Analysis of 3-sulfated and nonsulfated bile acids by one-step solvolysis and high performance liquid chromatography

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Summary A facile solvolysis procedure of 3-sulfated bile acid was devised using trifluoroacetic acid, tetrahydrofuran, and methanol. The sulfate esters were completely solvolyzed within only 2 hr by the present method. The clinical utility of the solvolysis procedure and high performance liquid chromatography using immobilized 3α-hydroxysteroid dehydrogenase was demonstrated in the analysis of bile acids in serum of patients with obstructive jaundice. The quantities of 3-sulfated bile acids were calculated from the difference in the amount of bile acids before and after solvolysis. A significantly large proportion of 3-sulfated glycochenodeoxycholic acid, i.e., 21.9 to 31.3% of total glycochenodeoxycholic acid, was found in the serum of patients with obstructive jaundice. Thus, the present method permits simultaneous quantitation of 3-sulfated as well as nonsulfated bile acids in biological samples.—**Hirano, Y., H. Miyazaki, S. Higashidate, and F. Nakayama.** Analysis of 3-sulfated and nonsulfated bile acids by one-step solvolysis and high performance liquid chromatography. *J. Lipid Res.* 1987. **28:** 1524–1529.

Supplementary key words obstructive jaundice • immobilized 3α-hydroxysteroid dehydrogenase

Biological fluids such as serum, urine, and bile provide information on the metabolism of bile acids. In recent years, bile acid metabolism in man in relation to hepatobiliary diseases has been vigorously investigated using various analytical techniques, for example, thin-layer chromatography (TLC), high performance liquid chromatography (HPLC), gas–liquid chromatography (GLC); gas–liquid chromatography–mass spectrometry (GLC-MS), and radioimmunoassay (RIA). Since a considerable proportion of bile acid exists in biological samples in the sulfated form, especially in urine, prior solvolysis is essential to its quantitation. Several solvolysis procedures have been published. Of these, the methods of Palmer and Bolt (1), Parmentier and Eyssen (2), and Kornel (3) have been widely used. The profile analysis of bile acids in serum or urine of various patients can be performed on a routine basis by HPLC without derivatization. Unconjugated and glycine- and taurine-conjugated bile acids can easily be separated and quantitated by HPLC with immobilized 3α-hydroxysteroid dehydrogenase (3α-HSD) in column form as devised by Okuyama et al. (4). However, 3-sulfated bile acids escape detection in the HPLC system. When individual bile acids are quantitated and compared before and after solvolysis, the profile of 3-sulfated bile acids can be calculated from the difference. The conventional methods for solvolysis presently available cannot be used routinely as pretreatment for the HPLC analysis of bile acid sulfates because of several disadvantages. Therefore, development of a simple and rapid solvolysis procedure which overcomes the disadvantages is very desirable.

Abbreviations: LCA, lithocholic acid; CDCA, chenodeoxycholic acid; UDCA, ursodeoxycholic acid; DCA, deoxycholic acid; CA, cholic acid; G-, glycine-conjugate; T-, taurine-conjugate; 3S- or -3S, 3-sulfate; THDCA, taurohyodeoxycholic acid; THF, tetrahydrofuran; TFA, trifluoroacetic acid; RIA, radioimmunoassay; TLC, thin-layer chromatography; GLC, gas–liquid chromatography; GLC-MS, gas–liquid chromatography–mass spectrometry; HPLC, high performance liquid chromatography; 3α-HSD, 3α-hydroxysteroid dehydrogenase; I. S., internal standard.

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This report describes a method of simultaneous determination of 3-sulfated and nonsulfated bile acids using the combination of a novel solvolysis procedure and HPLC with immobilized 3α-HSD, and its clinical application.

MATERIALS AND METHODS

All reagents were of analytical grade and distilled before use without further purification. Tetrahydrofuran (THF) was refluxed for 2 hr over potassium hydroxide and lithium aluminum hydride, since rigorous purification of THF is very important for later analysis. [24-^{14}C]Glycolithocholic acid-3-sulfate was synthesized from [24-^{14}C]lithocholic acid (sp act 55 mCi/mmol; Amersham International Limited, Buckinghamshire, UK) in our laboratory according to the method of Tserng and Klein (5). [11,12-$^{3}H_2$]Glycochenodeoxycholic acid-3-sulfate was prepared from [11,12-$^{3}H_2$]chenodeoxycholic acid (sp act 38.6 Ci/mmol; New England Nuclear Corporation, Boston, MA) according to the method of Parmentier and Eyssen (6). Glycolithocholic acid-3-sulfate was kindly supplied by Dr. G. A. D. Haslewood and glycochenodeoxycholic acid-3-sulfate by Dr. T. Nambara, Tohoku University, Sendai, Japan. Taurohyodeoxycholic acid was synthesized from hyodeoxycholic acid as described by Lack et al. (7). The purity of all bile acids was checked by TLC and GLC, and found to be more than 95%. A Bond-Elut C_{18} cartridge was from Analytichem International, Harbor City, CA.

A BA 110 HPLC system (Japan Spectroscopic Co., Ltd. (Jasco), Tokyo, Japan) was employed for the profile analysis of conjugated and unconjugated bile acids. This system was equipped with a trirotar pump, a gradient elution accessory, and fluorometer. A Jasco Bilepak column was used for the separation of bile acids and their conjugates. The operating conditions of the separation of individual bile acids were as follows: mobile phase A, acetonitrile–10 mM phosphate buffer (pH 7.80) 40:60; B, acetonitrile–30 mM phosphate buffer (pH 7.80) 20:80; gradient A/B ratio, 0:100 at start and 55:45 at 64 min; slope, linear; flow rate, 1 ml/min; detection, fluorescence, λex = 345 nm, λem = 470 nm; pressure, 70 kg/cm²; and temperature, 25°C. A Jasco Enzymepak-HSD, immobilized 3α-HSD in column form, was used in the HPLC system and reduced NAD^+ produced from bile acids by this enzyme was fluorimetrically determined with a fluorometer. This commercially available HPLC system for bile acid analysis was devised and described by Okuyama et al. (4).

Solvolysis

The serum (0.1 ml) was added with 3 ml of 0.1 N NaOH, and the mixture was incubated at 80°C for 20 min, cooled to room temperature, and extracted with a Bond-Elut cartridge. The extract was completely dried and submitted to solvolysis by Kornel's procedure (3) and by the present method. In the present method, the extract from the serum was thoroughly dissolved in 1 ml of methanol and 9 ml of THF and 0.1 ml of 1 M trifluoroacetic acid (TFA) in dioxane were added. After introducing a nitrogen stream into a reaction vessel to purge out air, the mixture was incubated at 45°C for 2 hr. The reaction mixture after solvolysis was cooled to room temperature and directly evaporated to dryness with a rotary evaporator below 25°C or under a nitrogen stream without neutralization. In Kornel's method (3), the reaction mixture, after solvolysis, was neutralized with 5% NaOH and evaporated to dryness. The residue was then dissolved in water and the solution was percolated through a Bond-Elut column with the mobile phase and eluted with methanol.

Analysis of bile acids in serum

Sera from thirteen patients with obstructive jaundice were obtained just before the biliary tract decompression by percutaneous transhepatic cholangiodrainage and kept frozen at −20°C until used. In the first experiment (see Table 1), five samples were used. One half of the extract from a serum sample was solvolyzed by the present method and the other half was treated by Kornel's method. In the second experiment (see Table 2), eight different samples were used. One half of the extract from a serum sample was solvolyzed by the present method and the other half was not solvolyzed. All samples were analyzed by the HPLC procedure.

RESULTS AND DISCUSSION

The methods of Palmer and Bolt (1), Parmentier and Eyssen (2), and Kornel (3) are currently available for solvolysis of sulfated bile acids. In the first two methods, a large amount of ester is formed during solvolysis. The formation of esters is undesirable for later analysis, since it necessitates hydrolysis of the ester prior to further analytical steps such as HPLC or RIA. A byproduct may be produced in their methods as pointed out by Cohen, Budai, and Javitt (8). In Kornel's method (3), the mild reaction and very little formation of ester is characteristic, but a large amount of alkaline solution has to be used to neutralize the excess acids after solvolysis, followed by tedious desalting procedure necessary for removal of the large amount of salt produced.

In order to circumvent these disadvantages, we attempted a new procedure for solvolysis. We thought that TFA with strong acidity and high volatility could be used with advantage to have the solvolyzing effect of inorganic acids and yet be easily removed by evaporation (9). The report on

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solvolysis of steroid hydrogen sulfates by Burstein and Lieberman (10) was informative in selecting THF as a new solvent for solvolysis. A mixture of TFA and ethyl acetate was used for the solvolysis of sulfatides by Shimomura and Kishimoto (11), but sulfated bile acids were not at all solvolyzed by their method probably due to a low solubility of double conjugates in their solvent mixture. The procedure selected for the present solvolysis was as follows: sulfated bile acids were completely dissolved in 1 ml of methanol, 9 ml of THF and 0.1 ml of 1 TFA were added, and the mixture was incubated at 45°C for 2 hr. The validity of the present solvolysis procedure was confirmed by using isotopically labeled bile acid sulfates added to serum. The recoveries of glycolithocholic acid and glycochenodeoxycholic acid from their 3-sulfates were satisfactory, 97.1 ± 0.7% (n = 10) and 97.2 ± 1.0% (n = 10), respectively, and no artefact was found after the solvolysis. The present method offers the following advantages. *1*) The reaction takes places rapidly and smoothly. *2*) Neutralization and desalting procedures are not needed. *3*) There is no formation of esters. *4*) There is no alteration of bile acid structure. The only problem

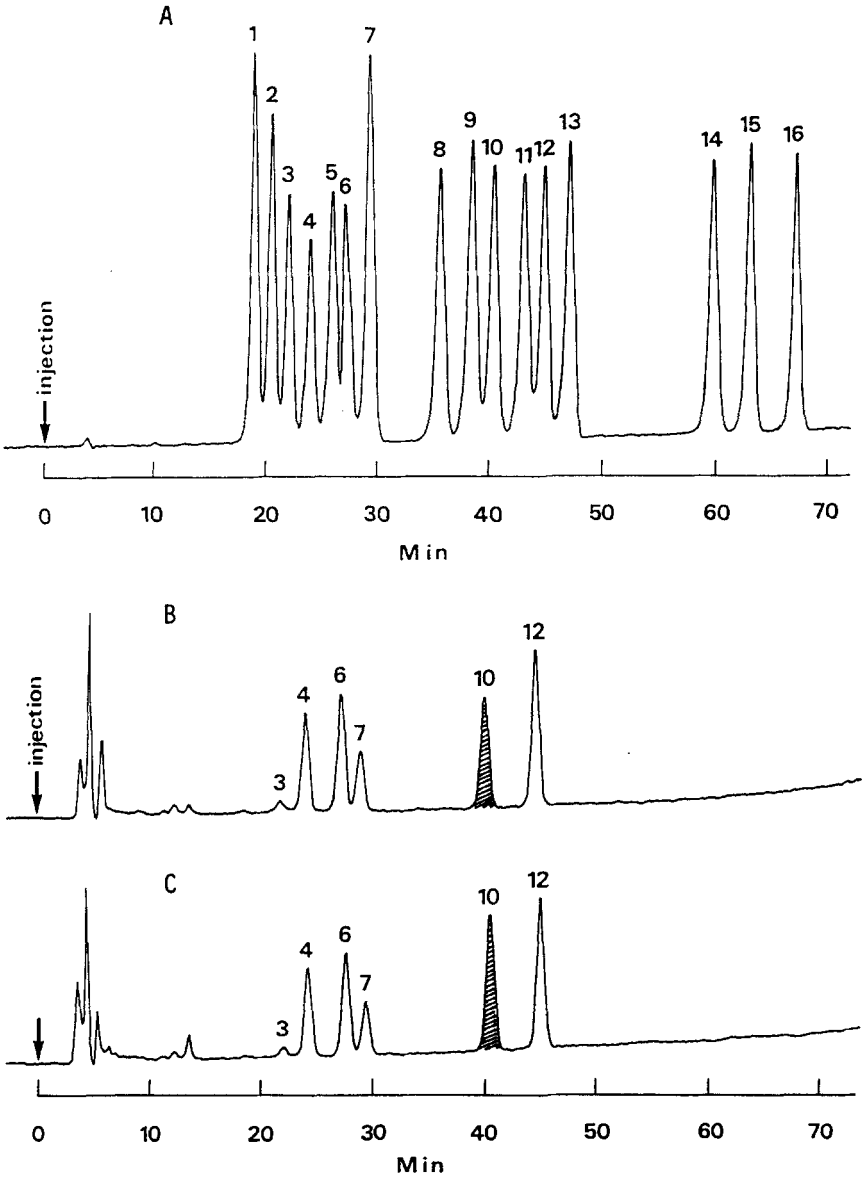

Fig. 1. A: Separation of an authentic mixture of free and glycine- and taurine-conjugated bile acids by HPLC. B and C: Representative chromatograms obtained from the same serum of a patient with obstructive jaundice; B, before solvolysis; C, after solvolysis. Peaks: 1, ursodeoxycholic acid; 2, cholic acid; 3, glycoursodeoxycholic acid; 4, glycocholic acid; 5, tauroursodeoxycholic acid; 6, taurocholic acid; 7, taurohyodeoxycholic acid (internal standard); 8, chenodeoxycholic acid; 9, deoxycholic acid; 10, glycochenodeoxycholic acid; 11, glycodeoxycholic acid; 12, taurochenodeoxycholic acid; 13, taurodeoxycholic acid; 14, lithocholic acid; 15, glycolithocholic acid; 16, taurolithocholic acid.

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in this method is the formation of a small amount of sludgy substances probably derived from THF. The formation of this undesirable product could be reduced by prior refluxing and distillation of THF in the presence of potassium hydroxide and lithium aluminum hydride and by introduction of a nitrogen stream into the reaction vessel.

HPLC with immobilized 3α-HSD can sufficiently separate unconjugates and glycine-(G-) and taurine- (T-) conjugates of cholic acid (CA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA), ursodeoxycholic acid (UDCA), and lithocholic acid (LCA), and detect 10 ng each of bile acid (4). An internal standard (I.S) was not used in this HPLC analysis (4). However, the use of an internal standard is essential in order to enhance the precision and accuracy of determination of bile acids by HPLC. Taurohyodeoxycholic acid (THDCA) was found to be suitable as an I.S. for this HPLC procedure. The separation of an authentic mixture of sixteen bile acids containing THDCA is demonstrated in **Fig. 1 A**.

The levels of bile acid in serum of five patients with obstructive jaundice were determined using this HPLC system with THDCA as I.S. after solvolysis by Kornel's procedure (3) and the present method. Each serum sample (0.1 ml) contained THDCA (1 μg). There was no statistical difference between these two methods as listed in **Table 1**.

Sulfated bile acids have been fractionated by ion exchange chromatography or TLC and submitted to HPLC, GLC, or GLC–MS analysis. However, the fractionation by these chromatographic procedures is a tedious and troublesome operation. Therefore, it is not easy for them to be performed precisely and accurately. In contrast, using the present solvolysis procedure, the profile analysis of 3-sulfated bile acids can be obtained easily by comparing the difference in the amounts of bile acids before and after solvolysis using HPLC with immobilized 3α-HSD. 7-Sulfated and disulfated bile acids reported to be resistant to solvolysis (8, 12, 13) were not investigated in our study. However, the principle of our method should be applicable to the analysis of 7- and 12-sulfated bile acids if a simple and effective method of solvolysis and use of immobilized 7α-HSD and 12α-HSD columns were to become available.

A clinical utility of the present method was demonstrated by the quantitation of sulfated and nonsulfated bile acids in sera of eight patients with obstructive jaundice. Figs. 1 B and C show representative chromatograms of serum bile acids of a patient with obstructive jaundice by HPLC before and after solvolysis. Five peaks besides the I.S. were identified to be GUDCA, GCA, TCA, GCDCA, and TCDCA using 0.1 ml of serum. The quantitative values are listed in **Table 2.** Glycine and taurine conjugates of primary bile acids were predominant and secondary bile acids were not detected except for GUDCA in this sample size because of the complete obstruction of the extrahepatic bile duct as demonstrated by percutaneous transhepatic cholangiography, and, therefore, complete interruption of enterohepatic circulation. However, the reason why GUDCA was found in these cases in spite of the failure of detecting other secondary bile acids is not well understood. Unconjugated bile acids were not observed in sera of these patients. A considerable proportion, ranging from 21.9 to 31.3%, of 3-sulfates was present in GCDCA but less than 7.4% in GCA, TCA, and TCDCA. A trace amount of GUDCA was observed but could not be quantified in this sample size. Our results are in good agreement with several reports on

TABLE 1. Comparison of levels of bile acid in serum of patients with obstructive jaundice after solvolysis by Kornel's procedure (3) and the present method

Patient	Solvolysis	CA	GUDCA	GCA	TCA	GCDCA	TCDCA	Total
				μg/ml of serum				
1	A[a]	1.2	3.1	44.7	71.5	25.2	24.6	170.3
1	B[b]	1.3	3.2	45.0	71.6	21.4	23.0	165.5
2	A	> 0.1	4.1	17.7	26.7	12.0	14.4	74.9
2	B	> 0.1	4.0	17.2	27.4	11.7	15.4	75.7
3	A	> 0.1	0.7	9.9	21.6	9.1	10.8	52.1
3	B	> 0.1	0.7	9.8	21.5	8.3	11.0	51.3
4	A	> 0.1	2.8	12.1	10.1	10.6	4.6	40.2
4	B	> 0.1	2.5	12.0	10.2	10.5	4.8	40.0
5	A	> 0.1	1.8	10.6	19.2	6.4	7.6	45.6
5	B	> 0.1	1.7	10.3	18.9	6.0	7.9	44.8

CA, cholic acid; GUDCA, glycoursodeoxycholic acid; GCA, glycocholic acid; TCA, taurocholic acid; GCDCA, glycochenodeoxycholic acid; TCDCA, taurochenodeoxycholic acid.
[a] Present method.
[b] Kornel's method.

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TABLE 2. Levels of 3-sulfated and nonsulfated bile acids in serum of patients with obstructive jaundice

Patient	GUDCA	GCA	GCA-3S	TCA	TCA-3S	GCDCA	GCDCA-3S	TCDCA	TCDCA-3S	Total
					μg/ml of serum					
6	tr	52.2	3.7 (6.6)[a]	70.2	2.6 (3.6)	20.2	5.6 (21.9)	28.7	2.3 (7.4)	185.3
7	tr	19.5	0 (0)	55.3	0 (0)	8.5	3.8 (30.9)	27.8	0.7 (2.5)	115.6
8	tr	11.3	0 (0)	22.7	0 (0)	7.2	2.1 (22.6)	14.0	0 (0)	57.3
9	tr	15.1	0 (0)	10.7	0 (0)	8.9	2.5 (21.9)	5.4	0 (0)	42.6
10	tr	13.0	0.4 (3.0)	20.8	0 (0)	5..0	1.6 (24.2)	9.6	0.6 (5.9)	51.0
11	tr	8.9	0 (0)	11.4	0 (0)	7.6	2.7 (26.2)	11.8	0.1 (0.8)	42.5
12	tr	18.5	0 (0)	12.9	0.4 (3.0)	11.0	5.0 (31.3)	8.7	0.2 (2.2)	56.7
13	tr	11.9	0.6 (4.8)	16.4	0.5 (3.0)	8.8	3.4 (27.9)	13.6	1.0 (6.8)	56.2

GUDCA, glycoursodeoxycholic acid; GCA, glycocholic acid; TCA, taurocholic acid; GCDCA, glycochenodeoxycholic acid; TCDCA, taurochenodeoxycholic acid; -3S, 3-sulfates; tr, trace.

[a] Percentage of 3-sulfates (3-sulfates/3-sulfates + nonsulfates) in parentheses.

levels of serum bile acids in patients with obstructive jaundice or extrahepatic cholestasis (14–17), though our procedure requires much smaller sample size, i.e., one twentieth to one fiftieth. The conjugate form of each bile acid could not be known in the other studies because analysis by GLC was used, necessitating prior hydrolysis of conjugates. The proportion of secondary bile acids found was much lower or not detected in our study, probably because of the complete obstruction of the extrahepatic bile duct with complete interruption of enterohepatic circulation as a result of cancer. Most of the sulfated bile acids were found to be present in the CDCA fraction in agreement with previous studies. Fifty ng each of bile acid, as injection weight, are required for satisfactory quantitation by HPLC with immobilized 3α-HSD.

Thus, the present method permits simultaneous analysis of 3-sulfated and nonsulfated bile acids as well as conjugated forms in biological samples. A quantitative determination of the bile acid profiles in various hepatobiliary diseases using the present procedure will be published elsewhere.

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