Biliary Excretion of Cyclohexylphenyl 4-[³⁵S]Sulphate in the Guinea Pig

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The metabolic fate and mode of excretion of cyclohexylphenyl 4-[³⁵S]sulphate were studied in the guinea pig. Up to 54.8% of the dose appeared in the bile, the majority as unchanged ester. Substantial amounts of hydroxylated cyclohexylphenyl 4-[³⁵S]sulphate were also excreted in the bile together with minor amounts of the corresponding glucuronic acid conjugate. When isolated guinea-pig livers were perfused with cyclohexylphenyl 4-[³⁵S]sulphate the biliary components were the same as those in the intact animal, although the relative concentration of the hydroxylated derivative was significantly greater. When the hydroxylated derivative was re-injected into guinea pigs it was excreted almost entirely unchanged in the bile. However, in the rat, it was excreted in the bile as a glucuronic acid conjugate. These findings are discussed in relation to studies carried out in the rat [Hearse, Powell, Olavesen & Dodgson (1969) *Biochem. Pharmacol.* 18, 181–195] and to differences in enzyme activities in rat and guinea-pig liver. The results are also discussed in terms of the molecular-weight threshold for the excretion of anions in guinea-pig bile.

The metabolic fates and modes of excretion of a series of structurally related arylsulphate esters have been studied in the rat (Hearse *et al.*, 1969b). One of the compounds, cyclohexylphenyl 4-[³⁵S]sulphate, was extensively metabolized and eliminated via the bile as cyclohexylphenyl 4-[³⁵S]sulphate glucuronide. The biliary elimination of this double conjugate has been rationalized (Millburn, 1970) on the grounds that the addition of the glucuronic acid moiety increases the molecular weight of the ester so that it exceeds the molecular-weight threshold for biliary elimination in the rat (325 ± 50 ; Hirom *et al.*, 1972). For this reason, it might be forecast that if cyclohexylphenyl 4-sulphate is metabolized in a similar fashion in the guinea pig, the metabolic product would also appear in the bile, since the glucuronic acid conjugate meets the molecular-weight requirement (400 ± 50 ; Hirom *et al.*, 1972) in that species. However, it has been shown that the metabolism of the mono- and di-sulphate esters of diethylstilboestrol are quantitatively and qualitatively different in rats (Barford *et al.*, 1977a) and guinea pigs (Barford *et al.*, 1977b). These differences have been related to differences in the enzyme activities in rat and guinea-pig liver. Diethylstilboestrol di-[³⁵S]sulphate is not desulphated in guinea pigs and is excreted unchanged in the bile. If the guinea pig is unable to desulphate cyclohexylphenyl 4-[³⁵S]sulphate, the route of excretion of the unmetabolized ester is unlikely to be biliary, since the molecular weight of the anion (255) is below the threshold for biliary elimination. These predictions are investigated in this study.

Materials and Methods

Potassium cyclohexylphenyl 4-[³⁵S]sulphate

The preparation method was essentially that of Hearse *et al.* (1969a). Sulphation of 4-cyclohexylphenol was carried out by using chloro[³⁵S]sulphonic acid (specific radioactivity 9.3 mCi/mmol; obtained from The Radiochemical Centre, Amersham, Bucks., U.K.). The product was homogeneous by t.l.c., paper chromatography and paper electrophoresis. The i.r. spectrum was identical with that reported previously (Hearse *et al.*, 1969a). The product was stored as a frozen aqueous solution (5 mg/ml).

β -Glucuronidase (type B1 from bovine liver) was obtained from Sigma (London) Chemical Co., Kingston upon Thames, Surrey, U.K.

Chromatography and paper electrophoresis

Descending paper chromatography was carried out on Whatman no. 1 paper for 16 h in butan-1-ol/acetic acid/water (3:1:1, by vol.). Horizontal paper electrophoresis was carried out on Whatman no. 1 paper for 3 h at a potential gradient of 10 V/cm in 0.1 M-sodium acetate/acetic acid buffer, pH 4.5. Radioactive areas on chromatograms and electrophoretograms were detected by radioautography for suitable periods on X-ray film or by scanning. The relative amount of radioactivity associated with each spot was measured as described by Jones & Dodgson (1965).

Experimental animals

Guinea pigs (random bred strain; Animal Suppliers London, Welwyn, Herts., U.K.) were maintained on a standard diet. Animals were anaesthetized with urethane [ethyl carbamate, 25% (w/v), in 0.9% NaCl] at a dose of 5 ml/kg body wt., administered intraperitoneally. After cannulation of the left jugular vein similar amounts of anaesthetic were administered to maintain anaesthesia throughout the experiment. Ureters were cannulated; bile ducts were cannulated after gall-bladder ligation.

In experiments with rats (adult male, MRC hooded) with bile-duct cannulae, animals were anaesthetized with diethyl ether, and Nembutal (15 mg/kg body wt.) was administered via the cannulated jugular vein. Animals were maintained under Nembutal anaesthesia throughout the experiments. Test materials were administered to rats and guinea pigs via a cannulated jugular vein.

Isolated-liver perfusion

The method used was essentially that described by Curtis *et al.* (1971).

Measurement of radioactivity

The radioactive contents of solutions were determined by liquid-scintillation counting. Samples (usually 50 μ l) were added to 10 ml of scintillation 'cocktail' [toluene (1 litre), Triton X-100 (500 ml), diphenyloxazole (5 g)], and radioactivity was determined in a liquid-scintillation spectrometer (Beckman LS 100). Sufficient radioactive counts were recorded to give a standard error of $\pm 2\%$.

Isotope-dilution analysis

The procedure of Dodgson *et al.* (1961) was adopted.

Experimental and Results

Intravenous administration

Guinea pigs received intravenous injections of potassium cyclohexylphenyl 4-[35 S]sulphate (1 mg/200 g body wt.). Urine and bile were collected after 30 min and 1 h and thereafter at 45 min intervals up to 6 h 15 min after injection and assayed for total [35 S]sulphate content. The results are shown in Table 1. Most of the excreted radioactivity (up to 54.8% of the radioactive dose) was excreted in the bile, although substantial amounts (up to 37.1% of the dose) appeared in urine.

Qualitative analysis of urine and bile

Urine and bile samples obtained from guinea pigs receiving potassium cyclohexylphenyl 4-[35 S]sulphate were analysed by paper chromatography and by electrophoresis together with appropriate controls, i.e. bile and urine samples to which authentic

Table 1. Distribution of 35 S in urine and bile of male guinea pigs injected intravenously with potassium cyclohexylphenyl 4-[35 S]sulphate (1 mg/200 g body wt.)

For experimental details see the text. Results are mean values for three animals, with ranges in parentheses.

Time (h)	Distribution of 35 S (% of 35 S injected)	
	Bile	Urine
30 min	19.3 (14.8–21.8)	0.0
1 h	10.6 (9.2–11.4)	1.7 (0.0– 4.8)
1 h 45 min	8.4 (6.8–10.4)	8.1 (0.0–20.5)
2 h 30 min	3.6 (2.8– 4.6)	5.5 (3.9– 6.7)
3 h 15 min	2.0 (1.4– 2.7)	3.5 (1.0– 6.4)
4 h	1.7 (1.3– 2.2)	3.6 (0.1– 9.1)
4 h 45 min	1.3 (0.9– 1.9)	0.8 (0.0– 1.7)
5 h 30 min	1.2 (0.8– 1.7)	4.0 (0.3–11.0)
6 h 15 min	1.3 (0.0– 1.7)	1.7 (0.0– 2.9)
Total 35 S content	48.9 (44.0–54.8)	28.9 (17.8–37.1)
Recovery (% of injected dose)	77.8 (72.6–84.9)	

cyclohexylphenyl 4-[35 S]sulphate had been added. Electrophoretograms of both bile and urine revealed the presence of a single spot with the same mobility as the injected ester.

Radioautograms prepared from paper chromatograms showed the presence of three radioactive spots: two major components and one trace component. The mobility of one of the major components (component I) was identical with that of cyclohexylphenyl 4-[35 S]sulphate (R_F 0.77); the other major component (component II) had R_F 0.63, and the minor component (component III) had R_F 0.31.

Quantification of bile components

The relative concentrations of the three radioactive components as revealed by chromatography were determined in all bile samples. The results were expressed as percentages of the radioactivity applied to paper chromatograms of each bile sample developed in butan-1-ol/acetic acid/water (3:1:1, by vol.). Fig. 1 illustrates the results obtained in a typical experiment. In all bile samples unchanged cyclohexylphenyl 4-[35 S]sulphate (component I) accounted for the majority of the biliary radioactivity and the proportions of the three components were relatively constant in all bile samples collected after 1 h.

Isolated liver perfusion

Heparinized whole guinea-pig blood (145 ml) was used and a male guinea pig (400 g body wt.) served as the liver donor. Potassium cyclohexylphenyl 4-[35 S]sulphate (15.75 mg) was added to the perfusate and the liver was perfused for 5 h 15 min. Bile samples were collected after 30 min, 1 h and thereafter at

45 min intervals. The radioactive contents of bile samples were determined. In addition, the proportions of each radioactive component were determined by paper chromatography and subsequent scanning. The amount of radioactivity within the first 30 min represented 1.2% of the added radioactivity (see Fig. 2a). Thereafter, the radioactive content increased to some extent, reaching a maximum in the 2.5 h collection (3.7% of the added radioactivity). Over the entire experimental period 20.8% of the radioactivity appeared in the bile. Analysis of bile samples showed (Fig. 2b) that initially, at 30 min, the amounts of components I and II were approximately equal.

As the experiment progressed the relative amount of component II increased, reaching approx. 85% of the biliary radioactivity in the 3 h 15 min bile collection. In contrast, the amounts of radioactivity associated with component I decreased with time. Component III could not be detected in some bile samples, but the amounts of radioactivity associated with component III in other samples were similar to those already recorded in whole animals receiving the ester intravenously.

At the end of the experiment, plasma prepared from the perfusate by centrifuging was analysed by paper chromatography and scanning. Component II was the major radioactive constituent, representing 65.7% of the radioactivity present in the plasma.

Purification of biliary component I

Bile (approx. 3 ml) was collected for 30 min from a guinea pig that had received intravenously potassium cyclohexylphenyl 4-[³⁵S]sulphate at a dose of 1 mg/200 g body wt. Portions (400 μ l) were applied as streaks to Whatman no. 1 chromatography paper and developed for 18 h in butan-1-ol/acetic acid/water (3:1:1, by vol.). Radioactive areas on chromatograms were detected by radioautography and areas of chromatograms corresponding to component I were eluted with water (approx. 200 ml), and freeze-dried to a solution (4.8 ml) containing 1.31×10^6 c.p.m. Chromatography [in butan-1-ol/acetic acid/water (3:1:1, by vol.)] with subsequent radioautography of this solution showed the presence of a single radioactive spot which co-chromatographed with cyclohexylphenyl 4-[³⁵S]sulphate. Authentic potassium cyclohexylphenyl 4-sulphate (100 mg) was added to a solution of component I and the whole recrystallized from water. Crystals were isolated and dried and a weighed sample (approx. 12 mg) was retained for determination of specific radioactivity. The procedure was

Fig. 1. Time course of excretion of cyclohexylphenyl 4-[³⁵S]sulphate and its metabolite in guinea-pig bile

The animal received cyclohexylphenyl 4-[³⁵S]sulphate intravenously at a dosage of 1 mg/200 g body wt. Paper chromatograms of bile samples were scanned for radioactivity and the amount of each radioactive component [component I, R_F 0.77 (●); component II, R_F 0.63 (■); component III, R_F 0.31 (▲)] is expressed as % of the total radioactivity on the chromatogram. For details see the text.

Fig. 2. Time course of excretion of radioactivity in bile produced by an isolated perfused guinea-pig liver

Cyclohexylphenyl 4-[³⁵S]sulphate (15.75 mg) was added to the perfusate (145 ml): (a) total ³⁵S present in bile expressed as percentage of radioactivity added to the perfusate; (b) the relative amounts of component I (●) and component II (■) present in each bile sample expressed as percentage of radioactivity in each bile sample. For details see the text.

repeated a further five times and material of constant specific radioactivity was obtained after the second recrystallization.

Purification of biliary component II

Component II was purified from bile collected at 30 min from guinea pigs that had received potassium cyclohexylphenyl 4-[³⁵S]sulphate at a dose of 1 mg/200 g body wt. The method of purification was essentially that described for component I. Chromatography of the solution of purified component II with subsequent scanning showed the presence of a single radioactive spot.

Re-injection of biliary component II into guinea pigs

Guinea pigs (two) each received intravenously a solution (0.5 ml containing approx. 90000 c.p.m.) of purified component II. Bile was collected every 15 min for 1 h and then after 2 and 3 h. Urine was collected as a single fraction over the 3 h experimental period. In both animals (see Table 2) most of the excreted radioactivity appeared in the bile (56.7 and 60.9%) and biliary excretion of radioactivity was greatest in the first 15 min after injection. A sample (100 μ l) of the 15 min bile was subjected to paper chromatography. Radioautograms revealed one major radioactive component which co-chromatographed with purified component II. In addition, traces of a further radioactive substance were detected with the same mobility as component III.

Re-injection of biliary component II into rats

Rats (two) each received intravenously a solution (0.4 ml) containing approx. 95000 c.p.m. of purified component II. Bile was collected hourly for 3 h and urine was collected as a single fraction over 3 h. The results are recorded in Table 2. Biliary excretion of

radioactivity was almost entirely confined to the first hour, and over 3 h 28.9 and 39.1% of the injected radioactivity was excreted in the bile. Significant amounts of ³⁵S were also found in the urine (21.9 and 23.5% respectively). A sample (100 μ l) of the bile collected at 1 h was subjected to paper chromatography, together with appropriate markers, including a 1 h bile sample (50 μ l) obtained from a male rat receiving potassium cyclohexylphenyl 4-[³⁵S]sulphate at a dose of 1 mg/200 g body wt. Radioautograms revealed one radioactive spot, the chromatographic mobility of which did not correspond to that of the injected component II but which was the same as component III and the major metabolite of cyclohexylphenyl 4-[³⁵S]sulphate in rat bile (already identified as cyclohexylphenyl 4-[³⁵S]sulphate glucuronide; Hearn *et al.*, 1969b).

Identification of component II

The possibility that component II could be identified with a glucuronic acid conjugate was investigated by incubating bile samples (0.2 ml) obtained from guinea pigs receiving potassium cyclohexylphenyl 4-[³⁵S]sulphate with an equal volume of a solution of β -glucuronidase [1000 Fishman units (Talalay *et al.*, 1946)/ml in 0.1 M-sodium phosphate buffer, pH 6.5] and incubated at 37°C for 3 h. Portions (20 μ l) of the incubation mixture, together with untreated bile, were subjected to paper chromatography. Scans of these chromatograms demonstrated that β -glucuronidase treatment did not affect the amount of component II present in the bile.

Rat bile obtained from an animal receiving potassium cyclohexylphenyl 4-[³⁵S]sulphate was also incubated with β -glucuronidase under identical conditions and the incubation mixture was chromatographed as described in the Materials and Methods section. Scans of these chromatograms demonstrated a diminution in concentration of cyclohexylphenyl 4-[³⁵S]sulphate glucuronide, the major constituent present in rat bile, and the appearance of a new radioactive spot, which corresponded in mobility with component II of guinea-pig bile. This suggested that component II may be identified with a hydroxylated derivative of cyclohexylphenyl 4-[³⁵S]sulphate.

A solution of purified component II, analysed by u.v. spectroscopy in 0.1 M-HCl, showed an absorption maximum at 281 nm. When the solution was analysed in 0.1 M-KOH the absorption maximum was displaced to 290 nm. This phenoxide shift represents confirmatory evidence of the presence of a free phenolic group in component II. Further evidence for the presence of a free hydroxy group in component II was provided when purified component II was subjected to paper chromatography before and after hydrolysis with 2 M-HCl. Radioautograms were prepared and the paper chromatograms were viewed

Table 2. Distribution of ³⁵S in urine and bile of male guinea pigs and rats injected intravenously with purified component II obtained from bile of guinea pigs receiving potassium cyclohexylphenyl 4-[³⁵S]sulphate intravenously

For experimental details see the text. Results are values obtained for individual animals; *collections not made.

		Distribution of ³⁵ S (% of ³⁵ S injected)	
	Time	Guinea pig	Rat
Bile	15 min	40.1, 27.0	* *
	30 min	6.5, 8.8	* *
	45 min	3.5, 7.0	* *
	1 h	1.3, 3.7	26.7, 34.9
	2 h	2.6, 7.9	1.6, 3.4
	3 h	2.7, 6.5	0.6, 0.8
	Total ³⁵ S in bile	56.7, 60.9	28.9, 39.1
Urine	Total ³⁵ S in urine (3 h)	31.3, 16.0	21.9, 23.5
Recovery (% of injected dose)		88.0, 76.9	50.8, 62.6

in short-wave u.v. light (254 nm). Before hydrolysis a single radioactive u.v.-absorbing spot was seen, whereas after hydrolysis radioactivity was found at the chromatographic origin only and a new u.v.-absorbing spot was seen with mobility (R_F 0.85) different from both that of the parent component II and authentic 4-cyclohexylphenol (R_F 0.95). This was again indicative of the introduction of a polar group into the parent cyclohexylphenyl 4-[³⁵S]-sulphate to form component II. The identity of the polar group with an hydroxy group, resulting in the formation of a phenolic compound, was strongly inferred by a positive reaction with FeCl_3 /ferri-cyanide reagent. The position of the hydroxy group on the benzene ring was investigated by use of the Gibbs (1927) test. When the aqueous solution of cyclohexylphenyl 4-sulphate was hydrolysed in 2M-HCl for 30 min at 100°C no reaction was obtained in the Gibbs (1927) test, demonstrating the negative response expected, since the hydroxy group revealed by acid hydrolysis is *para* to the cyclohexyl group on the aromatic ring. In contrast, when component II was tested with Gibbs (1927) reagent before and after acid hydrolysis a positive reaction was obtained. The colour (purple) obtained was the same as that obtained with 4-hexylresorcinol and 4-cyclohexylresorcinol and different from that obtained (blue) with the 4-sulphate ester of dopamine, suggesting that the hydroxy group present in component II occupies the position *meta* to the sulphate moiety.

Discussion

This study has demonstrated a significant species difference in the metabolism and excretion of cyclohexylphenyl 4-[³⁵S]sulphate in rats and guinea pigs. The major differences are the extent of biliary excretion and the nature of the biliary components. In the guinea pig, up to 54.8% of the radioactive dose appears in the bile, whereas the corresponding value for the rat is 81% (Hearse *et al.*, 1969b). In addition, although the major metabolite in the rat bile is cyclohexylphenyl 4-[³⁵S]sulphate glucuronide, in the guinea pig most of the biliary radioactivity was unchanged parent ester, with substantial amounts of hydroxylated cyclohexylphenyl 4-[³⁵S]sulphate and minor amounts only of the corresponding glucuronic acid conjugate. The hydroxylated cyclohexylphenyl 4-[³⁵S]sulphate was tentatively identified as 2-hydroxycyclohexylphenyl 4-[³⁵S]sulphate.

The qualitative and quantitative differences between guinea pigs and rats with respect to cyclohexylphenyl 4-[³⁵S]sulphate metabolism and excretion are usefully compared with those already recorded for the sulphate esters of diethylstilboestrol in the same species (Barford *et al.*, 1977a,b). The differences in metabolism were explained in terms of the enzyme activities of guinea-pig liver and rat liver

respectively. It seems possible that similar explanations may account for the observed differences in cyclohexylphenyl 4-sulphate metabolism. Thus the biliary elimination of unchanged ester in the guinea pig may be explained by the relatively low hepatic sulphohydrolase activity in this animal. However, it should be noted that although the sulphohydrolase in the rat is much more active, desulphation occurs to a minor extent only. In the rat the initial metabolic conversion is hydroxylation, but this is not a major event in the guinea pig, since the major biliary constituent is unmetabolized cyclohexylphenyl 4-[³⁵S]-sulphate. However, significant amounts of the hydroxylated derivative also appear in the guinea-pig bile and it is not readily explained why only small amounts of the glucuronic acid conjugate are present. It seems possible that the observations may be rationalized in terms of the activities of the appropriate hydroxylase and glucuronyltransferase in the guinea pig. It is therefore highly significant that when hydroxycyclohexylphenyl 4-[³⁵S]sulphate purified from guinea-pig bile obtained from animals receiving cyclohexylphenyl 4-[³⁵S]sulphate was re-injected into guinea pigs, it was excreted virtually unchanged in the bile. By contrast, when it was administered to rats, it was almost entirely metabolized to the glucuronic acid conjugate before biliary elimination.

Experiments with isolated perfused guinea-pig liver demonstrated unequivocally that the liver was the site of hydroxylation of cyclohexylphenyl 4-[³⁵S]sulphate. Although only 20.8% of the radioactivity added to the perfusate appeared in bile, a much lower value than those recorded in intact animals, it should be noted that this does not reflect a limitation on the capacity of the liver to eliminate cyclohexylphenyl 4-[³⁵S]sulphate. On the contrary, the absolute amounts of the ester appearing in the bile at the times of maximum excretion in intact animals and in perfusion experiments were comparable.

Bile samples obtained from experiments with isolated perfused livers were quantitatively different from those obtained from intact animals. The most striking difference was the concentration of parent ester in relation to its hydroxylated derivative. The concentration of the latter in bile increased with time in the isolated liver system and represented the majority of the biliary radioactivity after 30 min. The reason for this apparent discrepancy is probably accounted for by the 'closed system' conditions of the perfusion experiments. Thus the hydroxylated derivative cannot be removed from the plasma by renal filtration as in the intact animal, but is repeatedly presented to the liver, which secretes it into the bile. In this connection, it is pertinent that at the end of the perfusion most of the radioactivity in plasma was present as hydroxycyclohexylphenyl 4-[³⁵S]sulphate.

Cyclohexylphenyl 4-[^{35}S]sulphate has a molecular weight for the anion of 255, and elimination of significant amounts of unchanged ester in the bile would not be forecast and does not accord with the suggested molecular-weight threshold (400 ± 50 ; Hirom *et al.*, 1972). For this compound it seems that the molecular-weight criterion is not a significant predetermining factor for significant biliary excretion and that the appearance of the ester in substantial amounts in the bile is more likely to be due to the enzyme complement of the guinea-pig liver. The present study was carried out on male guinea pigs, and it should be noted that with some compounds there is a sex difference in the extent of biliary elimination in rats, but this has not been shown in guinea pigs or rabbits (see, for example, Hearse *et al.*, 1969b; Bertagni *et al.*, 1972; Gregson *et al.*, 1972).

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