Biliary Excretion of some Anionic Derivatives of Diethylstilboestrol and Phenolphthalein in the Guinea Pig

By PATRICIA A. BARFORD,* ANTHONY H. OLAVESSEN, C. GERALD CURTIS and GILLIAN M. POWELL

Department of Biochemistry, University College, P.O. Box 78, Cardiff CF1 1XL, Wales, U.K.

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The metabolic fates and modes of excretion of diethylstilboestrol mono[^{35}S]sulphate and diethylstilboestrol di[^{35}S]sulphate were studied in the guinea pig. Comparative studies were also made with [G- ^3H]diethylstilboestrol and phenolphthalein di[^{35}S]sulphate. Diethylstilboestrol di[^{35}S]sulphate was extensively eliminated in the bile unchanged. After administration of diethylstilboestrol mono[^{35}S]sulphate, extensive biliary elimination of radioactivity was also recorded. Radioactive components were identified as diethylstilboestrol disulphate, diethylstilboestrol monosulphate monoglucuronide and unchanged diethylstilboestrol monosulphate. When [G- ^3H]diethylstilboestrol was administered, ^3H -labelled diethylstilboestrol monoglucuronide, diethylstilboestrol monosulphate monoglucuronide and diethylstilboestrol disulphate appeared in the bile. Phenolphthalein di[^{35}S]sulphate was excreted unchanged in bile. These findings are discussed in relation to studies carried out in the rat [Barford, Olavesen, Curtis & Powell (1977) *Biochem. J.* 164, 423–430] and species differences are related to differences in enzyme activities in rat and guinea-pig liver.

In the rat, the metabolic fates and modes of excretion of the synthetic oestrogens diethylstilboestrol monosulphate and diethylstilboestrol disulphate have been explained (Barford *et al.*, 1977) on the basis of the enzyme complement of rat liver. The esters are desulphated to some extent *in vivo*, a finding consistent with the fact that both are substrates for microsomal arylsulphatase C. In contrast, phenolphthalein disulphate, which is not a substrate for arylsulphatase C, is eliminated unchanged in rat bile (Hirom *et al.*, 1972).

Significant biliary elimination of radioactivity was recorded after the administration of either diethylstilboestrol di[^{35}S]sulphate or diethylstilboestrol mono[^{35}S]sulphate. However, neither ester was excreted unchanged and the major metabolite in bile in each case was diethylstilboestrol mono[^{35}S]sulphate monoglucuronide. This metabolite arises by glucuronidation of the free hydroxyl group of the monosulphate ester and the hydroxyl group made available by desulphation of diethylstilboestrol disulphate.

It is known that the concentrations of arylsulphatase C are approx. 100-fold less in guinea-pig liver (Dolly *et al.*, 1972b) than in rat liver (Dolly *et al.*, 1972a), accounting for the observation that little or no desulphation of oestrone sulphate occurs in the guinea pig and the oestrogen is eliminated mainly in

the bile as a sulphate conjugate (Dolly *et al.*, 1972b). It has also been reported that guinea pigs are poor excretors of some glucuronides (Abou-El-Makarem *et al.*, 1967) and, further, the suggested molecular-weight threshold of 400 ± 50 (Hirom *et al.*, 1972) would lead one to forecast that, with respect to the sulphate esters of diethylstilboestrol, the bile would not be the major excretory route. These observations suggest that the fate of the sulphate esters of diethylstilboestrol in the guinea pig would be substantially different from their fate in the rat. By contrast, the fate of phenolphthalein disulphate might be expected to be the same in both species. The present investigation was undertaken to investigate these predictions.

Materials and Methods

Potassium diethylstilboestrol mono[^{35}S]sulphate (8.9 mCi/mmol) and potassium diethylstilboestrol di[^{35}S]sulphate (11.0 mCi/mmol) were prepared by the methods of Barford *et al.* (1977). Potassium phenolphthalein di[^{35}S]sulphate (8.9 mCi/mmol) was prepared as described by Powell *et al.* (1975). ^{35}S -labelled sulphate esters were stored as dilute frozen aqueous solutions (5 mg/ml). Diethylstilboestrol monoglucuronide was prepared by the method of Dodgson *et al.* (1948). [G- ^3H]Diethylstilboestrol (10 Ci/mmol) was obtained as a solution in benzene from The Radiochemical Centre, Amersham, Bucks., U.K. Before use, the solvent was removed by evaporation and unlabelled diethylstilboestrol was added so

* Present address: Department of Chemistry, University of Aston in Birmingham, Gosta Green, Birmingham B4 7ET, U.K.

that the specific radioactivity was 10 $\mu\text{Ci}/\text{mg}$. This material was dissolved in 50% (v/v) ethanol at a concentration of 7 mg/ml. Diethylstilboestrol mono-[^{35}S]sulphate monoglucuronide was obtained from rat bile (Barford *et al.*, 1977).

Chromatography

Thin-layer chromatography was carried out with activated silica-gel plates (Kieselgel G; Merck, Darmstadt, W. Germany). Solvent systems used were (A) butan-1-ol/acetic acid/water (50:12:25, by vol.) and (B) benzene/ethyl methyl ketone/ethanol/water (3:3:3:1, by vol.). Radioactive areas on the chromatograms were detected and quantified as previously described (Barford *et al.*, 1977).

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. Bile ducts were cannulated after gall-bladder ligation and similar amounts of anaesthetic were administered to maintain anaesthesia throughout the experiment. Test materials were injected via a cannulated jugular vein.

Determination of radioactivity in bile

Bile was suitably diluted and samples (50 μl) 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) calibrated for ^{35}S and ^3H to give a standard error of $\pm 2\%$.

Isotope-dilution analysis

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

Experimental and Results

Experiments with diethylstilboestrol di[^{35}S]sulphate

Guinea pigs (two male and one female) with bile-duct fistulae received potassium diethylstilboestrol di[^{35}S]sulphate (1 mg/0.2 ml of water per 200 g body wt.). Bile was collected after 30 min, 1 h and then at hourly intervals for a further 5 h. Samples were analysed for total [^{35}S]sulphate and the results are recorded in Table 1. Between 64.1% and 90.7% of the administered radioactivity was eliminated in the bile over 6 h, and substantial amounts of the radioactivity in bile appeared within the first 30 min after administration of the ester.

The bile samples obtained 30 min, 1 h, 2 h and 3 h after administration of diethylstilboestrol di[^{35}S]sulphate were subjected to t.l.c. in solvents A and B together with authentic potassium diethylstilboestrol di[^{35}S]sulphate and potassium diethylstilboestrol mono-[^{35}S]sulphate as markers. Radioactive areas on chromatograms were detected by radioautography and scanning. Chromatograms of all bile samples

Table 1. Excretion of radioactivity in bile of guinea pigs injected intravenously with labelled diethylstilboestrol, diethylstilboestrol monosulphate, diethylstilboestrol disulphate or phenolphthalein disulphate at a dosage of 1 mg/200 g body wt. For details, see the text.

Compound administered	Animals	Distribution of radioactivity in bile (% of radioactivity injected) after:							Radioactivity recovered (% of that administered)
		30 min	1 h	2 h	3 h	4 h	5 h	6 h	
[G- ^3H]Diethylstilboestrol	Male	42.8	9.9	6.4	4.0	*	*	*	63.1
	Male	48.8	3.2	5.6	2.2	*	*	*	59.8
	Female	34.1	10.5	6.5	2.8	*	*	*	53.9
	Female	41.2	6.0	4.3	2.0	*	*	*	53.5
Potassium diethylstilboestrol mono[^{35}S]sulphate	Male	63.0	9.4	*	*	*	*	*	72.4
	Male	53.7	15.4	6.9	2.6	0.8	0.6	0.5	80.5
	Female	10.1	18.3	22.4	9.8	6.2	3.6	3.7	74.1
Potassium diethylstilboestrol di[^{35}S]sulphate	Male	65.7	9.6	7.5	3.7	1.6	1.8	0.8	90.7
	Male	37.6	2.8	36.1	4.1	0.3	0.2	0.2	81.3
	Female	51.4	6.0	3.2	1.3	1.0	0.5	0.7	64.1
Potassium phenolphthalein di[^{35}S]sulphate	Male	52.9	15.9	7.4	4.1	3.6	2.0	1.5	87.4
	Male	31.8	15.9	11.7	1.9	2.0	0.6	0.4	64.3
	Female	38.7	21.7	10.6	4.0	2.4	1.1	0.8	79.3

* Collections not made.

showed the presence of one major radioactive component, which was chromatographically identical with authentic diethylstilboestrol di[^{35}S]sulphate. This was confirmed by isotope-dilution analysis. For this analysis bile was collected for 6 h from three guinea pigs that had received potassium diethylstilboestrol di[^{35}S]sulphate (1 mg/0.2 ml of water per 200 g body wt.). Bile samples were pooled and concentrated (approx. 5 ml) at 40°C *in vacuo*. The concentrate was diluted with acetone/water (10 ml; 1:1, v/v) and, after centrifuging (2000g) to clarify, applied as streaks to preparative t.l.c. plates, which were developed in solvent B. The position of the metabolite found in bile was located by using short-wave u.v. light (254 nm), the corresponding areas of the gel were removed from the plates and eluted (3 × 20 ml with acetone/water (1:1, v/v). Eluates were pooled and concentrated to dryness *in vacuo*. Authentic potassium diethylstilboestrol disulphate (50 mg) was added and the whole re-crystallized with water. Crystals were isolated and dried and a weighed sample (approx. 3 mg) was retained for the determination of specific radioactivity. The procedure was repeated a further four times and material of constant specific radioactivity was obtained after the first crystallization.

Experiments with diethylstilboestrol mono[^{35}S]sulphate

Guinea pigs (two male and one female) with bile-duct fistulae received potassium diethylstilboestrol mono[^{35}S]sulphate (1 mg/0.2 ml of water per 200 g body wt.). Bile was collected after 30 min, 1 h and then at hourly intervals for a further 5 h. Samples were analysed for total [^{35}S]sulphate. The results (Table 1) demonstrated that between 72.4% and 80.5% of the administered radioactivity appeared in bile after 6 h.

The bile samples obtained 30 min, 1 h, 2 h and 3 h after administration of the ester were subjected to t.l.c. in solvents A and B in the presence of suitable marker compounds. Radioactive areas were located by radioautography and scanning. All bile samples contained three main radioactive components designated A, B and C. The mobility of component A (R_F 0.41 in solvent A; 0.14 in solvent B) was identical with diethylstilboestrol mono[^{35}S]sulphate monoglucuronide and co-chromatographed with it. Component B was identical in mobility and co-chromatographed with diethylstilboestrol di[^{35}S]sulphate (R_F 0.41 in solvent A; 0.23 in solvent B). Component C co-chromatographed with diethylstilboestrol mono[^{35}S]sulphate (R_F 0.65 in solvent A; 0.80 in solvent B). The identity of component B (the major component) as diethylstilboestrol di[^{35}S]sulphate was confirmed by isotope-dilution analysis. For this analysis bile was collected for 6 h from a guinea pig receiving injections of diethylstilboestrol mono[^{35}S]sulphate (5 mg) at hourly intervals. Component B was purified and

subjected to isotope-dilution analysis as described above. Material of constant specific radioactivity was obtained after the second crystallization.

Experiments with [G- ^3H]diethylstilboestrol

Guinea pigs (two male and two female) with bile-duct fistulae received [G- ^3H]diethylstilboestrol (1 mg/200 g body wt.) and bile was collected after 30 min, 1 h, 2 h and 3 h. Bile samples were analysed for total radioactivity and between 53.5% and 63.1% of the administered radioactivity appeared in the bile within 3 h (Table 1). Most of the biliary radioactivity appeared in bile collected over the first 30 min.

Bile samples obtained 30 min and 1 h after administration of [G- ^3H]diethylstilboestrol were subjected to t.l.c. in solvents A and B, in the presence of appropriate marker compounds. Radioactive areas were located by scanning. All bile samples contained three radioactive components. The mobility of one of the components was consistent with its identification as [G- ^3H]diethylstilboestrol monosulphate monoglucuronide. The mobilities of the other components were consistent with their identification as [G- ^3H]diethylstilboestrol disulphate and [G- ^3H]diethylstilboestrol monoglucuronide (R_F 0.47 in solvent B).

To confirm the presence of the glucuronic acid moieties and the identities of the glucuronic acid conjugates, bile samples (0.5 ml) were mixed with an equal volume of a solution of β -glucuronidase [Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K.; type BI from bovine liver; 2000 Fishman units (Talalay *et al.*, 1946)/ml] in 0.1 M-sodium phosphate buffer, pH 6.5, and incubated at 37°C for 6 h. Portions of the incubation mixtures, together with suitable marker compounds, were subjected to t.l.c. in solvent B. Radioactive areas were located by scanning. Chromatograms showed the appearance of two new radioactive components which were chromatographically identical with [G- ^3H]diethylstilboestrol and diethylstilboestrol mono[^{35}S]sulphate.

Experiments with phenolphthalein di[^{35}S]sulphate

Guinea pigs (two male and one female) with bile-duct fistulae received potassium phenolphthalein di[^{35}S]sulphate (1 mg/0.2 ml of water per 200 g body wt.). Bile was collected at suitable intervals over 6 h. Analysis of bile samples for total [^{35}S]sulphate showed that between 64.3% and 87.4% of the administered radioactivity was eliminated in the bile over 6 h (see Table 1).

Bile samples collected up to 2 h after administration of the ester were subjected to t.l.c. in solvent A and radioactive areas were located by radioautography and scanning. All bile samples contained a single radioactive component that was chromatographically

Scheme 1. Schematic representation of the biliary excretion of diethylstilboestrol mono[³⁵S]sulphate, diethylstilboestrol di[³⁵S]sulphate and their metabolites in rat and guinea-pig bile

St-SO₄, diethylstilboestrol mono[³⁵S]sulphate; St-(SO₄)₂, diethylstilboestrol di[³⁵S]sulphate; St-SO₄-GlcA, diethylstilboestrol mono[³⁵S]sulphate monoglucuronide; SO₄, sulphate.

identical with authentic phenolphthalein di[³⁵S]-sulphate (*R_F* 0.47 in solvent A).

Discussion

The metabolic fates of diethylstilboestrol mono[³⁵S]sulphate and diethylstilboestrol di[³⁵S]sulphate in the guinea pig differ markedly from those already established in the rat (Barford *et al.*, 1977). The salient differences shown in Scheme 1 are explicable in terms of the enzyme activities of guinea-pig liver and rat liver respectively.

In the guinea pig, the relatively low sulphohydrolase activity accounts for the limited release of inorganic sulphate from both diethylstilboestrol monosulphate and diethylstilboestrol disulphate. Consequently, the disulphate is eliminated unchanged in the bile, whereas the administered monosulphate is available for conjugation. Although conjugation in the rat is exclusively with glucuronic acid, in the guinea pig sulphation of the free hydroxyl group is quantitatively important. A similar situation occurs with respect to stilboestrol itself. In the rat, although the liver possesses the facility for sulphating diethylstilboestrol (Payne & Mason, 1963) the glucuronide derivative is the major biliary component, whereas in the guinea pig both glucuronide and sulphate conjugation occur. Again, these results may be explained in terms of the relative activities of the glucuronyltransferase and the sulphotransferase systems in the two species. In spite of the fact that rat liver is known to possess an enzyme capable of sulphating diethylstilboestrol, it appears that the combination of the sulphohydrolase and the effective liver glucuronyltransferase favours the formation and secretion of the glucuronide derivative in the bile.

A comparison of the extents to which the compounds studied are metabolized and eliminated via the bile in the guinea pig and the rat illustrate an important principle with respect to an understanding of the mechanisms involved in the biliary secretion of conjugates. It is clear from the present studies that a mechanism for the biliary secretion of sulphuric acid esters is present in the guinea pig, because diethylstilboestrol disulphate is excreted unchanged via this route. Furthermore, diethylstilboestrol monosulphate is excreted as the mono- and disulphate conjugates. The apparent inability of the rat to excrete diethylstilboestrol disulphate unchanged is almost certainly not due to the absence of an appropriate biliary mechanism, but rather to the fact that it is desulphated. This concept derives support from the observations that phenolphthalein disulphate, which is not a substrate for arylsulphatase C (Powell *et al.*, 1975), is excreted unchanged in rat bile.

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