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STUDIES ON STEROIDS

CLXX. SEPARATION AND DETERMINATION OF BILE ACID 3-SULFATES IN HUMAN BILE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A method for the simultaneous determination of sulfated bile acids in human bile without prior hydrolysis and solvolysis is described. The sulfate fraction was obtained from a bile specimen by passing it through a Sep-Pak C_{18} cartridge, followed by group separation by ion-exchange chromatography on a lipophilic gel, piperidinohydroxypropyl Sephadex LH-20. Subsequent resolution into the 3-sulfates of unconjugated, glycine- and taurine-conjugated ursodeoxycholate, cholate, chenodeoxycholate, deoxycholate and lithocholate was attained by high-performance liquid chromatography (HPLC) on an SC-02 column. Separation of these sulfates was effected when acetonitrile—0.5% ammonium carbonate (8:31, 8:26 and 8:23, v/v) was used as mobile phase. The sulfated bile acids in human bile were unequivocally identified on the basis of their behaviour in HPLC using mobile phases of various pH values. The present method proved to be applicable to the characterization and quantitation of sulfated bile acids in human bile.

INTRODUCTION

In recent years considerable attention has been focused on the metabolism of unsulfated and sulfated bile acids in man in connection with hepatobiliary diseases [1—4]. The separation and determination of sulfated bile acids has hitherto been performed by gas—liquid chromatography (GLC) with prior hydrolysis and/or solvolysis [3, 4]. This method, however, has the inevitable disadvantages of lack of reliability of the analytical results owing to incomplete deconjugation [5, 6] and formation of artifacts, as well as the loss of information about the conjugated form. In the previous paper of this series we described the high-performance liquid chromatography (HPLC) of the

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3-sulfates of unconjugated, glycine- and taurine-conjugated bile acids [7]. The present paper deals with a method for the simultaneous determination of bile acid 3-sulfates in human bile, which involves clean-up with a Sep-Pak C_{18} cartridge, group separation by ion-exchange chromatography on a lipophilic gel, and subsequent resolution into individual sulfated bile acids by HPLC. In addition, characterization of these sulfates by HPLC using mobile phases of various pH values is also described.

EXPERIMENTAL

High-performance liquid chromatography

The apparatus used for this work was a Waters 6000A solvent delivery system (Waters Assoc., Milford, MA, U.S.A.) equipped with a Model Uvidec-100 II ultraviolet (UV) detector (Japan Spectroscopic Co., Tokyo, Japan) for monitoring the absorbance at 205 nm. The test samples were applied to the chromatograph by a Waters U6K sample loop injector (Waters Assoc.) with an effective volume of 2 ml. A column (25 cm × 4 mm I.D.) packed with ODS SC-02 (10 μm) (Japan Spectroscopic Co.), an octadecyl-bonded silica, was used under ambient conditions.

Materials

The standard bile acid 3-, 7- and 12-sulfates and glycocholic acid were synthesized in these laboratories by the methods previously reported [8, 9]. All the chemicals employed were of analytical-reagent grade. Solvents were purified by distillation prior to use. Sephadex LH-20, and Amberlite XAD-2 and XAD-4 were supplied by Pharmacia Fine Chemicals (Uppsala, Sweden) and Rohm and Haas Co. (Philadelphia, PA, U.S.A.), respectively. Amberlite XAD resins were washed successively with methanol, 6% hydrochloric acid in 70% ethanol, water, 6% sodium hydroxide in 70% ethanol, water and methanol before use. Piperidinohydroxypropyl Sephadex LH-20 (PHP-LH-20) (0.8 mequiv./g was prepared in the manner previously reported [10]. A Sep-Pak C_{18} cartridge (Waters Assoc.) was also washed successively with methanol (5 ml) and water (10 ml) before use. Sulfoethyl Sephadex LH-20 (SE-LH-20) was prepared according to the method of Setchell et al. [11].

Procedure for determination of bile acid 3-sulfates in human bile

A bile specimen (100 μl) was diluted with 0.5 *M* phosphate buffer (pH 7.0, 4 ml), heated at 60°C for 1 h and passed through a Sep-Pak C_{18} cartridge. After washing with water (12 ml), unsulfated and sulfated bile acids were eluted with 90% ethanol (4 ml). The eluate was applied to a column (20 × 6 mm I.D.) of PHP-LH-20 (acetate) (110 mg). After removal of neutral compounds by washing with 90% ethanol (4 ml), unsulfated bile acids were separated into the unconjugated, glycine- and taurine-conjugated fractions by stepwise elution with 0.1 *M* acetic acid in 90% ethanol (4 ml), 0.2 *M* formic acid in 90% ethanol (4 ml) and 0.3 *M* acetic acid—potassium acetate in 90% ethanol (pH 6.3, 4 ml) in the manner previously reported [10, 12]. The sulfated bile acids were then eluted with 1% ammonium carbonate in 70% ethanol (4 ml). To the eluate were added glycocholic acid (2.0 μg) and 7α-

acetoxy-12α-hydroxy-3-oxo-5β-cholan-24-oic acid 12-sulfate (40 μg) as internal standards, and the whole was subjected to a SE-LH-20 column (50 mm × 10 mm I.D.) for elimination of inorganic salts. After washing with 70% ethanol (4 ml), the effluent and washing were combined and concentrated in vacuo below 40°C. The residue was redissolved in water (100 μl), a 10—30 μl aliquot of which was subjected to HPLC.

Recovery test for bile acid 3-sulfates added to human bile

The test samples were prepared by dissolving the 3-sulfates (50 μg each of unconjugated and 5 μg each of glycine- and taurine-conjugated cholate, chenodeoxycholate, deoxycholate and lithocholate) in human hepatic bile (100 μl). The bile specimen was diluted with 0.5 *M* phosphate buffer (4 ml) and subjected successively to clean-up with a Sep-Pak C_{18} cartridge, group separation on PHP-LH-20, desalting on SE-LH-20 and determination by HPLC in the manner described above.

Extraction of bile acid sulfates by Amberlite XAD resin or Sep-Pak C_{18} cartridge

Amberlite XAD resin. A synthetic mixture of 50 μg each of 3-sulfates of glycine- and taurine-conjugated cholate and chenodeoxycholate was dissolved in water (50 ml), adjusted to pH 2—11 with dilute hydrochloric acid or sodium hydroxide solution and then applied to a column (12.5 cm × 18 mm I.D.) of Amberlite XAD-2 or XAD-4 (10 g). After washing with water (50 ml), the sulfates were eluted with 50—100% ethanol (100 ml) with or without concentrated hydrochloric acid (0.05 ml) or concentrated ammonia (1 ml). The eluate was added with an internal standard and then subjected to HPLC.

Sep-Pak C_{18} cartridge. A synthetic mixture of 50 μg of chenodeoxycholate 3-sulfate, 25 μg each of 3-sulfates of taurochenodeoxycholate, glycine-conjugated cholate, chenodeoxycholate, deoxycholate and lithocholate was dissolved in phosphate buffer (pH 7.0, 4 ml) and applied to the cartridge. After washing with water (8 ml), the sulfates were eluted with 90% ethanol. The effluent was fractionally collected (each 0.5 ml), to which was added an internal standard, and then subjected to HPLC.

RESULTS AND DISCUSSION

Group separation of sulfated bile acids on PHP-LH-20

In the previous paper of this series, we demonstrated the separation of unsulfated bile acids into unconjugated, glycine- and taurine-conjugated fractions on a lipophilic ion-exchange gel, PHP-LH-20 [10, 12]. Almé et al. [4] reported the group separation on diethylaminohydroxypropyl Sephadex LH-20 where acetate buffer in 72% ethanol (pH 7.6) was used for the elution of bile acid monosulfates. In this study, 1% ammonium carbonate in 70% ethanol (pH 9.0) was chosen as a suitable eluent for sulfated bile acids. A synthetic mixture of 50 μg each of 3-sulfates of unconjugated, glycine- and taurine-conjugated cholate and lithocholate dissolved in 90% ethanol was applied to a column of PHP-LH-20. After washing with 90% ethanol, unsulfated bile acids were eluted successively with 4 ml each of 0.1 *M* acetic acid in 90% ethanol, 0.2 *M* formic

acid in 90% ethanol and 0.3 *M* acetic acid—potassium acetate in 90% ethanol (pH 6.3). The desired sulfate fraction was then eluted with 1% ammonium carbonate in 70% ethanol and subjected to HPLC. As illustrated in Fig. 1, each of the bile acid 3-sulfates was recovered at a rate of more than 90% in an initial 2-ml effluent. Other 3-sulfates also showed a similar elution pattern.

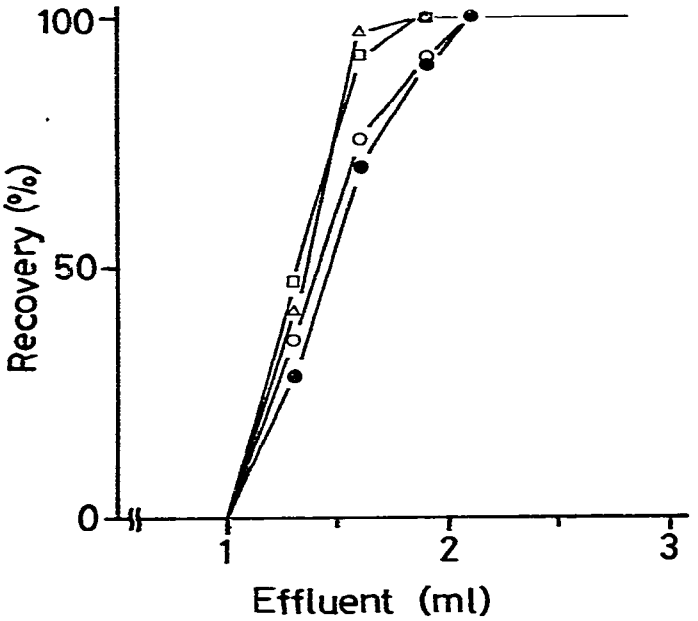

Fig. 1. Cumulative elution curves of bile acid 3-sulfates on PHP-LH-20. (○), Cholate 3-sulfate; (□), glycocholate 3-sulfate; (△), taurocholate 3-sulfate; (●), lithocholate 3-sulfate.

Clean-up procedure for sulfated bile acids in human bile

Amberlite XAD resin is widely used for the separation of polar compounds in biological fluids. Recently, Bradlow [13] reported that steroid conjugates such as dehydroepiandrosterone sulfate are quantitatively eluted with methanol from Amberlite XAD-2 when the conjugates are converted to the triethylamine salts by washing with 0.5 *M* triethylamine sulfate (pH 7.2). By this method, however, sulfated bile acids were not quantitatively recovered. The effect of pH on adsorption and desorption on Amberlite XAD-2 or XAD-4 was investigated with various bile acid 3-sulfates. A synthetic mixture of 50 μg each of 3-sulfates of cholate, glycocholate, taurocholate, chenodeoxycholate, glycochenodeoxycholate and taurochenodeoxycholate was dissolved in water, adjusted to pH 2—11, and then applied to an Amberlite XAD-2 or XAD-4 column. The sulfates eluted with ethanol were separated and determined by HPLC. In the case of Amberlite XAD-4, the sulfates were effectively adsorbed when the solution was adjusted to pH 8—10 (Fig. 2). The use of an acidified eluent was suitable for desorption of the sulfates. The content of ethanol in the eluent was also important (Fig. 3). As for Amberlite XAD-2, the use of pH 4 for the sample solution and pH 9 for the eluent was found most suitable for extraction of sulfated bile acids and this result was almost identical with that

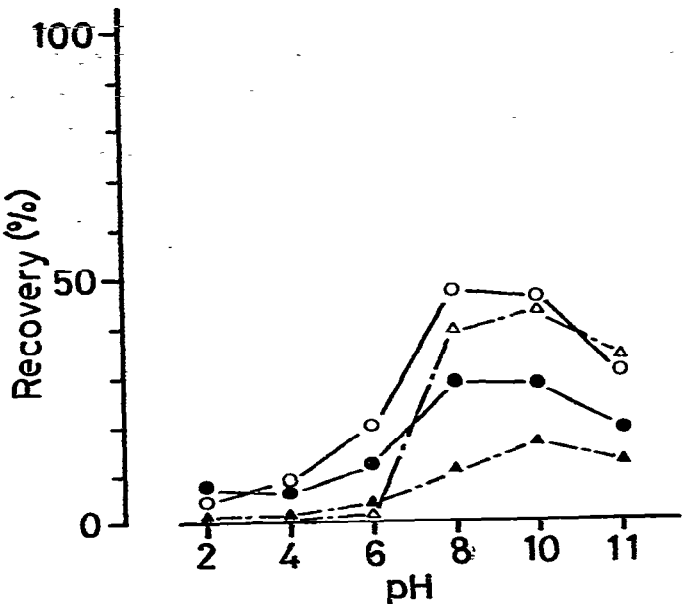

Fig. 2. Effect of pH on the adsorption of bile acid 3-sulfates on Amberlite XAD-4. (○), Glycocholate 3-sulfate; (●), taurocholate 3-sulfate; (△), glycochenodeoxycholate 3-sulfate; (▲), taurochenodeoxycholate 3-sulfate.

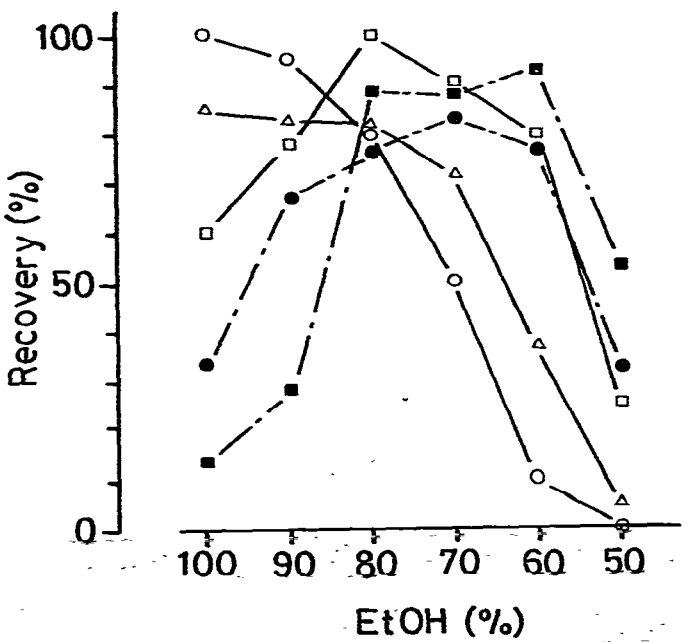

Fig. 3. Effect of ethanol (EtOH) concentration on the desorption of bile acid 3-sulfates on Amberlite XAD-4. (○), Deoxycholate 3-sulfate; (△), glycodeoxycholate 3-sulfate; (□), taurodeoxycholate 3-sulfate; (●), taurochenodeoxycholate 3-sulfate; (■), taurocholate 3-sulfate.

reported previously [4]. However, the recovery rates of the sulfates by these procedures were dependent upon their structures and, hence, the appropriate extraction conditions for all the sulfates could not be established. Moreover, the use of Amberlite XAD resins required removal of interfering inorganic acid or base in the eluent prior to separation on a lipophilic ion-exchange gel [4]. Accordingly, the use of a Sep-Pak C_{18} cartridge [12, 14] for this purpose was then undertaken. A synthetic mixture of 3-sulfates of chenodeoxycholate, taurochenodeoxycholate, glycine-conjugated cholate, chenodeoxycholate, deoxycholate and lithocholate was dissolved in phosphate buffer (pH 7.0) and applied to the cartridge. After elimination of inorganic salts by washing with water, the desired sulfates were eluted with 90% ethanol and then determined by HPLC. The sulfated bile acids were recovered at a rate of more than 90% in an initial 2-ml effluent. When 1.5% ethanol was used to remove co-existing polar substances as previously reported [12], a small amount of the polar sulfate was lost. The use of a Sep-Pak C_{18} cartridge was much more efficient and convenient for extraction of sulfated bile acids in biological fluids than Amberlite XAD resin.

Determination of sulfated bile acids in human bile

A standard procedure for the separation and determination of sulfated bile acids in human bile is shown in Fig. 4. The sulfates were separated by HPLC on the ODS SC-02 column under the conditions previously reported [7] with a minor modification. First, 3-sulfates of unconjugated, glycine- and taurine-conjugated ursodeoxycholate and cholate were separated with acetonitrile—0.5% ammonium carbonate (8:31, v/v) and then the 3-sulfates of unconjugated and conjugated chenodeoxycholate and deoxycholate were resolved with acetonitrile—0.5% ammonium carbonate (8:26, v/v). Finally, acetonitrile—0.5% ammonium carbonate (8:23, v/v) was chosen as a suitable mobile phase for the separation of lithocholate 3-sulfates. For quantitation of ursodeoxycholate and cholate 3-sulfates, 7α-acetoxy-12α-hydroxy-3-oxo-5β-cholan-24-oic acid 12-

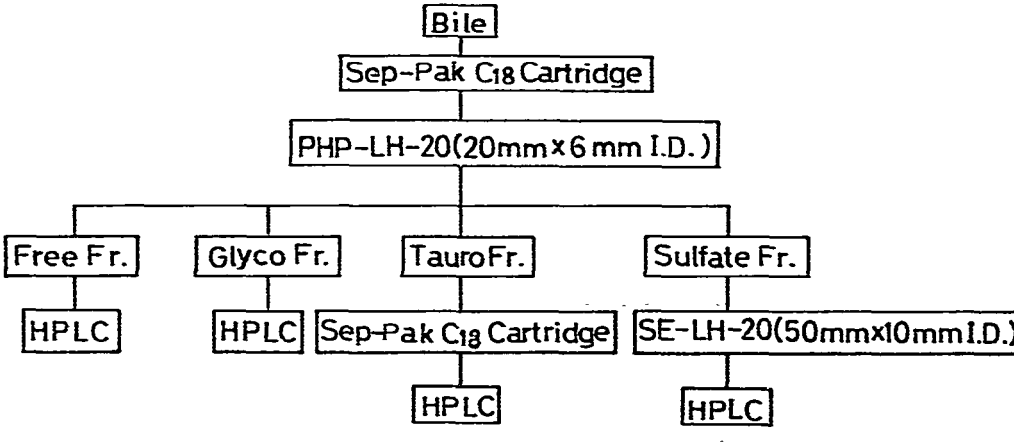

Fig. 4. General scheme for separation and determination of bile acid 3-sulfates in human bile.

sulfate was used as an internal standard, while for other sulfates glycocholic acid was used.

The calibration curve was constructed by plotting the ratio of peak area of each bile acid 3-sulfate to that of the internal standard against the amount of 3-sulfate. The quantitation limits of glycine- and taurine-conjugated and unconjugated bile acid 3-sulfates obtainable by monitoring the absorbance at 205 nm were 0.5, 1.0 and 5.0 μg, respectively. Applying the standard procedure to human bile, 3-sulfates of conjugated bile acids were determined with a satisfactory reproducibility (Table I). The known amounts of representative bile acid 3-sulfates were added to human bile and their recovery rates were estimated. As listed in Table II, almost all bile acid 3-sulfates were recovered at a rate of more than 90%, the only exception being lithocholate 3-sulfate (88%).

TABLE I

REPRODUCIBILITY OF THE PRESENT METHOD FOR DETERMINATION OF BILE ACID 3-SULFATES IN HUMAN BILE

Bile acid 3-sulfate	Found (μg per 0.1 ml, ± S.D.)*	
	G**	T**
Ursodeoxycholate	5.46 ± 0.42	0.88 ± 0.18
Cholate	2.10 ± 0.35	1.33 ± 0.24
Chenodeoxycholate	5.63 ± 0.19	5.59 ± 0.13
Deoxycholate	7.59 ± 0.17	8.20 ± 0.30
Lithocholate	2.38 ± 0.21	1.01 ± 0.28

*$n = 9$.
**G = glycine conjugate; T = taurine conjugate.

TABLE II

RECOVERY OF UNCONJUGATED AND CONJUGATED BILE ACID 3-SULFATES ADDED TO HUMAN BILE

Bile acid 3-sulfate	Bile	Added (μg per 0.1 ml)	Expected (μg per 0.1 ml)	Found (μg per 0.1 ml)	Recovery (%, ± S.D.*)
Cholate	0	47.0	47.0	45.2	96.4 ± 7.5
Chenodeoxycholate	0	49.1	49.1	48.6	99.0 ± 0.8
Deoxycholate	0	46.7	46.7	45.0	96.4 ± 7.0
Lithocholate	0	34.0	34.0	30.0	88.2 ± 6.2
Glycocholate	0	6.4	6.4	6.1	95.3 ± 5.1
Glycochenodeoxycholate	2.0	4.8	6.8	6.7	98.5 ± 1.9
Glycodeoxycholate	0	4.5	4.5	4.4	97.8 ± 3.3
Glycolithocholate	0	4.0	4.0	3.8	95.0 ± 2.5
Taurocholate	0	5.9	5.9	5.6	94.9 ± 5.1
Taurochenodeoxycholate	1.0	5.3	6.3	6.2	98.4 ± 2.6
Taurodeoxycholate	0	5.3	5.3	5.1	96.2 ± 1.6
Taurolithocholate	0	5.1	5.1	4.9	96.1 ± 2.7

*$n = 8$.

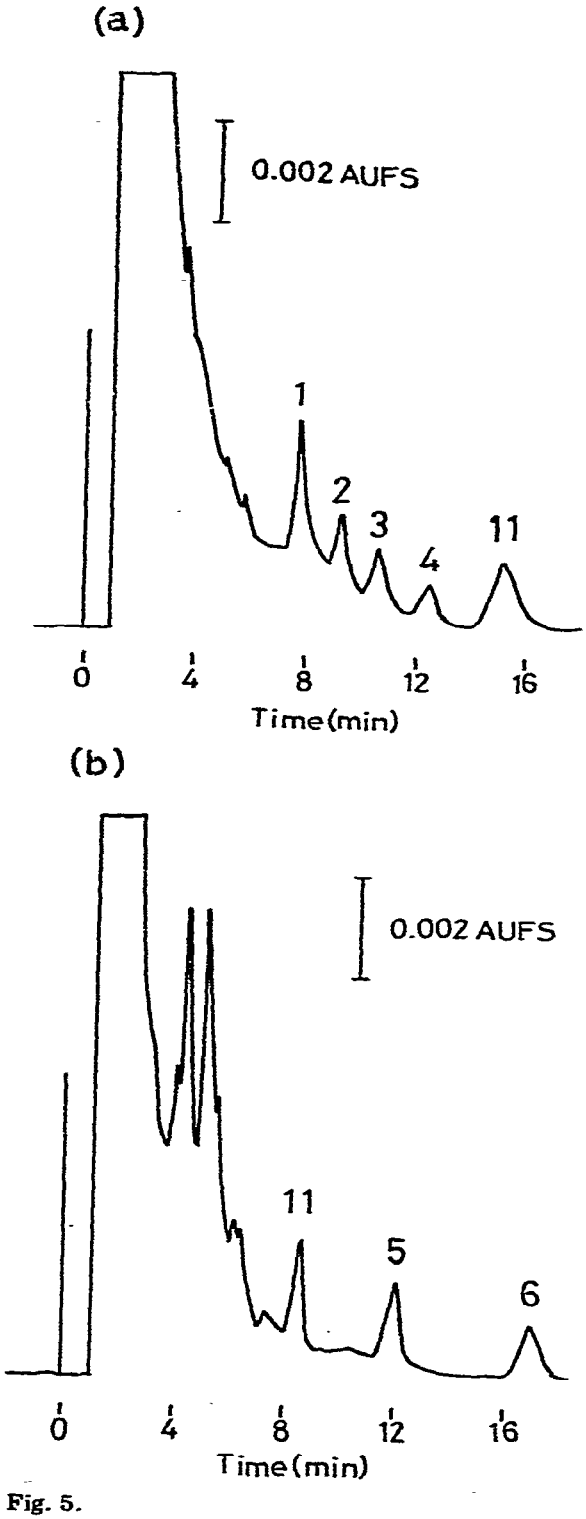
(a)
0.002 AUFS
1
2
3
4
11
0
4
8
12
16
Time(min)
(b)
0.002 AUFS
11
5
6
0
4
8
12
16
Time(min)

Fig. 5.

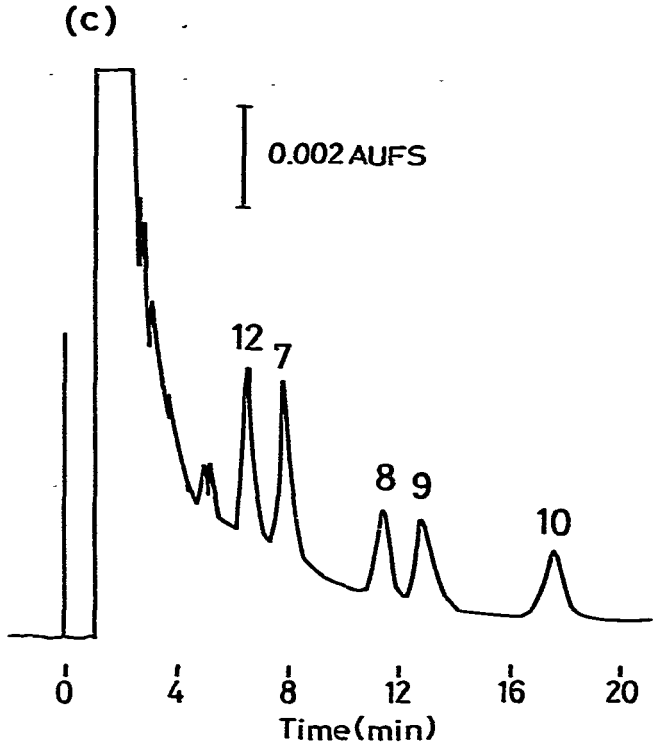

Fig. 5. Separation of bile acid 3-sulfates in human bile by HPLC. Conditions: SC-02 column; mobile phase, acetonitrile—0.5% ammonium carbonate (a) 8:26 (v/v), (b) 8:23 (v/v), (c) 8:31 (v/v), 2 ml/min. 1 = Glycochenodeoxycholate 3-sulfate, 2 = glycodeoxycholate 3-sulfate, 3 = taurochenodeoxycholate 3-sulfate, 4 = taurodeoxycholate 3-sulfate, 5 = glycolithocholate 3-sulfate, 6 = taurolithocholate 3-sulfate, 7 = glycoursodeoxycholate 3-sulfate, 8 = tauroursodeoxycholate 3-sulfate, 9 = glycocholate 3-sulfate, 10 = taurocholate 3-sulfate, 11 = glycocholate (internal standard), 12 = 7α-acetoxy-12α-hydroxy-3-oxo-5β-cholan-24-oic acid 12-sulfate (internal standard).

A chromatogram of bile acid 3-sulfates in human bile which had been processed in the manner described above is illustrated in Fig. 5. Simultaneous determination of bile acid 3-sulfates was carried out with ten bile specimens collected from post-operative patients with obstructive jaundice. The results obtained are listed in Table III. It should be noted that in almost all the cases 3-sulfates of conjugated ursodeoxycholate were found in a larger amount, corresponding to 5—38% of the total bile acid 3-sulfates. No unconjugated bile acid 3-sulfates could be detected even when 1 ml of human bile was subjected to HPLC.

Characterization of bile acid 3-sulfates in human bile

The disadvantage of HPLC in structural elucidation, because the information provided is insufficient, has already been pointed out. For this purpose GLC—mass spectrometry is widely used. This technique, however, is not applicable to the characterization of sulfated bile acids. Recently, a new monitoring system using dual wavelengths has been devised [15, 16]. This method is applicable to compounds having a chromophore but not to sulfated bile acids. In a

TABLE III

AMOUNTS OF BILE ACID 3-SULFATES IN BILE OF PATIENTS WITH OBSTRUCTIVE JAUNDICE

Results are given in μg/ml.

Subject	Cholate		Chenodeoxy-cholate		Deoxycholate		Lithocholate		Ursodeoxy-cholate	
	G*	T*	G	T	G	T	G	T	G	T
A**	29	14	62	46	69	69	10	8	40	9
B***	48	16	55	41	trace	trace	10	13	10	trace
C	18	13	17	17	n.d.	9	n.d.	n.d.	n.d.	n.d.
D***	n.d.§	n.d.	20	10	n.d.	n.d.	n.d.	n.d.	8	n.d.
E	n.d.	n.d.	24	25	n.d.	16	n.d.	n.d.	26	7
F	49	18	39	25	26	17	90	29	63	trace
G	51	13	20	16	n.d.	trace	10	16	25	trace
H	trace	n.d.	33	26	17	17	n.d.	n.d.	30	12
I	11	n.d.	11	12	5	5	n.d.	n.d.	10	17
J	13	n.d.	29	28	48	43	32	14	36	n.d.

*G = glycine conjugate; T = taurine conjugate.
**Pre-operative patient.
***Patient administered ursodeoxycholic acid.
§ n.d. = not detectable.

previous study we investigated the chromatographic behaviours of sulfated bile acids with mobile phases of varying pH and found that they were dependent upon the position of the sulfate and hydroxyl groups and the structure of the side-chain [17]. In the present study, this finding was applied to the structural characterization of bile acid 3-sulfates in human bile. The eluate corresponding to each peak on the chromatogram was collected and, after the addition of an internal standard, subjected to HPLC using three mobile phases of varying pH. As listed in Table IV, relative k' values of bile acid sulfates in bile were identical with those of authentic samples. Moreover, the peak area ratio of each sulfate to the corresponding internal standard showed almost the same value at pH 3.5, 5.5 and 7.5 with a standard deviation of 0.7—6.3 (Table V). These results imply that the present method undergoes no interferences with co-existing substances and is favorable for the determination and structural elucidation of sulfated bile acids in biological fluids.

It is hoped that the availability of an excellent method for the separation and determination of sulfated bile acids without prior hydrolysis and solvolysis may provide more precise knowledge on the metabolic profile of bile acids and may serve in the diagnosis of hepatobiliary diseases.

TABLE IV

RELATIVE k' VALUES OF SULFATED BILE ACIDS IN HUMAN BILE AND STANDARD SAMPLES*

Sulfated bile acid	pH 3.5		pH 5.5		pH 7.5	
	G**	T**	G	T	G	T
Ursodeoxycholate 3-S** (a)	1.95	0.68	0.53	0.70	0.42	0.62
Ursodeoxycholate 7-S	3.70	1.43	1.15	1.53	0.97	1.43
Bile	1.95	0.68	0.53	0.70	0.42	0.62
Cholate 3-S (a)	2.55	1.00	0.78	1.00	0.71	1.00
Cholate 7-S	2.10	0.89	0.66	0.83	0.62	0.84
Cholate 12-S	2.55	1.03	0.76	0.92	0.69	0.91
Bile	2.55	1.00	0.78	1.00	0.71	1.00
Chenodeoxycholate 3-S (b)	3.10	0.88	0.64	0.82	0.57	0.83
Chenodeoxycholate 7-S	3.18	0.96	0.72	0.81	0.67	0.98
Bile	3.10	0.88	0.64	0.82	0.57	0.83
Deoxycholate 3-S (b)	3.54	1.00	0.76	1.00	0.70	1.00
Deoxycholate 12-S	4.18	1.32	0.99	1.13	0.92	1.34
Bile	3.54	1.00	0.76	1.00	0.70	1.00
Lithocholate 3-S (c)	4.43	1.00	0.80	1.00	0.67	1.00
Bile	4.43	1.00	0.80	1.00	0.67	1.00

*The figures express k' values relative to the following internal standards: (a) taurocholate 3-sulfate; (b) taurodeoxycholate 3-sulfate; (c) taurolithocholate 3-sulfate.
**G = glycine conjugate; T = taurine conjugate; S = sulfate.

TABLE V

PEAK AREA RATIOS OF BILE ACID 3-SULFATES IN HUMAN BILE TO INTERNAL STANDARDS

Sulfated bile acid	Internal standard	pH			S.D. (%)
		3.5	5.5	7.5	
Glycoursodeoxycholate 3-S*	Glycocholate 3-S	1.73	1.68	1.74	1.9
Tauroursodeoxycholate 3-S	Taurocholate 3-S	0.25	0.24	0.27	6.3
Glycocholate 3-S	Glycoursodeoxycholate 3-S	0.60	0.66	0.60	5.6
Taurocholate 3-S	Tauroursodeoxycholate 3-S	1.33	1.28	1.32	2.0
Glycochenodeoxycholate 3-S	Glycodeoxycholate 3-S	0.72	0.75	0.76	2.9
Taurochenodeoxycholate 3-S	Taurodeoxycholate 3-S	0.76	0.83	0.78	4.6
Glycodeoxycholate 3-S	Glycochenodeoxycholate 3-S	1.49	1.51	1.47	1.3
Taurodeoxycholate 3-S	Taurochenodeoxycholate 3-S	0.98	0.99	0.99	0.7
Glycolithocholate 3-S	Glycocholate	0.42	0.38	0.40	5.0
Taurolithocholate 3-S	Glycocholate	0.37	0.38	0.39	2.6

*S = sulfate.

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REFERENCES

1 I. Makino, K. Shinozaki and S. Nakagawa, Lipids, 8 (1973) 47.
2 A. Stiehl, Eur. J. Clin. Invest., 4 (1974) 59.
3 I. Makino, H. Hashimoto, K. Shinozaki, K. Yoshino and S. Nakagawa, Gastroenterology, 68 (1975) 545.
4 B. Almé, A. Bremmelgaard, J. Sjövall and P. Thomassen, J. Lipid Res., 18 (1977) 339.
5 G.P. van Berge Henegouwen, R.N. Allan, A.F. Hofmann and P.Y.S. Yu, J. Lipid Res., 18 (1977) 118.
6 G. Lepage, A. Fontaine and C.C. Roy, J. Lipid Res., 19 (1978) 505.
7 J. Goto, H. Kato and T. Nambara, J. Liquid Chromatogr., 3 (1980) 645.
8 J. Goto, H. Kato, F. Hasegawa and T. Nambara, Chem. Pharm. Bull., 27 (1979) 1402.
9 J. Goto, H. Kato, K. Kaneko and T. Nambara, Chem. Pharm. Bull., 28 (1980) 3389.
10 J. Goto, M. Hasegawa, H. Kato and T. Nambara, Clin. Chim. Acta, 87 (1978) 141.
11 K.D.R. Setchell, B. Almé, M. Axelson and J. Sjövall, J. Steroid Biochem., 7 (1976) 615.
12 J. Goto, H. Kato, Y. Saruta and T. Nambara, J. Liquid Chromatogr., 3 (1980) 991.
13 H.L. Bradlow, Steroids, 30 (1977) 581.
14 J.O. Whitney and M.M. Thaler, J. Liquid Chromatogr., 3 (1980) 545.
15 F.K. Trefz, D.J. Byrd and W. Kochen, J. Chromatogr., 107 (1975) 181.
16 J.Q. Rose and W.J. Jusko, J. Chromatogr., 162 (1979) 273.
17 J. Goto, H. Kato, K. Kaneko and T. Nambara, J. Liquid Chromatogr., in press.