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Measurement of sulfated and nonsulfated bile acids in human serum and urine

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Abstract Amberlite XAD-2 was used to extract bile acids from urine or diluted serum of patients with hepatobiliary diseases. Columns containing Sephadex LH-20 were then used to separate the sulfated and nonsulfated bile acids. Thin-layer chromatography of the sulfated bile acid fraction obtained from urine revealed several spots with R_F values different from those of the taurine or glycine conjugates. According to thin-layer chromatographic mobilities, gas-liquid chromatographic analyses, infrared spectra, and elementary analysis of the sulfated material, one of these sulfated bile acids was identified as glycochenodeoxycholic acid monosulfate, and the others were presumed to be taurochenodeoxycholic acid sulfate and glycocholic acid sulfate.

A large amount of bile acid sulfate was found in urine of patients with hepatobiliary diseases. They accounted for 35.5–93.3% of total urinary bile acids and consisted of both di- and trihydroxycholanoic acids, with chenodeoxycholic acid as the major acid.

Sulfated bile acids were also found in serum, and accounted for 1.8–21.2% of the total bile acids. Only dihydroxycholanoic acids (mainly chenodeoxycholic) were identified.

Supplementary key words Amberlite XAD-2 · Sephadex LH-20 · glycochenodeoxycholic acid monosulfate · glycocholic acid sulfate · taurochenodeoxycholic acid sulfate · hepatobiliary diseases

It is well known that in patients with hepatobiliary diseases there are increased concentrations of serum bile acids and large amounts of bile acid are excreted in urine; these bile acids consist of either glycine or taurine conjugates as well as free bile acids (1–7).

In the course of our studies on bile acid metabolism, the presence of sulfated bile acids in urine and serum of patients with hepatobiliary diseases was noted (8, 9).

This paper describes the chromatographic analysis of sulfated and nonsulfated bile acids in human serum and urine. Amberlite XAD-2 was used for the extraction of bile acids (10–13), and the separation of sulfated and nonsulfated bile acids was carried out on Sephadex LH-20 (14, 15).

MATERIALS AND METHODS

Reagents

All solvents were analytical grade. Amberlite XAD-2 resin (Rohm and Haas, Philadelphia, Pa.) was washed with 5–10 vol of water, methanol, acetone, methanol, and water, in that order. Sephadex LH-20 (25–100 μm) was purchased from Pharmacia, Uppsala, Sweden, and alumina (activity 1) from Merck A.G., Darmstadt, Germany. [24-^{14}C]Chenodeoxycholic acid was kindly supplied by Professor J. Sjövall (Karolinska Institutet, Stockholm, Sweden) and Tokyo Tanabe Co., Ltd., Tokyo, Japan. Standard bile acids were obtained from commercial sources.

Gas–liquid chromatography

A gas chromatograph, model GC-2C (Shimazu Manufacturing Co., Kyoto, Japan), with a hydrogen flame detector was used. The glass U-shaped column (150 cm × 4 mm ID) was packed with a commercial preparation (Shimazu Manufacturing Co.) of 1.5% QF-1 on Chromosorb W. The column temperature was 215°C.

Extraction of urine

30 ml of urine was percolated through 10 g of Amberlite XAD-2 in a column (1.0 × 20.0 cm) at a rate of about one drop every 2 sec. The column was washed with 60 ml of water, and bile acids were eluted with 60 ml of methanol (10).

Separation of sulfated and nonsulfated bile acids on Sephadex LH-20

The eluate from the Amberlite XAD-2 column was evaporated to dryness. The residue was dissolved, with ultrasonic agitation, in 2 ml of chloroform–methanol 1:1 (v/v) containing 0.01 M sodium chloride; this solution was then added to a column (1.0 × 25.0 cm), containing 4 g of Sephadex LH-20, which had been packed and equilibrated overnight with the same solvent. The glycine or

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taurine conjugates (nonsulfated fraction) were eluted with 70 ml of the solvent. The column was then eluted with 50 ml of methanol to obtain sulfates of either glycine or taurine conjugates (sulfated fraction) (15).

Solvolysis, hydrolysis, and methylation of bile acids

The sulfated fraction was subjected to solvolysis in 30 ml of ethanol–acetone 1:9 (v/v) acidified with a few drops of 2 N HCl to pH 1. After 2 days at room temperature, the mixture was neutralized by addition of 1 N NaOH, and the solvent was evaporated (16). The residue was hydrolyzed with 15 ml of 15% NaOH for 4 hr at 120°C, and, after acidification to pH 1 with 6 N HCl, the free bile acids were extracted with 3 × 50 ml of ether. The pooled extracts were washed to neutrality with 5-ml portions of water and then reextracted with 20 ml of ether. The ether phases were pooled and the solvent was evaporated. The residue was dissolved in 5 ml of ether–methanol 9:1 (v/v), and diazomethane was added in excess. This mixture was allowed to stand 15 min, and then the solution was evaporated to dryness.

The nonsulfated bile acid fraction was treated in the same way except for the initial solvolysis.

Purification of urinary bile acids on aluminum oxide and analysis by gas–liquid chromatography

The methyl esters of the urinary bile acids were dissolved in 2 ml of benzene and applied to a column containing 15 g of aluminum oxide (grade V) (10). The column was washed with 100 ml of benzene–hexane 9:1 (v/v), and the bile acid methyl esters were then eluted with 100 ml of methanol. The eluate was evaporated to dryness and the methyl esters were converted to the trifluoroacetate derivatives, which were then analyzed by gas–liquid chromatography on QF-1 (5). The amount of bile acid was determined by comparing the peak area with that of a standard reference bile acid.

Extraction of serum

2 ml of serum was diluted with 9 vol of 0.1 N NaOH in 0.9% NaCl. The sample was percolated through a small column of Amberlite XAD-2 (0.5 × 20.0 cm) at a rate of about one drop every 2 sec. The column was washed with water until the effluent was neutral, and then bile acids were eluted with 10 ml of ethanol (13).

The separation of sulfated and nonsulfated bile acids on Sephadex LH-20 and the solvolysis, hydrolysis, and methylation procedure were as described above for urinary bile acids.

The methyl esters of the serum bile acids were purified by the method of Sandberg et al. (5) on columns containing 2 g of aluminum oxide. The purified materials were analyzed by gas–liquid chromatography as described above.

RESULTS

Synthesis of monosulfated sodium glycochenodeoxycholate

Glycochenodeoxycholic acid was prepared by the method of Norman (17), and the sulfate ester of glycochenodeoxycholic acid was synthesized according to the method of Palmer (18, 19).

A 25-ml stoppered tube containing 1 ml of dry pyridine was held slanting in ice while 0.1 ml of chlorosulfonic acid was added in small drops. About 300 mg of glycochenodeoxycholic acid was dissolved in 1.0 ml of dry pyridine and transferred to the reaction tube. The reaction mixture was allowed to stand at room temperature for 7 days. Water was added to terminate the reaction, and the mixture was evaporated in vacuo to remove excess pyridine. The mixture was diluted with 20 ml of water, transferred to a separatory funnel, and extracted with *n*-butanol. The butanol was washed with water and then evaporated. The residue was dissolved in a small amount of chloroform–methanol 1:1 (v/v) containing 0.01 M NaCl and applied to a Sephadex LH-20 column. The bile acids were eluted as described above (15). The methanol eluate (sulfate fraction) was evaporated, and the residue was dissolved in 20 ml of 0.1 N NaOH. This sample was applied to an Amberlite XAD-2 column, which was then washed with water. The bile acid sulfate was eluted with methanol. This synthetic bile acid sulfate gave one spot on thin-layer plates developed in *n*-butanol–acetic acid–water 10:1:1 (see Fig. 3).

Elementary analysis of the synthetic sulfate gave the following results.

Analysis: $C_{26}H_{41}O_8NSNa_2 \cdot H_2O$;
calculated: C, 52.78; H, 7.33; N, 2.37; S, 5.42
found: C, 52.18; H, 7.29; N, 2.62; S, 5.20

The infrared spectrum of the synthetic bile acid sulfate was determined using a potassium bromide disc (Fig. 1). The characteristic strong band at 1060 cm^{-1} indicates the presence of the R—S=O radical (20).

According to these results, this synthetic standard bile acid was confirmed as monosulfated sodium glycochenodeoxycholate, which seems to be sulfated at the C-3 position, but there was no further investigation into the position of the sulfate ester.

Separation of sulfated and nonsulfated bile acids on Sephadex LH-20

Monosulfated sodium [24-^{14}C]glycochenodeoxycholate (specific activity about 220,000 cpm/mg) was synthesized according to the method given above for unlabeled sodium glycochenodeoxycholate monosulfate ester. An aliquot of this labeled material (about 53,000 cpm) was dissolved in 2 ml of chloroform–methanol 1:1 (v/v) containing 0.01 M sodium chloride; this solution was then applied to a col-

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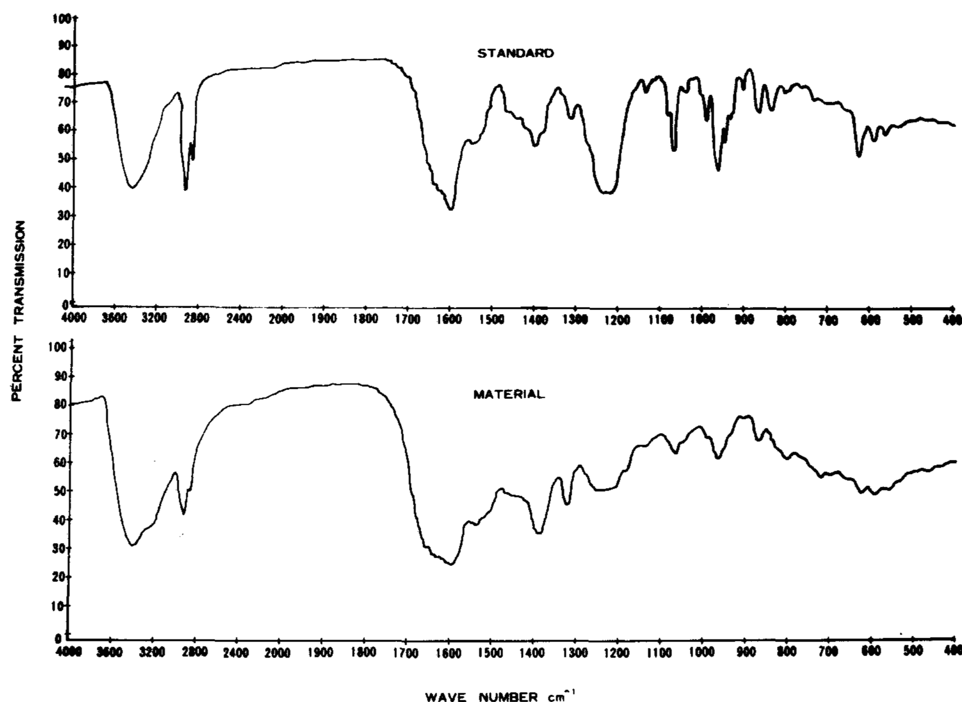

Fig. 1. Infrared spectra of monosulfated sodium glycochenodeoxycholate. *Top,* synthetic bile acid sulfate; *bottom,* the material isolated from area *b* in Fig. 3.

umn (1.0 × 25.0 cm) containing 4 g of Sephadex LH-20, and the sulfated bile acid fraction was eluted as described above. As shown in Fig. 2, only one radioactive peak was eluted with methanol (sulfate fraction). The radioactive fractions were pooled and evaporated, and the residue was subjected to solvolysis as described above. The resulting material had the mobility of nonsulfated bile acid as judged by rechromatography on Sephadex LH-20 (Fig. 2). According to these results, the complete separation of sulfated and nonsulfated bile acids on Sephadex LH-20 column was confirmed.

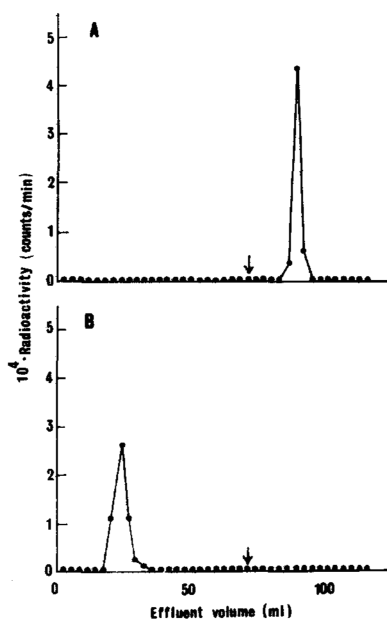

Fig. 2. Chromatography of sodium [24-^{14}C]glycochenodeoxycholate monosulfate ester on Sephadex LH-20 column. Chloroform–methanol 1:1 (v/v), 0.01 M with respect to NaCl, was used as the first solvent (0–70 ml of effluent), followed by methanol (70–120 ml). *A,* before solvolysis; *B,* after solvolysis of radioactive peak in *A*.

Identification of sulfated bile acid in human urine

An aliquot from each of the two fractions (sulfated and nonsulfated) obtained from the Sephadex LH-20 separation of the urinary bile acids of patient M.W. (acute hepatitis) was evaporated to dryness, and the residue was dissolved in 20 ml of 0.1 N NaOH solution. Each sample was applied to a column containing Amberlite XAD-2, which was washed with water. Bile acids were eluted with methanol, and the purified sulfated and nonsulfated bile acids were analyzed by thin-layer chromatography (silica gel G) using *n*-butanol–acetic acid–water 10:1:1 (21). After development, the plate was sprayed with phosphomolybdic acid–ethanol solution and heated to visualize the spots. The chromatograms are shown in Fig. 3. A large spot corresponding to standard glycocholic acid (column 8) was observed for the nonsulfated fraction (column 1), while four spots *(a, b, c,* and *d)* with R_F values different from those of either glycine or taurine conjugates were detected in the sulfated fraction (column 2). Spot *b* corresponded to standard synthetic monosulfated sodium glycochenodeoxycholate. The silica gel in zones *a, b, c,* and *d*

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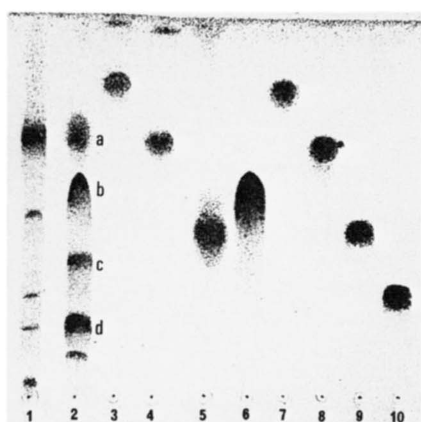

Fig. 3. Thin-layer chromatograms of nonsulfated and sulfated bile acids in urine of patient M.W. *1*, nonsulfated fraction; *2*, sulfated fraction; *3*, spot *b* from sulfated fraction after solvolysis; *4*, spot *c* from sulfated fraction after solvolysis; *5*, spot *d* from sulfated fraction after solvolysis; *6*, synthetic sodium glycochenodeoxycholate monosulfate ester; *7*, standard glycochenodeoxycholic acid; *8*, standard glycocholic acid; *9*, standard taurochenodeoxycholic acid; *10*, standard taurocholic acid.

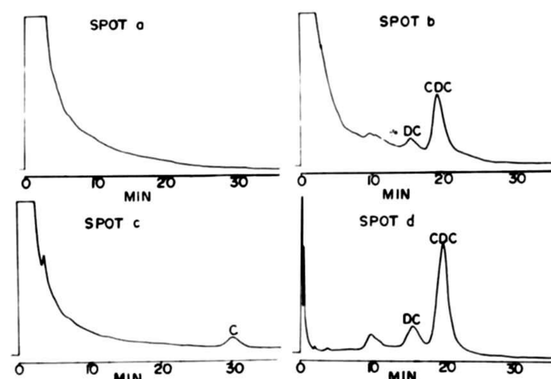

Fig. 4. Gas–liquid chromatograms of the *a*, *b*, *c*, and *d* materials eluted with methanol in the zones corresponding to spots *a*, *b*, *c*, and *d* in Fig. 3 (after solvolysis, hydrolysis, methylation, and trifluoroacetylation). Column, 1.5% QF-1; sensitivity, 10^3; range, 0.4 V. DC, deoxycholic acid; CDC, chenodeoxycholic acid; C, cholic acid.

was scraped off and eluted with methanol, and the eluate from each area was divided into several aliquots. One aliquot was subjected to solvolysis, and analysis of the reaction product by thin-layer chromatography revealed materials with the same R_F values as glycochenodeoxycholic, glycocholic, and taurochenodeoxycholic acids (columns 3, 4, and 5 of Fig. 3). Thin-layer chromatographic mobilities of sulfated and nonsulfated bile acids are listed in Table 1.

Samples of eluates from areas *a*, *b*, *c*, and *d* were subjected to solvolysis, alkaline hydrolysis, methylation, and trifluoroacetylation (without purification on an aluminum oxide column) and then analyzed by gas–liquid chromatography as described above. No bile acid was observed for area *a* material (Fig. 4). Areas *b* and *d* were composed mainly of chenodeoxycholic acid, with deoxycholic acid as a minor component, and area *c* contained cholic acid (Fig. 4).

In order to confirm the presence of sulfur in the material eluted from spot *b* in Fig. 3, infrared spectra were obtained. As shown in Fig. 1, the spectrum of the sample was similar to that of synthetic monosulfated sodium glycochenodeoxycholate, and the characteristic band for the R—S=O radical (1060 cm^{-1}) was also observed (20). This material contained 3.30% sulfur. Although this value was lower than the calculated value of 5.42%, it is clear that spot *b* in Fig. 3 contains sulfur. It was very difficult to obtain sufficient amounts of glycochenodeoxycholic acid monosulfate from urine for recrystallization, and it seems likely that the isolated glycochenodeoxycholic acid monosulfate was not completely pure. According to these results, spot *b* was monosulfated sodium glycochenodeoxycholate which was more polar than the nonsulfated original glycodihydroxycholanoic acid.

One aliquot of eluate *b* was subjected to alkaline hydrolysis without solvolysis and then analyzed by gas–liquid chromatography after methylation and trifluoroacetylation. No bile acid was detected except a very small peak with a retention time equal to that of the trifluoroacetate of chenodeoxycholic acid methyl ester.

Recovery of bile acid from urine and serum using this method

To determine the recovery of urinary bile acids on columns of Amberlite XAD-2, [24-^{14}C]cholic acid and monosulfated sodium [24-^{14}C]glycochenodeoxycholate were added to urine of a patient with severe jaundice, and these samples were applied to an Amberlite XAD-2 column. The radioactivity in aliquots of eluates was determined by liquid scintillation counting, and the recoveries were calculated to be 90.0% and 76.3%, respectively, for cholic acid and the sulfated acid.

Recovery tests employing ^{14}C-labeled cholic acid and various unlabeled bile acids added to serum were also carried out. The recovery of ^{14}C-labeled cholic acid was tested at various stages throughout the entire procedure by counting aliquots of the various samples; the results are listed in Table 2. When unlabeled deoxycholic, cheno-

TABLE 1. Thin-layer chromatographic mobilities of sulfated and nonsulfated bile acids

	R_F
Glycochenodeoxycholic acid	0.83
Glycocholic acid	0.67
Taurochenodeoxycholic acid	0.45
Taurocholic acid	0.27
Glycochenodeoxycholic acid monosulfate	0.58
Glycocholic acid sulfate (probably)	0.36
Taurochenodeoxycholic acid sulfate (probably)	0.21

Solvent system was *n*-butanol–acetic acid–water 10:1:1.

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TABLE 2. Recovery of [24-^{14}C]cholic acid added to serum[a]

Stage of Procedure	cpm	
Added to serum	143,800	
After Amberlite XAD-2 column	139,200	
After Sephadex LH-20 column	138,200	(Nonsulfated fraction)
	180	(Sulfated fraction)
After ether extraction	128,700	(Nonsulfated fraction)
After formation of trifluoroacetate of bile acid methyl ester	121,100	(Nonsulfated fraction)

[a] The overall recovery of added ^{14}C-labeled cholic acid (nonsulfated fraction) was 84.2%.

TABLE 3. Recoveries of unlabeled bile acids added to normal serum and carried through the entire procedure

Added Bile Acid	Amount Added	Recovery
	μg	%
Deoxycholic acid	120	96.1
Chenodeoxycholic acid	110	79.8
Cholic acid	130	91.2

The amount of serum used was 2 ml in each case.

deoxycholic, and cholic acids were added to serum, the recoveries were measured by gas–liquid chromatography; these data are listed in Table 3. According to these results, the recovery for the entire procedure was 80–96%.

Sulfated and nonsulfated bile acids in urine of patients with hepatobiliary diseases

Table 4 gives the results of quantitative estimation of urinary bile acids in eight patients with hepatobiliary diseases. A large amount of bile acid sulfate was found in these urine samples. The daily urinary excretion of total bile acids is indicated in the table as well as the percentages of the sulfated bile acids. The excretion of these sulfated acids was unexpectedly high. The major sulfated bile acid was chenodeoxycholic acid, and deoxycholic and cholic acids were only minor constituents.

Sulfated and nonsulfated bile acids in serum of patients with hepatobiliary diseases

In patients with hepatobiliary diseases, levels of both sulfated and nonsulfated bile acids in serum rose, but the sulfated acids did not increase as much as the nonsulfated bile acids (Table 5). The levels of nonsulfated acids determined in this study were similar to levels of total serum bile acids reported by previous workers (1–7). The sulfated bile acids in serum consisted of only the dihydroxycholanoic acids, deoxycholic acid and chenodeoxycholic acid.

DISCUSSION

Extraction of serum and urinary bile acid by Amberlite XAD-2

Sandberg et al. (5) have established the method of separation and measurement of plasma bile acids by using an anion exchanger, Amberlite A-26, and gas–liquid chromatography. However, there has been a need for a simpler method for clinical use. It has been shown that Amberlite XAD-2 adsorbs steroids quite well (11, 12), and Makino and Sjövall tried to utilize this resin for extraction of serum bile acids (13).

In serum, conjugated bile acid is bound to albumin. The extent of binding is affected by the pH of the serum (22). The affinity of albumin for bile acids decreases rapidly above pH 9 and is nearly nonexistent above pH 11. Makino and Sjövall (13) studied the effect of concentration of aqueous or saline solutions of sodium hydroxide added to serum on the adsorption of serum bile acids by Amberlite XAD-2. When serum was diluted 10 times with 0.1

TABLE 4. Daily excretion of nonsulfated and sulfated bile acids in urine of patients with hepatobiliary diseases

Patient	N.K.	Y.I.	M.G.	S.S.	M.W.	T.S.	J.S.	U.D.
Diagnosis	Cancer of gallbladder	Cancer of Vater's papilla	Metastatic cancer of the liver	Acute hepatitis	Acute hepatitis	Acute hepatitis	Cirrhosis	Cirrhosis
	Excretion of bile acids in urine (mg/24 hr)							
Nonsulfated								
DC[a]			0.22	0.03				
CDC	0.06	0.14	1.38	1.08	0.47	0.28	0.18	0.47
C	2.34	5.53	3.94	2.97	2.11	1.82	0.42	1.64
	2.40	5.67	5.54	4.08	2.58	2.10	0.60	2.11
Sulfated								
DC			2.17	1.33	3.57	0.38		
CDC	4.79	6.21	11.00	53.53	23.26	6.04	3.21	1.16
C	4.06	1.33	0.83	2.16	8.14	0.80		
	8.85	7.54	14.00	57.02	34.97	7.22	3.21	1.16
Total	11.25	13.21	19.54	61.10	37.55	9.32	3.81	3.27
% Sulfated	78.7	57.1	71.6	93.3	93.1	77.5	84.3	35.5

[a] DC, deoxycholic acid; CDC, chenodeoxycholic acid; C, cholic acid.

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TABLE 5. Levels of nonsulfated and sulfated bile acids in serum of patients with hepatobiliary diseases

Patient	S.S.	Y.F.	J.S.	Y.I.	H.W.	M.G.	I.M.	H.Y.
Diagnosis	Acute hepatitis	Chronic hepatitis	Cirrhosis	Cancer of Vater's papilla	Primary biliary cirrhosis	Metastatic cancer of the liver	Normal	Normal
				μg/ml				
Nonsulfated								
DC[a]	tr	1.37				0.46	0.18	tr
CDC	28.03	14.30	17.98	14.00	28.20	4.81	0.07	0.92
C	11.98	3.47	9.95	55.85	34.61	4.24	tr	0.14
	39.91	19.14	27.93	69.85	62.81	9.51	0.25	1.06
Sulfated								
DC		0.06			0.94	0.30	0.03	
CDC	1.50	0.44	0.51	3.24	11.00	2.26		0.16
C			tr					
	1.50	0.50	0.51	3.24	11.94	2.56	0.03	0.16
Total	41.41	19.64	28.44	73.09	74.75	12.07	0.28	1.22
% Sulfated	3.6	2.5	1.8	4.6	16.0	21.2	10.7	13.1

[a] DC, deoxycholic acid; CDC, chenodeoxycholic acid; C, cholic acid.

N sodium hydroxide in saline solution, the maximum affinity of bile acid for Amberlite XAD-2 was observed. The degree of adsorption of bile acid by Amberlite XAD-2 was in inverse relationship to the affinity of serum bile acid for albumin. Although only free bile acid was tested, the recovery experiments (Tables 2 and 3) were quite satisfactory. Amberlite XAD-2 is usually used for the extraction of steroids from urine (11, 12). Norman and Strandvik succeeded in extracting urinary bile acids by direct application of urine to an Amberlite XAD-2 column (10). The results of recovery tests on an Amberlite XAD-2 column employing the addition of ^{14}C-labeled cholic acid and ^{14}C-labeled glycochenodeoxycholic acid monosulfate to urine in the present experiment were satisfactory. Urinary bile acid had been previously extracted with butanol, but this extract was highly contaminated. Thin-layer chromatograms of such an extract revealed severe tailing, and bile acids could not be seen as discrete spots. In the present experiment, sulfated and nonsulfated bile acids obtained after Sephadex LH-20 fractionation of the urinary extract of patient M.W. were repurified on an Amberlite XAD-2 column. As shown in Fig. 3, the purified materials yielded spots with slight tailing on thin-layer chromatograms. Thus, it is possible to analyze urinary bile acids by thin-layer chromatography after extraction using Amberlite XAD-2.

Separation of sulfated and nonsulfated bile acids on Sephadex LH-20

Since it is not yet possible to do direct gas–liquid chromatographic determinations of conjugated bile acids, separations must be done by liquid–gel chromatography. Sephadex LH-20 has been used for the separation of free and mono- and disulfated steroids, and Cronholm, Makino, and Sjövall (23) have shown that an extract of bile chromatographed on Sephadex LH-20 yielded two fractions of bile acid having the chromatographic properties of sulfated and nonsulfated compounds. This technique encouraged us to try separation of sulfated and nonsulfated bile acid from human serum and urine. Based on studies of the mobility of labeled synthetic bile acid sulfate, monosulfated sodium glycochenodeoxycholate has a much larger elution volume than nonsulfated bile acid (Fig. 2), and the separation of the two fractions (nonsulfated and sulfated) was complete. Solvolysis under the conditions of the present experiments was also confirmed by using ^{14}C-labeled glycochenodeoxycholic acid monosulfate (Fig. 2), and solvolysis of the sulfated fraction obtained from urine resulted in a nonsulfated glycine- or taurine-conjugated bile acid (Fig. 3). However, sulfate was not cleaved by alkaline hydrolysis.

Sulfated bile acids in urine and serum of patients with hepatobiliary diseases

It is well known that the concentration of serum taurine or glycine-conjugated bile acid increases in patients with jaundice (1–7) and that these materials are excreted in the urine. However, there have been no detailed reports about the presence of bile acid sulfate. It was therefore surprising to find large amounts of bile acid sulfate in urine of patients with hepatobiliary diseases. As no solvolysis was carried out in the earlier studies, bile acid sulfate in urine and serum as described in this paper was not determined.

Admirand, Stiehl, and Thaler (24) have recently reported that a patient with intrahepatic biliary atresia excreted 25% of the daily bile acid production in the urine as sulfated metabolites, and within 12 hr of the oral administration of labeled cholate and chenodeoxycholate, half of the label was excreted in the urine as the sulfated fraction. Recently, ^{14}C-labeled glycolithocholic acid sulfate and ^{14}C-labeled taurolithocholic acid sulfate were recovered from bile after the oral administration of ^{14}C-la-

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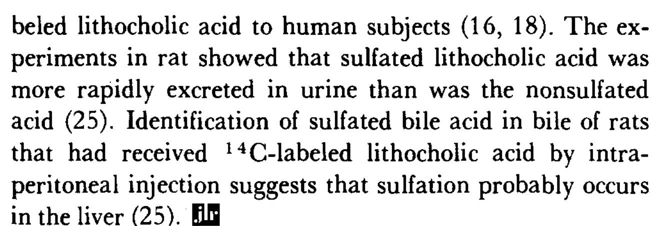

beled lithocholic acid to human subjects (16, 18). The experiments in rat showed that sulfated lithocholic acid was more rapidly excreted in urine than was the nonsulfated acid (25). Identification of sulfated bile acid in bile of rats that had received ^{14}C-labeled lithocholic acid by intraperitoneal injection suggests that sulfation probably occurs in the liver (25).

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