrazone, benzoic acid benzylidene-hydrazide (I) and hepatic washed rat microsomal preparations fortified with NADPH. The aim was to establish whether (I) produced any phenolic and/or hydrolytic metabolites.

MATERIALS AND METHODS

Synthesis and characterisation of (I) and its potential metabolites

This work required the preparation of (I) and certain potential phenolic metabolites, i.e. p-hydroxybenzoic acid benzylidene hydrazide (II), benzoic acid p-hydroxybenzylidenehydrazide (III) and p-hydroxybenzoic acid p-hydroxybenzylidene hydrazide (IV). Benzoic hydrazide, p-hydroxybenzoic hydrazide and the corresponding p-hydroxybenzaldehyde were purchased from Aldrich Chemical Company, Poole, Dorset, UK. Benzaldehyde was obtained from Merck, Darmstadt, Germany. The substrate (I) and its potential metabolites (II), (III) and (IV) were prepared as described in previous reports /1,2,5/ (Fig. 1).



(I) (X and X_1 = -H)
(II) (X = -OH and X_1 = -H)
(III) (X = -H and X_1 = -OH)
(IV) (X and X_1 = -OH)

Fig. 1: Structures of (I) and certain potential metabolites (see text for abbreviations).

Equimolar amounts of aldehyde and the corresponding hydrazide (0.05 mol) were refluxed for 30 minutes, then the crystalline product was filtered off, washed with cold water and recrystallized from ethyl alcohol /1,2,5/. The prepared compounds were characterised by use of mass (Fig. 2), UV and NMR spectroscopic techniques and by their melting points /1-5/ (Table 1).

Analytical procedures for the detection and identification of (I) and its potential metabolites

The separation techniques were based on TLC and an isocratic HPLC system (Table 2). All chromatographic solvents were obtained from Merck Chemical Company. TLC was carried out using prepared silica gel GF254 0.25 mm glass plates (E. Merck, Darmstadt) with chloroform:methanol (90:30 v/v) as solvent system. Table 2 shows TLC characteristics of (I) and its potential metabolites. The plate after development was examined under UV light (254 nm) and then sprayed with "diazotized p-nitroaniline" followed by sodium carbonate. The HPLC column, Spherisorb C18 (5 μm, 25 cm length x 4.6 mm i.d.), was purchased from Phase Separations Limited, Deeside, UK. The guard column packing material (Whatman Pellicular ODS) was purchased from Whatman International Ltd., Maidstone, Kent, UK. The HPLC chromatograph consisted of an isocratic system comprising one LCD analytical constaMetric 3200 solvent delivery system, a Rheodyne syringe loading sample injector valve (model 7125) fitted with a 20 μl sample loop, a Milton ROY spectroMonitor-3100 variable wavelength UV detector, and a Milton ROY electronic integrator. The reaction products were eluted under isocratic conditions with a mobile phase of acetonitrile:water (20:80, v/v), at a flow rate of 1 ml/min. The metabolic products were detected by their absorbance at 300 nm. The above system allowed detection of metabolites representing less than 1% conversion of the substrate. Retention times of compounds under these conditions are given in Table 2.

Incubation and extraction procedures

Glucose-6-phosphate dehydrogenase was purchased from the Boehringer Mannheim Corporation (London) Ltd. Nicotinamide adenine dinucleotide phosphate monosodium salt (NADP) and glucose-6-phosphate disodium salt were obtained from Sigma Ltd. All

Fig. 2: Mass spectral fragmentation pathways for (I) and certain potential metabolites.

TABLE 1

Analytical and spectral data for (I) and certain potential metabolites

Compound*	M.Wt.	m.p. (°C)	Yield (%)	UV λmax (nm)	H^1 NMR
(I)	224	202-205	85	299	7.40-8.05 (m,10H, ar. protons); 8.51 (s,1H,= CH-)
(II)	240	230-235	90	310	6.89 (d,2H,meta prot. to benzylidene ring) 7.40-8.10 (m,7H, other ar. protons); 8.44 (s,1H,=CH-); 9.98 (S,1H,-OH); 11.74 (s,1H,NH)
(III)	240	218-224	88	302	6.90 (d,2H, meta prot. to benzoyl ring); 7.35-7.80 (m,5H,ar.prot. to benzylidene ring); 7.86 (d,2H,ortho prot. to benzoyl ring); 8.46 (s,1H.=CH-); 10.12 (s,1H, -OH); 11.68 (s,1H,-NH)
(IV)	256	254-257	75	314	6.81 (d,2H,meta prot. to benzylidene ring); 6.91 (d,2H,orto prot. to benzylidene ring); 7.56 (d,2H,meta prot. to benzylidene ring); 7.84 (d,2H,ortho prot. to benzoyl ring); 8.36 (s,1H.=CH-); 11.46 (s,1H,NH)

* For abbreviations see text.

UV spectra were obtained on a Kontron-Uvikon 860 UV spectrophotometer; the samples were prepared as 10^{-3} molar solutions in methanol.

NMR spectra were determined in DMSO-d6 as solvent on a Perkin Elmer R32 90 Mz Instrument.

M.p.s were uncorrected.

TABLE 2

Chromatographic characteristics of (I) and certain potential metabolites

Compound	TLC Rf x 100 value*	HPLC Rt value* (min)
Benzaldehyde	-	3
Benzoic hydrazide	44	5
(I)	70	49
(II)	57	21
(HI)	63	26
(IV)	48	11

* For TLC and HPLC solvent systems and abbreviations see text.

other chemicals used in the experiments were obtained as mentioned earlier. The animals used in the investigations were male rats. Hepatic microsomes were prepared at 0°C using the calcium chloride precipitation method of Schenkman and Cinti /7/. Incubations were carried out for 30 min in a shaking water bath at 37°C using a standard NADPH generating co-factor solution at pH 7.4, consisting of NADP (2 μmol), glucose-6-phosphate (10 μmol), glucose-6-phosphate dehydrogenase (1 unit), $MgCl_2$ (20 μmol) prepared in phosphate buffer (2 ml, 0.2 M, pH 7.4) pre-incubated for 5 minutes before addition of substrate (5 μmol) in methanol (50 μl) and microsomes equivalent to 0.5 g original liver in phosphate butter (pH 7.4, 0.2 M, 1.0 ml). Metabolic reactions were stopped by extraction with dichloromethane (2 x 5 ml). Organic extracts were bulked and evaporated to dryness at 20°C using a stream of N_2. The dry organic residues were reconstituted in 200 μl of methanol for HPLC and 100 μl of methanol for TLC analysis. Control experiments in which either microsomes, co-factors or substrate were omitted were carried through the incubation procedure and extracted and analysed as described for the complete metabolic incubation system.

RESULTS AND DISCUSSION

The reaction of hydrazides with the appropriate aldehydes gave rise to the corresponding hydrazones in a pure state. Analytical data confirmed the purity and authenticity of the compounds synthesised.

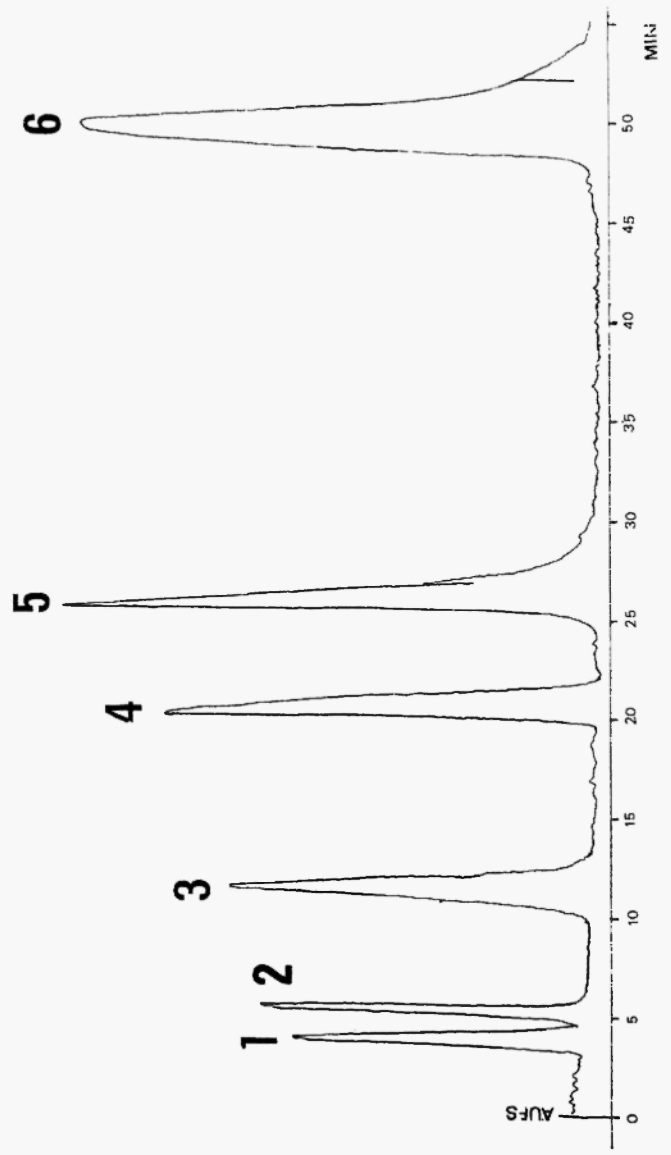


Fig. 3a: HPLC chromatogram of (I) and its potential metabolites.

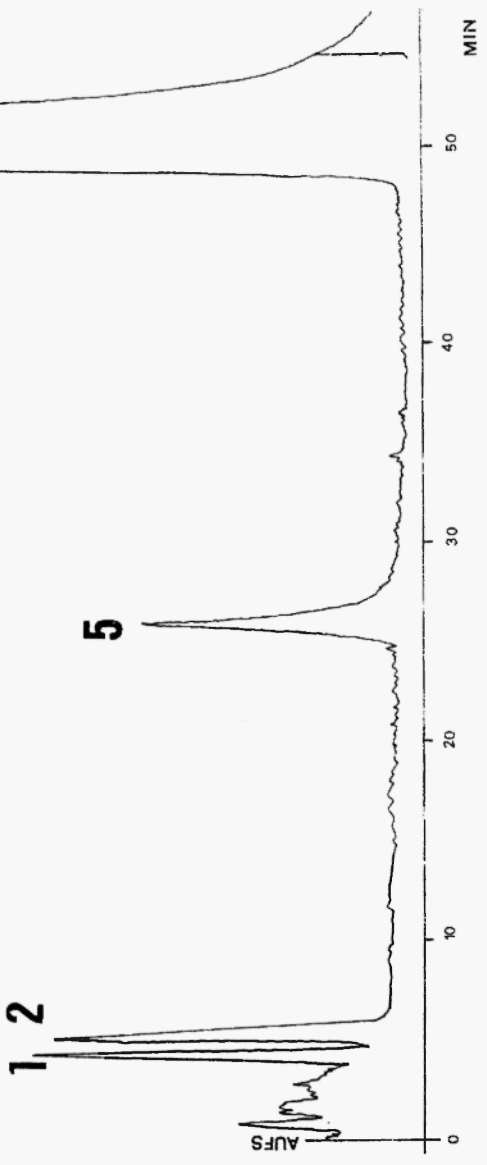


Fig. 3b: HPLC chromatogram obtained following extraction of metabolites from a rat microsomal incubation mixture with (I) as a substrate. 1= Benzaldehyde; 2 = Benzoic hydrazide; 3 = (IV); 4= (II); 5= (III); 6= (I). (See text for abbreviations and HPLC conditions.)

The UV spectra exhibited the characteristic K bands of a chromophoric C=N group around 300 nm /1,5/. NMR analysis confirmed the structures (Table 1) /5/. In the mass spectrometer the main fragmentation pattern was derived from the removal of the hydrazone group. Cleavage of the N-N bond gave both amide and cyanide moieties (Fig. 2). The MS and UV spectra were consistent with the literature data for other hydrazone type compounds /1,2,5/. The *in vitro* metabolism of (I) by rat microsomes showed the formation of hydrolytic products, benzoic hydrazide and benzaldehyde, together with one p-hydroxylated metabolite which gave a positive reaction on TLC with diazotised p-nitroaniline and sodium carbonate solution. This metabolite had identical chromatographic properties on TLC as authentic benzoic acid p-hydroxybenzylidenehydrazide (III) (Fig. 3).

This route of hydroxylation, i.e. p-hydroxylation of the benzylidene ring but not the benzoyl ring, is not unexpected due to the stronger electron withdrawing influence of the former substituent group. In this respect it is also interesting that cleavage occurred by an oxidative mechanism at the -N=CH- bond rather than by hydrolytic cleavage of the amide bond. The latter process would be expected to occur in the absence of co-factors but no metabolites were observed in control experiments. The chemical stability of the hydrazone molecule may be important for the toxicological properties of this kind of structure, as instability would yield the hydrazine moiety which would be expected to be more toxic than the hydrazone. Established metabolic products of (I) are presented in Figure 4. Further experiments are required on this

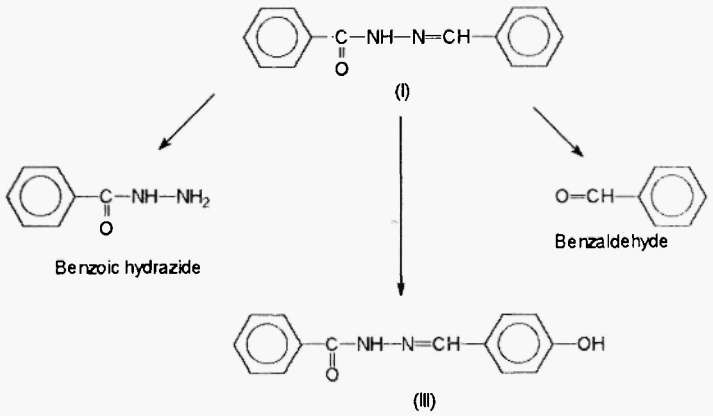


Fig. 4: Established metabolic profile of (I).

group of compounds to establish complete structure-metabolic activity relationships and the enzymology involved. This may lead us to develop safer antimicrobial products based on hydrazide hydrazones.

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