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Note

Thin-layer chromatography of bile salt sulphates

G. PARMENTIER* and H. EYSSEN
The Rega Institute, University of Leuven, 10 Minderbroedersstraat, B-3000 Leuven (Belgium)
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Appreciable quantities of sulphate esters of bile salts are produced in hepatobiliary diseases in man[1–4]. Bile salt sulphates also occur in bile and the intestinal contents of mice[5] and rats[6–8]. However, unless these substances are solvolysed prior to analysis, they are not detected by the common methods for bile salt determination. Analytical methods for the sulphates of the common bile salts are still inadequate. Chromatography on Sephadex LH-20 separates bile salt sulphates into mono-, di- and trisulphates[9], but fails to resolve the respective isomers within each group. Information on thin-layer chromatography (TLC) of bile salt sulphates is scarce[10–15] and only deals with a limited number of compounds, some of which are chemically undefined. In previous papers we have described the synthesis of the isomeric monosulphates of cholic acid[16] and of unconjugated and conjugated chenodeoxycholic and deoxycholic acid[17]. We now report the TLC characteristics, in six solvent systems, of 33 bile salt sulphates, representing most of the possible sulphate esters of the common bile salts.

MATERIALS AND METHODS

Bile salt sulphates

Bile salts were sulphated by treatment with a pyridine-SO_3 complex prepared by addition of chlorosulphonic acid to dry pyridine[18]. Details of the preparation and purification of the isomeric monosulphates of dihydroxy and trihydroxy bile acids have been reported previously[16,17]. The monosulphates of 3β-hydroxy-5-cholenoic acid, and of lithocholic acid and its taurine or glycine conjugates, were similarly prepared by sulphation of these monohydroxy bile salts for at least 24 h. The disulphates of unconjugated or conjugated chenodeoxycholic and deoxycholic acid, and the trisulphates of unconjugated or conjugated cholic acid, were prepared by sulphation of the parent substances for periods from 24 h to 5 days. After desalting by passage over Amberlite XAD-2 and purification by chromatography on Sephadex LH-20 with chloroform–methanol (1:1), all products were obtained as the sodium salts.

Cholate 3,7-disulphate was prepared by sulphation of 3α,7α-dihydroxy-12-oxo-5β-cholanoic acid and reduction of the resulting trisodium 3α,7α-disulphoxy-12-oxo-5β-cholanoate with sodium borohydride. Cholate 3,12-disulphate was similarly

* To whom reprint requests should be addressed.

obtained from 3α,12α-dihydroxy-7-oxo-5β-cholanoic acid. Cholate 7,12-disulphate was prepared by sulphation of the 3-ethoxycarbonyl derivative[19] of methyl cholate; after sulphation, the protecting 3-ethoxycarbonyl group was removed by mild alkaline hydrolysis[17]. The products were chromatographed on Sephadex LH-20 with chloroform–methanol (1:1). The location of the sulphate ester groups was confirmed by oxidation of the free hydroxyl function followed by solvolysis and identification of the resulting dihydroxy-oxo-5β-cholanoic acid by gas–liquid chromatography, as described for the monosulphates[16].

TLC procedure

Ascending TLC was carried out on 20 × 20 cm silica gel pre-coated plates with a layer thickness of 0.25 mm (Merck, Darmstadt, G.F.R.). Samples were dissolved in methanol at a concentration of 0.1–0.5%, and amounts of 10–20 μg of the bile salt sulphate were applied to the plate. The chromatography tank atmosphere was saturated with the solvent system for 1 h prior to development. After chromatography, the plates were allowed to dry for 30 min and the bile salt sulphates were detected by spraying with 10% sulphuric acid in glacial acetic acid and heating for 5 min at 110°.

Solvent systems

Six solvent systems were selected for TLC of the bile salt sulphates. System 1 and the systems 5 and 6 were developed in this laboratory. Solvent 2 was described by Briggs and Bussjaeger[20] for TLC of tauroallocholate and sulphated bile alcohols in fish bile. Solvents 3 and 4 were modifications of the *n*-butanol–acetic acid–water system (10:1:1) used by Gänshirt *et al.*[21] for chromatography of conjugated bile salts.

Solvent 1: methyl ethyl ketone–chloroform–methanol–propan-2-ol–acetic acid–water (20:6:2:2:4:1)

Solvent 2: ethyl acetate–*n*-butanol–acetic acid–water (8:6:3:3)

Solvent 3: *n*-butanol–acetic acid–water (10:2:1)

Solvent 4: *n*-butanol–acetic acid–water (10:5:1)

Solvent 5: methyl ethyl ketone–methanol–propan–2–ol–acetic acid–water (10:1:1:1:1)

Solvent 6: methyl ethyl ketone–methanol–propan–2–ol–acetic acid–water (10:1:1:3:1).

RESULTS AND DISCUSSION

The R_F values of unconjugated, glycine- or taurine-conjugated bile salt sulphates in six solvent systems are presented in Tables I, II and III, respectively. Bile salt sulphates are soluble in methanol and water, slightly soluble in ethanol, and nearly insoluble in less polar solvents. In consequence, the choice of solvent systems in which they migrate sufficiently is limited. Unconjugated bile salt monosulphates were well resolved in solvent 1. The systems containing *n*-butanol (solvents 2–4) were well suited to the separation of unconjugated or glycine-conjugated bile salt sulphates, but ran slowly (3 h for solvent 2, and 5 h for solvents 3 and 4). Taurine-conjugated bile salt sulphates could be chromatographed in systems 4–6.

Unconjugated bile salt sulphates (Table I) migrated according to their pre-

dicted polarity, and no overlapping occurred with the unconjugated non-sulphates. In all systems, the groups of bile salt monosulphates, of dihydroxy bile salt disulphates, of trihydroxy bile salt disulphates and the only trihydroxy bile salt trisulphate used, were separated. In system 1, the monosulphates of monohydroxy, of dihydroxy and of trihydroxy bile salts migrated in that order. However, in systems 2–4, deoxycholate 3-sulphate was incompletely resolved from lithocholate 3-sulphate and from 3β-hydroxy-5-cholenoate sulphate. According to their faster migration on TLC, bile salt 3-sulphates were less polar than the isomeric 7- or 12-sulphates. Chenodeoxycholate 7-sulphate was separated from deoxycholate 12-sulphate in system 1, but cholate 7-sulphate was always poorly resolved from cholate 12-sulphate. Chenodeoxycholate disulphate was incompletely separated from deoxycholate disulphate, but both compounds moved faster than the cholate disulphates. Cholate 3,7-disulphate and cholate 3,12-disulphate were not separated from each other but moved in front of cholate 7,12-disulphate in systems 3–6.

TABLE I

R_F VALUES OF UNCONJUGATED BILE SALTS AND THEIR SULPHATES

Compound	*Solvent system*					
	1	*2*	*3*	*4*	*5*	*6*
Lithocholic acid	0.95	0.98	0.93	0.95	0.96	0.98
3β-Hydroxy-5-cholenoic acid	0.95	0.98	0.92	0.95	0.96	0.98
Deoxycholic acid	0.93	0.96	0.91	0.92	0.94	0.98
Chenodeoxycholic acid	0.92	0.96	0.91	0.92	0.94	0.97
Cholic acid	0.85	0.93	0.86	0.89	0.92	0.95
Lithocholate 3-sulphate	0.75	0.73	0.73	0.83	0.95	0.96
3β-Hydroxy-5-cholenoate 3-sulphate	0.75	0.71	0.71	0.81	0.93	0.94
Deoxycholate 3-sulphate	0.66	0.69	0.73	0.82	0.91	0.94
Chenodeoxycholate 3-sulphate	0.63	0.68	0.71	0.79	0.88	0.92
Deoxycholate 12-sulphate	0.59	0.66	0.71	0.79	0.87	0.92
Chenodeoxycholate 7-sulphate	0.54	0.64	0.68	0.77	0.84	0.90
Cholate 3-sulphate	0.50	0.58	0.65	0.77	0.81	0.89
Cholate 7-sulphate	0.39	0.53	0.60	0.73	0.73	0.84
Cholate 12-sulphate	0.37	0.51	0.58	0.72	0.73	0.84
Deoxycholate disulphate	0.23	0.35	0.39	0.63	0.60	0.77
Chenodeoxycholate disulphate	0.22	0.35	0.37	0.61	0.58	0.76
Cholate 3,7-disulphate	0.17	0.25	0.31	0.56	0.47	0.68
Cholate 3,12-disulphate	0.16	0.25	0.31	0.55	0.46	0.66
Cholate 7,12-disulphate	0.14	0.23	0.26	0.49	0.41	0.57
Cholate trisulphate	0.06	0.11	0.14	0.34	0.26	0.48

Glycine-conjugated bile salt sulphates (Table II) migrated in the same order as described for the unconjugated sulphate esters. In system 1 and in systems 5 and 6, however, unsulphated glycocholate moved more slowly than glycolithocholate 3-sulphate. A similar result may be expected for glycocholate and glyco-3β-hydroxy-5-cholenoate sulphate. Taurine-conjugated bile salt sulphates (Table III) migrated very slowly in systems 1–3. However, an increase in the acetic acid content of the butanol–acetic acid–water systems, resulted in a considerable improvement in the mobility (solvent 4). A similar pattern of separation was obtained with systems 5 and 6, which had the advantage of a shorter development time.

TABLE II

R_F VALUES OF GLYCINE CONJUGATED BILE SALTS AND THEIR SULPHATES

Compound	*Solvent system*					
	1	*2*	*3*	*4*	*5*	*6*
Glycolithocholate	0.84	0.88	0.89	0.88	0.88	0.94
Glycodeoxycholate	0.68	0.79	0.75	0.82	0.81	0.88
Glycochenodeoxycholate	0.67	0.78	0.75	0.82	0.80	0.88
Glycocholate	0.40	0.62	0.61	0.74	0.65	0.78
Glycolithocholate 3-sulphate	0.45	0.53	0.57	0.73	0.73	0.83
Glycodeoxycholate 3-sulphate	0.31	0.42	0.48	0.67	0.59	0.76
Glycochenodeoxycholate 3-sulphate	0.28	0.42	0.48	0.67	0.59	0.75
Glycochenodeoxycholate 7-sulphate	0.20	0.38	0.42	0.62	0.47	0.68
Glycodeoxycholate 12-sulphate	0.18	0.38	0.41	0.62	0.44	0.65
Glycocholate 3-sulphate	0.18	0.30	0.32	0.56	0.41	0.64
Glycochenodeoxycholate disulphate	0.09	0.16	0.18	0.42	0.24	0.48
Glycodeoxycholate disulphate	0.08	0.15	0.16	0.39	0.21	0.46
Glycocholate trisulphate	0.02	0.04	0.05	0.19	0.05	0.26

All systems failed to separate adequately the 3-sulphates of conjugated chenodeoxycholate and deoxycholate. Similarly, the 7-sulphate of conjugated chenodeoxycholate was poorly separated from conjugated deoxycholate 12-sulphate. This was not entirely unexpected, as the resolution on TLC of chenodeoxycholic and deoxycholic acid has always been difficult, either in underivatized form or as the methyl esters. However, the different colours obtained after spraying with the sulphuric acid–acetic acid (1:9) reagent facilitated the identification of these two bile salts. Sulphated bile salts showed the same colour reactions as the non-sulphates. Cholate and deoxycholate sulphates, either unconjugated or conjugated, showed yellow fluorescent spots under UV light; spots of chenodeoxycholate and its sulphates were grey with a pink fluorescent fringe, whereas sulphated or unsulphated lithocholate and 3β-hydroxy-5-cholenoate could be distinguished from the other bile salts by their purple colour and fluorescence.

TABLE III

R_F VALUES OF TAURINE CONJUGATED BILE SALTS AND THEIR SULPHATES

Compound	*Solvent system*					
	1	*2*	*3*	*4*	*5*	*6*
Taurolithocholate	0.43	0.55	0.62	0.74	0.73	0.83
Taurodeoxycholate	0.29	0.48	0.54	0.67	0.65	0.75
Taurochenodeoxycholate	0.28	0.47	0.54	0.67	0.64	0.74
Taurocholate	0.13	0.36	0.38	0.55	0.41	0.55
Taurolithocholate 3-sulphate	0.17	0.29	0.34	0.55	0.48	0.65
Taurodeoxycholate 3-sulphate	0.11	0.20	0.27	0.47	0.41	0.57
Taurochenodeoxycholate 3-sulphate	0.10	0.20	0.27	0.47	0.41	0.56
Taurodeoxycholate 12-sulphate	0.08	0.19	0.24	0.39	0.29	0.47
Taurochenodeoxycholate 7-sulphate	0.07	0.18	0.23	0.39	0.29	0.46
Taurocholate 3-sulphate	0.05	0.12	0.17	0.31	0.28	0.46
Taurodeoxycholate disulphate	0.03	0.05	0.09	0.21	0.14	0.28
Taurochenodeoxycholate disulphate	0.02	0.04	0.09	0.21	0.13	0.28
Taurocholate trisulphate	start	start	0.03	0.06	0.04	0.12

The glycine- or taurine-conjugated cholate 7- or 12-monosulphates have not yet been obtained as chemically defined products and therefore are not included in the Tables. Their mobility may be expected to be inferior to that of the corresponding 3-sulphates but greater than that of conjugated dihydroxy bile salt disulphates. The isomeric glycine- or taurine-conjugated cholate disulphates were not prepared because the appropriate conjugated starting products were not available. However, their polarities would indicate that these compounds should migrate between the corresponding trisulphates and the dihydroxy bile salt disulphates.

Whether unconjugated bile salt disulphates can be conjugated with glycine or taurine, as described for unsulphated bile acids[22], remains to be established; it might greatly facilitate the preparation of specific mono- or di-sulphates of conjugated trihydroxy bile salts. On the other hand, unpublished experiments performed in our laboratory have shown that glycine- or taurine-conjugated bile salt monosulphates can be deconjugated enzymatically with cholylglycine hydrolase, or with an acetone-dried powder of *Clostridium perfringens*, to yield the corresponding unconjugated bile salt monosulphates which, in turn, can be identified by their mobility on TLC and their colour reactions after spraying with 10% sulphuric acid in glacial acetic acid. This procedure might contribute significantly to a more precise characterization of bile salt sulphates in the serum or in the urine of patients with hepato-biliary diseases.

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