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o-HYDROXYPHENYLACETALDEHYDE: A MAJOR NOVEL METABOLITE OF COUMARIN FORMED BY RAT, GERBIL AND HUMAN LIVER MICROSOMES

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SUMMARY: A major novel coumarin metabolite was isolated from rat hepatic microsomal incubations by high-performance liquid chromatography. In the presence of a rat liver cytosolic fraction and NADH it was rapidly metabolized to o-hydroxyphenylethanol. The metabolite co-chromatographed with an authentic sample of o-hydroxyphenylacetaldehyde and its identity was confirmed by mass spectral analysis. The formation of o-hydroxyphenyl acetaldehyde from coumarin was NADPH-dependent. It was the major metabolite formed by rat, gerbil and human liver microsomes at a coumarin concentration of 1mM.

Coumarin (2H-1-benzopyran-2-one) is a naturally-occurring constituent of many plants which is added to perfumes, toothpastes and tobacco products [1]. It was banned from use as a food flavouring agent in the USA in 1954 when it was shown to be hepatotoxic in the rat and dog [2,3]. Coumarin has recently been used for the treatment of various cancers [4-6], high protein oedema [7,8], brucellosis and certain other chronic infections [7]. Liver toxicity in patients receiving relatively high daily doses of coumarin is very rare [9].

Species differences in coumarin hepatotoxicity are thought to be metabolism-mediated [2]. The rat reportedly metabolizes coumarin via an initial 3-hydroxylation reaction followed by cleavage of the heterocyclic ring to give o-hydroxyphenylacetic acid (o-HPAA) and other ring-opened metabolites [10]. In humans 7-hydroxycoumarin (7-HC) is the major urinary metabolite, constituting about 80% of the administered dose [11]. We have shown that the Mongolian gerbil, unlike most other rodent species, is a good

Abbreviations: o-HPAA, o-hydroxyphenylacetic acid; HC, hydroxycoumarin; HPLC-MS, high-performance liquid chromatography-mass spectrometry; o-HPA, o-hydroxyphenylacetaldehyde; o-HPE, o-hydroxyphenylethanol.

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7-hydroxylator of coumarin, and therefore proposed that the gerbil may be a more appropriate animal model than the rat for pharmacological and toxicological studies relevant to the use of coumarin in man [12].

Coumarin is metabolized by rat liver microsomes to 3-HC and an unidentified major metabolite ('X'), which we previously postulated was coumarin 3,4-dihydrodiol [13]; 'X' is also formed by gerbil and human liver microsomal incubations [14]. Lake and coworkers have proposed that coumarin induced hepatotoxicity in the rat is due to the cytochrome P450-dependent formation of a reactive coumarin 3,4-epoxide intermediate during the 3-hydroxylation of coumarin [15], and subsequently they have also suggested that 'X' may be coumarin 3,4-dihydrodiol [16]. However, in recent studies employing HPLC-MS or phenyl boronic acid affinity columns for solid-phase extraction we have not found any evidence for the existence of a diol metabolite in rat hepatic microsomal incubations.

In the present study we have investigated the hepatic microsomal metabolism of coumarin in the rat in the presence of a liver cytosolic fraction and various cofactors. The novel coumarin metabolite, 'X', was isolated from rat liver microsomal incubations and has been identified as o-hydroxyphenylacetaldehyde (o-HPA). The formation of this ring-opened coumarin metabolite by gerbil and human liver microsomes has also been demonstrated.

MATERIALS AND METHODS

Reagents: Coumarin, o-hydroxyphenylacetic acid (o-HPAA), periodic acid and cofactors were purchased from Sigma Chemical Co. Ltd., Poole, UK. o-Hydroxy phenylethanol (o-HPE) was obtained from Aldrich Chemical Co. Ltd., Gillingham, UK, and 3-hydroxycoumarin (3-HC) was supplied by APIN Chemicals Ltd., Oxon, UK. 3-o-Hydroxyphenylpropane-1,2-diol was a generous gift of Dr. J.M. Bruce of the Department of Chemistry, University of Manchester, UK.
Synthesis: o-Hydroxyphenylacetaldehyde (o-HPA) was synthesized from 3-o-hydroxyphenylpropane-1,2-diol and periodic acid by the method of Bruce and Creed [17].
Animals: Adult male Wistar rats (120-150g) and Mongolian gerbils (Meriones unguiculatus; 40-50g) were obtained from the University of Nottingham Medical School Animal Unit. They had access to standard laboratory diet and tap water at all times.
Human liver samples: Microsomal fractions from 3 human liver samples obtained from renal transplant donors were generously provided by Drs. A.R. Boobis and B.P. Murray of the Department of Clinical Pharmacology, Royal Postgraduate Medical School, London, UK. A liver sample (with normal histology) was also obtained from a patient undergoing lobectomy for a well differentiated adenocarcinoma.
Preparation of liver microsomes: Microsomal fractions were prepared from pooled livers of 6 rats or 10 gerbils, and from the human liver sample, by the calcium aggregation method [18].
Preparation of rat liver cytosol: A 25%(w/v) liver homogenate in 0.25M sucrose was centrifuged at 10,000g for 20 min. The resulting supernatant was centrifuged at 127,000g for 60 min and the supernatant from this was designated the cytosolic fraction. To determine cofactor requirements for o-HPA metabolism the cytosol was dialyzed overnight at 4°C against 0.25M

sucrose and then 0.1M sucrose (1000 volumes each) to remove any endogenous cofactors. The non-protein sulphydryl content (mainly glutathione) of the cytosol after dialysis was negligible, as assayed by the method of Sedlak and Lindsay [19]. The liver microsomal and cytosolic protein contents were determined by the method of Lowry et al.[20].

Coumarin and o-HPA metabolism: The incubation mixture (1ml) contained liver microsomal and/or cytosolic fractions (both approx. 1mg protein), phosphate buffer, pH 7.4 (100mM), $MgSO_4$ (5mM), and NADPH (0.5 or 1.0 mM) and/or NADH (0.5mM). Coumarin (0.5 or 1.0 mM final concentration) was added in 5µl methanol and the samples incubated for 10-120 min. Reactions were terminated with 25%(w/v) trichloroacetic acid (0.25ml) and the metabolites present in the deproteinized supernatant analyzed directly by HPLC. Rat hepatic microsomal and cytosolic metabolism of o-HPA (1mM final concentration) was similarly determined using an incubation time of 10 min.

HPLC analysis: Coumarin metabolites were analyzed on a Spherisorb 5 ODS-2 column with formic acid-water (5/95, v/v) and methanol as the solvents, as described previously [14,21]. The eluent was monitored at 280nm, and metabolites were quantified by comparison of peak heights with those of standards prepared in aqueous methanol. A small amount of metabolite 'X' was isolated and partially purified from a large-scale rat liver microsomal incubation (20ml) by collecting appropriate fractions of the column eluent during HPLC. The fractions were pooled and extracted with diethyl ether immediately prior to mass spectral analysis.

Mass spectrometry: Mass spectra were obtained using a VG Micromass 70E mass spectrometer equipped with a VG11/250 data system. The isolated coumarin metabolite, 'X', and synthesized o-HPA were analyzed by positive electron ionization using direct probe insertion at 70eV and 180-200°C.

RESULTS AND DISCUSSION

Mass spectral analysis of a partially-purified sample isolated from a large-scale incubation of coumarin with rat liver microsomes, which contained the unknown metabolite ('X') as the major component, revealed a molecular ion peak at m/z 136 (Figure 1B); the peaks at m/z 118 and 107 probably reflect the loss of H_2O and CHO respectively. Experiments undertaken concurrently to investigate the effects of the addition of liver cytosol and various cofactors on the hepatic microsomal metabolism of coumarin in the rat indicated that 'X' was the precursor of the ring-opened metabolite o-HPE (Figure 2). This suggested that 'X' could be o-HPA which has the required molecular weight of 136. Synthesized o-HPA had a comparable retention time (approx. 11.8 min) to 'X' upon HPLC analysis, and the two components co-chromatographed when rat hepatic microsomal incubations containing 'X' were spiked with o-HPA. A mass spectral profile obtained with an authentic sample of o-HPA confirmed that 'X' was indeed o-HPA (Figure 1A).

Thus o-HPA was identified as the major coumarin metabolite formed by rat liver microsomes (2.7 ± 0.1 nmol/min/mg protein; mean ± SEM of triplicate determinations, using a coumarin concentration of 0.5mM and an incubation time of 10 min); o-HPE (0.4 ± 0.0) and 3-HC (0.1 ± 0.0) were also produced, and small amounts of o-HPAA were detected after incubating for 30 min (Figure 2). Metabolism of coumarin to o-HPA was NADPH-dependent and was

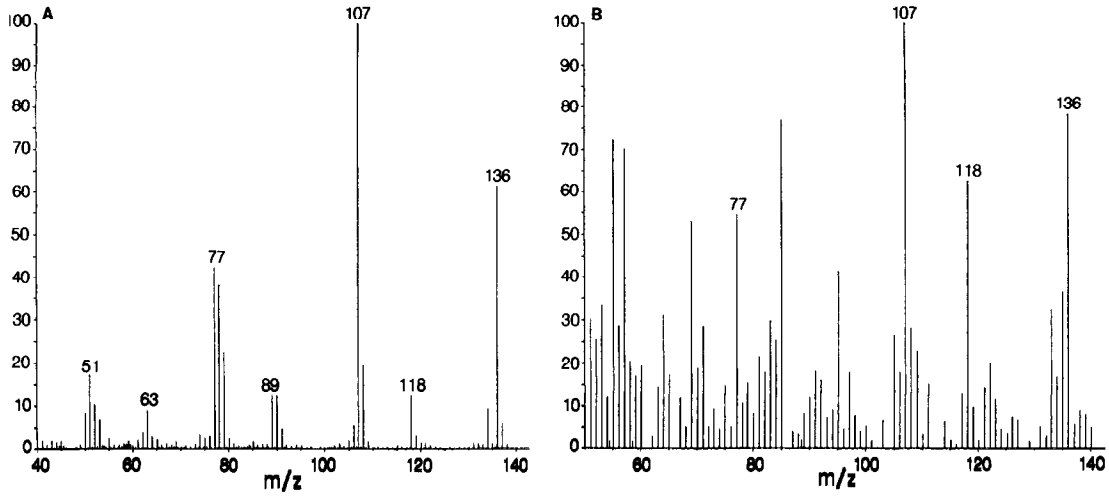

Figure 1. Mass spectra of (A) authentic o-hydroxyphenylacetaldehyde and (B) the coumarin metabolite 'X' isolated from rat liver microsomal incubations. The mass/charge ratio (m/z) has been normalized to the most abundant ion in the range shown. In (B) the other peaks represent material present in the HPLC solvent matrix.

stimulated by about 30% by NADH. The addition of rat liver cytosol to the microsomal incubations resulted in a decrease in the amount of o-HPA detected; this was accompanied by a marked increase in o-HPE formation. If both cytosol and NADH were added the decline in o-HPA levels was dramatic; it was rapidly metabolized to o-HPE and, possibly, small amounts of o-HPAA (Figure 2). Therefore, it appears that the hepatic microsomal metabolism of coumarin to o-HPA is cytochrome P450-mediated whilst the further metabolism of o-HPA to o-HPE is primarily cytosolic and is stimulated by NADH. These results agree with those of Norman and Wood [22,23] who initially identified o-HPE as a metabolite of coumarin formed by rat liver 10,000g postmitochondrial supernatant fractions but not by microsomes. However, these authors probably erroneously identified 'X' (i.e. o-HPA) as 6-HC because of the simple isocratic HPLC method employed for the analysis of coumarin and its metabolites.

At a coumarin concentration of 1mM and with an incubation time of 10 min the formation of o-HPA by rat, gerbil and human liver microsomes [mean $\pm$ SEM (n)] was 5.0 $\pm$ 0.4(10), 8.0 $\pm$ 0.5(7) and 1.4 $\pm$ 0.2(4) nmol/min/mg protein respectively. o-HPA was the major metabolite at this particular coumarin concentration in all three species. With rat liver microsomes 3-HC (0.24 nmol/min/mg protein) was the only other metabolite formed in significant amounts, whilst 3-, 5-, 6-, 7- and 8-HCs, and 6,7-diHC, were detected in gerbil liver microsomal incubations; the total production of all these hydroxy metabolites was about 3.9nmol/min/mg protein (i.e. together they accounted for about 33% of the coumarin metabolites formed with oHPA

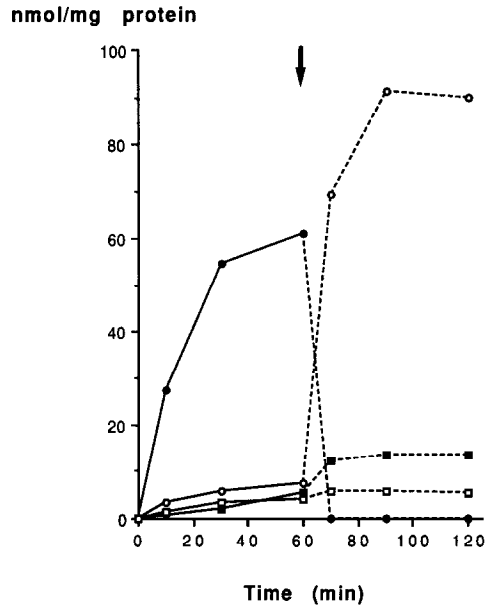

Figure 2. Effect of the addition of liver cytosol and NADH on the metabolism of coumarin by rat liver microsomes.
Incubations were carried out as described in 'Materials and Methods' using coumarin and NADPH concentrations of 0.5mM and 1.0mM respectively. The arrow indicates the point at which cytosol (approx. 1mg protein) and NADH (0.5mM final concentration) were added. Metabolites detected by HPLC were: o-HPA/'X' (●), o-HPE (○), o-HPAA (■) and 3-HC (□). Amounts are quoted as nmol metabolite formed/mg microsomal protein and each point represents the mean of triplicate determinations.

representing the other 67%). 7-HC was previously the major identified coumarin metabolite with human liver microsomes [0.23 ± 0.10(4) nmol/min/mg protein].

A preliminary investigation of the metabolism of o-HPA by rat liver cytosol and microsomes confirmed that it was primarily metabolized to o-HPE by the cytosolic fraction (approx. 6nmol/min/mg cytosolic protein); a small amount of o-HPAA was also detected which may have been formed directly from o-HPA or by further metabolism of o-HPE. The formation of o-HPE was greatest with NADPH as the cofactor but NADH also supported o-HPE production at about 40% of the rate observed with NADPH. Rat liver microsomes metabolized o-HPA to o-HPE but to a lesser extent than the cytosolic fraction (approx. 2nmol/min/mg microsomal protein); trace amounts of o-HPAA were again detected. NADH alone supported the formation of o-HPE by rat liver microsomes at the same rate as when both cofactors were present; with NADPH o-HPE formation was about 15% of that observed with NADH. Thus both hepatic cytosolic and microsomal enzymes, with different cofactor requirements, appear to be involved in the metabolism of o-HPA; these may include aldehyde reductase, alcohol dehydrogenase, retinol dehydrogenase and/or aldehyde dehydrogenase.

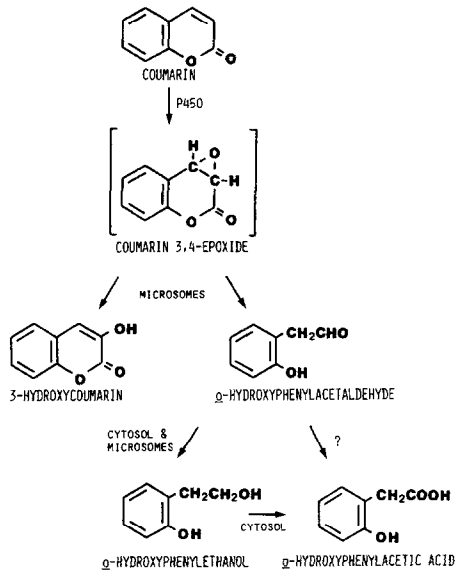

Figure 3. Postulated routes for the formation of 3-hydroxycoumarin, o-hydroxyphenylacetaldehyde and other ring-opened coumarin metabolites. The putative coumarin 3,4-epoxide intermediate may also conjugate with glutathione or bind covalently to cellular macromolecules resulting in hepatotoxicity.

These results are consistent with those reported by Norman and Wood who showed that optimal production of o-HPE from coumarin by rat liver 10,000g supernatants required both NADPH and NADH [22,23]. These authors also demonstrated that the 3,4-double bond of coumarin was necessary for the formation of o-HPE [22], and that o-HPE was further metabolized to o-HPAA by rat liver cytosol at a rate of 4.4nmol/min/mg protein [23]. The production of o-HPAA from coumarin *in vivo* has been reported to proceed via an initial cytochrome P450-dependent 3-hydroxylation reaction followed by oxidative decarboxylation at carbon 2 [10]. However, the metabolism of 3-HC by rat liver microsomes did not result in the formation of o-HPA, o-HPE or o-HPAA. Our results, like those of Norman and Wood, suggest that the formation of lactone ring-opened metabolites such as o-HPAA proceeds via an intermediate other than 3-HC; this is probably coumarin 3,4-epoxide (Figure 3).

In conclusion, we have identified the major coumarin metabolite ('X') formed by rat, gerbil and human liver microsomes as the novel lactone ring opened compound o-hydroxyphenylacetaldehyde. The enzymes involved in the formation of ring-opened coumarin metabolites, and the importance of o-HPA with regard to the marked species differences observed in coumarin-induced hepatotoxicity, now require investigation.

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