Separation of sulfated from non-sulfated serum bile acids without the use of Sephadex columns

Guy Lepage, Claude C. Roy, and Andree M. Weber

Department of Pediatrics, Hôpital Ste-Justine and l'Université de Montréal, Montréal, Canada

Summary Methods currently in use for the quantitative measurement of sulfated serum bile acids usually lead to low recoveries. The present technique uses two internal standards and involves enzymatic hydrolysis followed by extraction of the non-sulfated fraction in ether at pH 4.6. The sulfated fraction is then solvolysed before gas–liquid chromatographic analysis of the individual bile acids of both fractions. Using 200 μl of serum, recoveries of more than 81% of sulfated bile acids were achieved. This method is of particular significance in view of recent evidence showing that certain sulfated bile acids are cholestatic.—**Lepage, G., C. C. Roy, and A. M. Weber.** Separation of sulfated from non-sulfated serum bile acids without the use of Sephadex columns. *J. Lipid Res.* 1981. **22:** 705–711.

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Sulfate esters of bile acids were initially identified in 1967 by Palmer (1) who established sulfation as a pathway for the metabolism of bile acids, particularly in patients with cholestatic liver disease. The greater aqueous solubility of sulfated bile acids explains their higher proportion in urine than in blood (2). The sulfate group not only prevents their tubular reabsorption but also their absorption from the intestine (3). Furthermore, there is presumptive evidence that sulfation may represent a biotransformation of considerable protective advantage (4). This is especially so in man who lacks the capacity to hydroxylate lithocholate conjugates further (5, 6). The efficiency of this detoxification mechanism has been challenged recently. Carey (7) showed in vitro that the disodium salt of glycolithocholate sulfate is not more soluble than its non-sulfated form, suggesting that sulfated glycolithocholate may be potentially hepatotoxic. This suggestion was confirmed in studies by Yousef et al. (8), where the injection of glycolithocholic acid monosulfate was shown to be cholestatic in rats.

In view of the potential role of certain sulfated bile acids in the pathogenesis of cholestatic liver disease (8), there is a need for a technique for the measurement of sulfated bile acids in serum that is reliable and circumvents the problems associated with column work. The purpose of the present study is to describe a technique for the measurement of sulfated bile acids in serum which largely overcomes these drawbacks and uses small amounts of serum, making it eminently appropriate for the investigation and the followup of neonates and infants with cholestasis.

Abbreviations: SBA, sulfated bile acids; NSBA, non-sulfated bile acids; GLC, gas–liquid chromatography; TLC, thin-layer chromatography; IR, infrared spectrometry. "Sulfate" refers to the 3-sulfate ester of the corresponding bile acid, unless otherwise indicated.

MATERIALS AND METHODS

Reagents and materials

Analytical grade solvents were redistilled in glassware. Sephadex LH-20 was obtained from Pharmacia, Uppsala, Sweden; non-sulfated bile acids and sterols standards were from Steraloids, Wilton, NH; lithocholate 3-sulfate and its glycine and taurine conjugates were from Calbiochem-Behring Corp., La Jolla, CA; cholylglycine hydrolase (No. 8222, 7,200 units/mg protein) was from Schwarz/Mann, Orangeburg, NY; a crude extract of the same enzyme (No. C 3636, 12 units/mg solid) was from Sigma, St. Louis, MO.; EDTA was from Fisher, Fair Lawn, NJ and 2-mercaptoethanol was from Eastman, Rochester, NY. [24-^{14}C]Taurocholic acid (40 mCi/mol) was purchased from New England Nuclear, Boston, MA. Chenodeoxycholic sulfates and conjugates were kindly supplied by Dr. S. Barnes.

Thin-layer chromatography

Thin-layer chromatography was carried out using plates precoated with Sil G-25 HR from Macherey–Nagel and Co., Werkstrabe, Germany. Separation of individual bile acids into free, conjugated, and sulfated (free and conjugated) groups was readily achieved using three solvent systems (**Table 1**). Unconjugated bile acids were separated in solvent I, acetic acid–carbon tetrachloride–diisopropyl ether–isoamylacetate–n-propanol–benzene 5:20:30:40:10:10 (v/v) (9). Conjugated bile acids were identified in solvent II, propionic acid–isoamylacetate–water–n-propanol 15:20:5:10 (v/v) (9). Free or conjugated bile acid sulfates were analyzed in solvent III, ethyl acetate–n-butanol–acetic acid–water 40:30:15:15 (v/v) (10). Spots were detected by spraying the plates with 3.5% phosphomolybdic acid in isopropanol and heating them at 90°C for 20 min.

Infrared spectrometry

IR spectra were determined on a Perkin Elmer 137 spectrometer using 100 mg KBr pellets at 1% sample concentration. Polystyrene IR spectrum was used as reference for the apparatus accuracy.

TABLE 1. TLC mobilities (R_f's) of bile acids with three solvent systems

Bile Acid	R_f Values Solvent I[a]	Solvent II[b]	Solvent III[c]
NSBA			
Lithocholic acid	0.82	0.91	0.98
Nordeoxycholic acid	0.53	0.76	0.89
Deoxycholic acid	0.40	0.74	0.96
Chenodeoxycholic acid	0.42	0.76	0.96
Cholic acid	0.16	0.75	0.93
Glycolithocholic acid	0.40	0.72	0.88
Glycodeoxycholic acid	0.08	0.70	0.84
Glycochenodeoxycholic acid	0.09	0.61	0.88
Glycocholic acid	0	0.48	0.70
Taurolithocholic acid	0	0.30	0.51
Taurochenodeoxycholic acid	0	0.28	0.47
SBA			
Lithocholic acid 3-sulfate	0.01	0.31	0.70
Nordeoxycholic acid 3-sulfate	0.04	0.30	0.61
Deoxycholic acid 3-sulfate	0.03	0.31	0.68
Chenodeoxycholic acid 3-sulfate	0.03	0.31	0.68
Chenodeoxycholic acid 7-sulfate	0.01	0.27	0.67
Cholic acid 3-sulfate	0.01	0.26	0.65
Glycolithocholic acid 3-sulfate	0.01	0.24	0.60
Glycodeoxycholic acid 3-sulfate	0	0.11	0.46
Glycochenodeoxycholic acid 3-sulfate	0	0.19	0.48
Glycochenodeoxycholic acid 7-sulfate	0	0.18	0.45
Glycocholic acid 3-sulfate	0	0.06	0.34
Taurolithocholic acid 3-sulfate	0	0.02	0.20
Taurochenodeoxycholic acid 3-sulfate	0	0.01	0.16
Taurochenodeoxycholic acid 7-sulfate	0	0	0.14

[a] Acetic acid–carbon tetrachloride–diisopropyl ether–isoamyl-acetate–n-propanol–benzene 5:20:30:40:10:10.
[b] Propionic acid–isoamylacetate–water–n-propanol 15:20:5:10.
[c] Ethyl acetate–n-butanol–acetic acid–water 40:30:15:15.

Radioactivity determination

Radioactivity determination was made by liquid scintillation counting using Toluene 0.4% Omnifluor (New England Nuclear) as scintillation liquid on a Unilux II, Nuclear Chicago apparatus.

Gas–liquid chromatography

Bile acids were chromatographed as methyl ester acetates on 6-ft U-shaped glass columns with an internal diameter of 2 mm. The columns were packed with 2% QF_1 or 2% OV-225 on chromosorb W (HP) 100–120 mesh. Analyses were performed on a Hewlett-Packard 7610A gas chromatograph with a flame ionization detector. Temperature was programmed from 230 to 245°C at a rate of 4°/min, then to 265°C at a rate of 1°/min. The injection port temperature was 245°C and the detector 280°C. Peak area measurements were carried out during GLC using an Autolab 6300 automatic digital integrator. Reproducibility during the analysis was excellent based on recovery of the added internal standards as will be discussed later.

Synthesis of sulfated standards

The 3-monosulfates of nordeoxycholic acid, glycodeoxycholic acid, and glycocholic acid were synthesized with sulfur trioxide-triethylamine in dimethylformamide (11, 12). These synthesized 3-monosulfates were purified by TLC. As judged by IR, TLC, and elementary analysis of sulfur, the synthesis procedure produced the desired bile acid sulfates.

PROCEDURE

An outline of the method used for separation and quantitation of non-sulfated (NSBA) and sulfated bile acids (SBA) is shown in **Fig. 1.** In 20 ml of hot ethanol–ammonia water 800:1 (pH 11), two internal standards, 3α,7α-dihydroxy, 12-keto-cholanic acid and nordeoxycholic acid 3-sulfate, were added followed by the addition of 200–500 μl of serum in a drop by drop fashion to insure deproteinization of the serum. The mixture was then heated at 70°C for 10 min and centrifuged. The supernatant was collected and then 3 ml of 95% ethanol was added to the precipitate and the same procedure was repeated. The supernatants were pooled and evaporated under a stream of air. After solubilization of the residues with 3 ml of water, they were subjected to enzymatic hydrolysis for a period of 16 hr. Neutral sterols were extracted

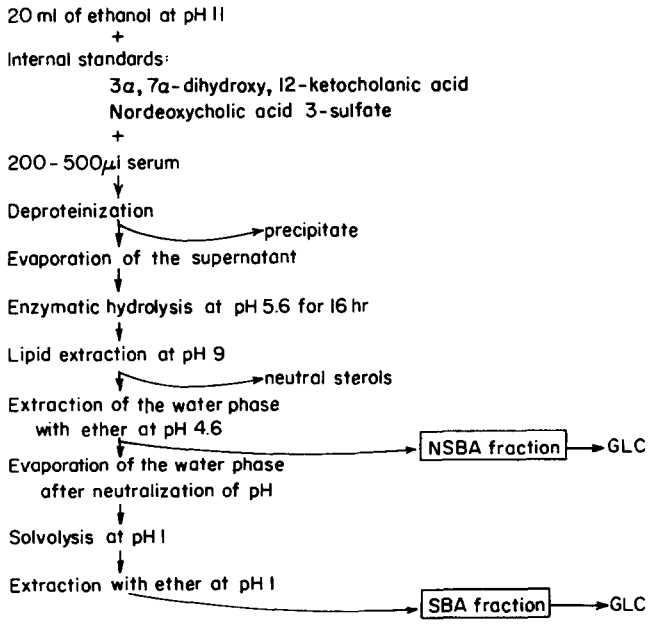

Fig. 1. Flowsheet for the analysis of nonsulfated and sulfated bile acids fractions in serum.

at pH 9 using a modification of the technique of Jeejeebhoy, Ahmad, and Kozad (13). The pH was adjusted to 4.6 and free bile acids were extracted five times with diethyl ether. The pH was then neutralized and the remaining water phase was evaporated under air. Unconjugated sulfated bile acids were subjected to solvolysis as described by Parmentier and Eyssen (14). Five ml of water was added and free bile acids were extracted with diethyl ether as previously described. NSBA and SBA fractions were methylated with an ethereal solution of freshly distilled diazomethane, acetylated, then analyzed by GLC as previously described.

RESULTS AND DISCUSSION

Validation of the technique described above was carried out for each of the individual procedural steps.

Internal standards

One other group has utilized two internal standards for the separation and recovery of NSBA from SBA (15). It appeared essential to control recoveries of both fractions independently. The late addition of an internal standard to the SBA fraction (16) preempts assessment of the efficiency of the procedure used for separation of the two fractions. 3α,7α-Dihydroxy,12-ketocholanic acid was selected as the internal standard for the NSBA and nordeoxycholic acid 3-sulfate for the SBA fraction.

Deproteinization

In serum, conjugated bile acids are bound to albumin, but the affinity of albumin for bile acids is nearly nonexistent above pH 11 (17). Lithocholic acid (LCA) is more strongly bound to albumin than other bile acids (18–20). However, Campbell, McGuffie, and Powell (21) showed that the loss of LCA was less than 5% when proteins and peptides were precipitated in hot alkaline ethanol. Recoveries for tested bile acids in the present study support these findings.

Enzymatic hydrolysis

In 1961, Levin, Irvin, and Johnston (22) suggested that enzymatic hydrolysis should replace alkaline hydrolysis which is known to destroy bile acids, certain species being more vulnerable than others (19, 20, 23–28). Few studies have been carried out on the hydrolysis of conjugated bile acid sulfates without solvolysis. In 1967, Nair, Gordon, and Reback (29) suggested that free hydroxyl groups were a prerequisite to enzymatic hydrolysis. Subsequently, Palmer and Bolt (30) found that incubation of taurine or glycine bile acid sulfates with cholylglycine hydrolase resulted in significant hydrolysis of the amide linkage while keeping intact the sulfate ester group. However the reaction was slower than with the unsulfated compounds (30) and Palmer and Bolt (30) did not define the experimental conditions necessary to achieve complete hydrolysis. Studies with the purified enzyme, cholylglycine hydrolase obtained from Schwarz/Mann as described by Nair et al. (29), confirmed the findings of Palmer and Bolt (30) regarding the glycine conjugated bile acid sulfates. However, the extent of hydrolysis after 30 min was unacceptably low for taurine conjugates (**Table 2**). Studies were then carried out on the same substrates with a crude extract of cholylglycine hydrolase (Sigma, St. Louis, MO). TLC and GLC results (Table 2) showed that hydroly-

TABLE 2. Enzymatic hydrolysis of bile acids with cholylglycine hydrolase

Bile Acid	Enzyme I[a] Deconjugation 30 min	Enzyme II[b] Deconjugation 30 min	Enzyme II[b] Deconjugation 5 hr	Enzyme II[b] Deconjugation 16 hr	Enzyme II[b] Solvolysis after 16 hr	Enzyme II[b] Artifactual products after 16 hr
			%			
Glycolithocholic acid	96	86	96	96	NA[c]	1
Glycocholic acid	89	92	96	97	NA	0
Taurolithocholic acid	71	91	98	97	NA	1
Taurocholic acid	13	73	91	94	NA	0
Lithocholic acid sulfate	NA	NA	NA	NA	4	2
Nordeoxycholic acid 3-sulfate	NA	NA	NA	NA	0	0
Glycolithocholic acid sulfate	94	98	93	96	3	0
Glycodeoxycholic acid 3-sulfate	87	90	92	95	0	0
Glycocholic acid 3-sulfate	84	82	96	96	0	0
Taurolithocholic acid sulfate	18	69	82	94	0	0

[a] Schwarz/Mann (500 units).
[b] Sigma (4 units).
[c] NA, not applicable.

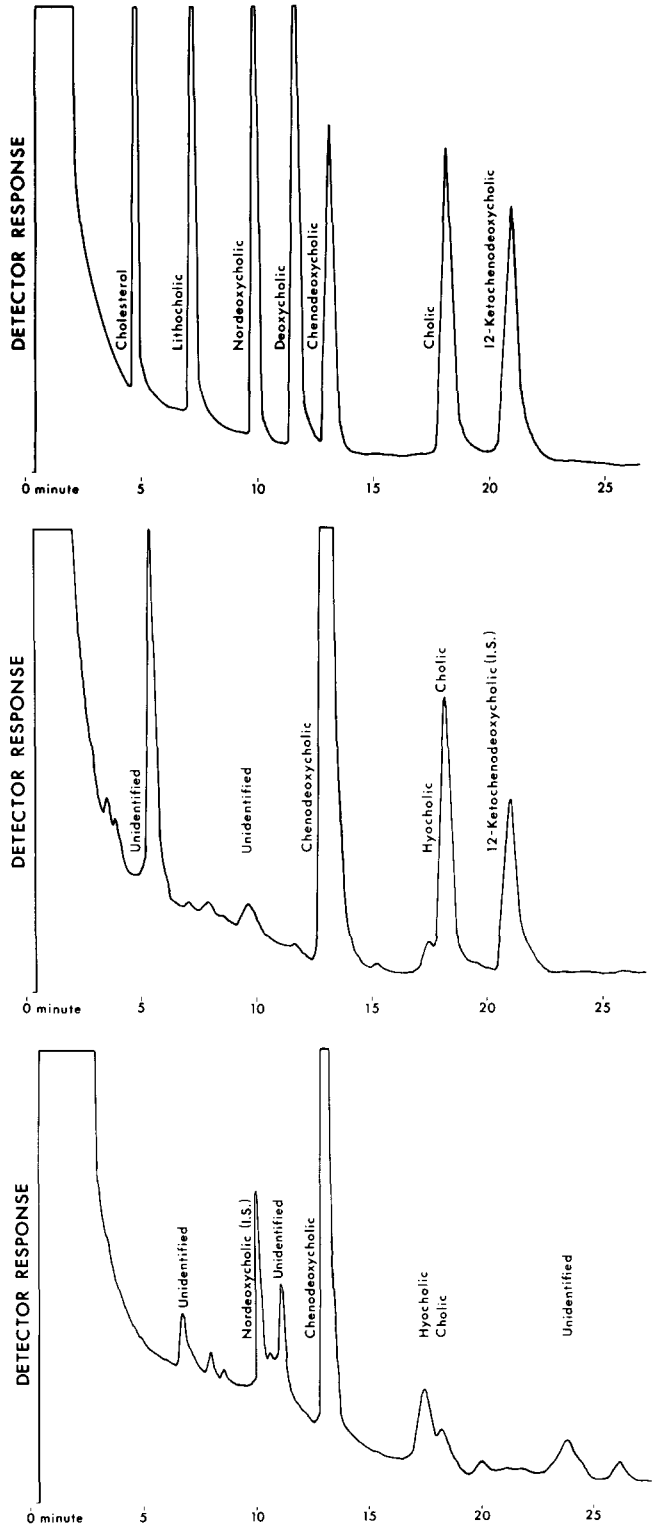

Fig. 2. The GLC pattern of a mixture of bile acids and cholesterol standards (100 ng of each) with their retention times obtained with a column packed with 2% QF_1 on chromosorb W (HP) 100–120 mesh (upper). The two other patterns are representative of the serum bile acid profile in a 2-month-old infant with hepatic ductular hypoplasia. The chromatogram of the nonsulfated (middle) and of the sulfated fraction (lower) were obtained from a 200 μl sample of fasting serum.

TABLE 3. Effect of enzymatic hydrolysis[a] on chenodeoxycholic acid derivatives

	Deconjugation	Solvolysis	Artifactual products
		%	
Chenodeoxycholic acid	NA[b]	NA	0
Glycochenodeoxycholic acid	100	NA	0
Taurochenodeoxycholic acid	98	NA	0
Chenodeoxycholic acid 3-sulfate	NA	2	0
Chenodeoxycholic acid 7-sulfate	NA	0	0
Glycochenodeoxycholic acid 3-sulfate	98	2	0
Glycochenodeoxycholic acid 7-sulfate	96	0	0
Taurochenodeoxycholic acid 3-sulfate	90	0	3
Taurochenodeoxycholic acid 7-sulfate	96	0	1

[a] Hydrolysis with cholylglycine hydrolase (4 units) from Sigma for 16 hr at 37°C on 400 μg of each.
[b] NA, not applicable.

sis of NSBA was nearly complete after 30 min, even for taurolithocholic acid. After a 5-hr incubation period, better results were observed, especially for taurocholic acid and SBA, but 16 hr seemed necessary for 94% hydrolysis of taurolithocholic acid sulfate. Even after 16 hr of hydrolysis, solvolysis of lithocholic acid sulfate and of glycolithocholic acid sulfate was minimal. Less than 2% of lithocholic acid sulfate had broken down to degradation products. Since chenodeoxycholic is often the predominant bile acid in cholestatic serum (**Fig. 2**), special attention has been given to its derivatives in the enzymatic hydrolysis procedure (**Table 3**). Because the present observations were derived from studies restricted to a limited number of bile acids, it would be of interest to verify the effects of a 16-hr period of enzymatic hydrolysis on di- and tri-sulfates, especially on those conjugated with taurine.

Extraction of lipids

When extracting lipids with heptane, recovery of lithocholic acid is lower than for other bile acids (31). Following the triple extraction used in our methodology, up to 92% of the cholesterol is extracted and less than 4% of lithocholic acid is lost. On human serum, better results in the extraction of lipids were observed when extractions were carried out after the enzymatic hydrolysis step and not before.

Separation of NSBA from SBA

To date the only methods used for separation of NSBA from SBA are the LH-20 and DEAP-LH-20 Sephadex columns. Despite many attempts, the satisfactory results of Campbell et al. (21) could not be reproduced. In our hands, almost 100% of the free lithocholic acid sulfate and more than 40% of the

glycolithocholic acid sulfate were eluted with the chloroform–methanol 1:1 (v/v) fraction. Although Makino et al. (32) with glycochenodeoxycholic acid monosulfate and Laatikainen, Lehtonen, and Hesso (33) with glycocholic acid monosulfate obtained recoveries of 90% in the methanol fraction, it is worth pointing out that these glycoconjugated sulfates are not representative of less polar bile acids such as lithocholic acid sulfate and glycolithocholic acid sulfate. Therefore, it is important that any separation procedure of SBA from NSBA be tested using these less polar bile acid sulfates, especially since Yousef et al. (8) recently established that both lithocholic acid sulfate and glycolithocholic acid sulfate may induce intrahepatic cholestasis in rats. Other well known problems are associated with LH-20 columns. Purifying 200 mg of commercial lithocholic acid, Kelsey and Sexton (34) routinely obtained satisfactory recoveries of 80 to 90%. On the other hand, when they used smaller amounts of mass, lithocholic acid adhered to the gel and led to poor recoveries. In 1976, Eyssen, Parmentier, and Mertens (16) found that when larger amounts of NSBA were present in the sample, the sulfated fraction could be contaminated by trace amounts of NSBA, which he called "memory effects" of Sephadex LH-20. In 1979, Pageaux et al. (19) showed that taurocholic acid and deoxycholic acid 12-sulfate overlapped each other using this fractionation procedure. Almé et al. (26) claimed that DEAP-LH-20 columns solved the problem but purification using Amberlite XAD-2 was essential since inorganic and organic compounds in urine interfered with the anion exchange chromatography and acted as eluting buffers. Recoveries on XAD-2 as on XAD-7 are difficult (19, 28, 32), since they are pH- , flow rate- , and solvent-dependent. Even under the best of conditions defined by Almé et al. (26), Pageaux et al. (19) are of the opinion that such a procedure does not seem to be suitable for serum samples.

Because of the drawbacks associated with column

TABLE 4. Recovery on GLC analysis of unlabeled bile acids and sterols run through the entire procedure[a]

	Amount Added (μg)	Unsulfated Fraction Recovery (%)	Sulfated Fraction Recovery (%)
Cholesterol	15	8	0
Coprostanol	15	2	0
Cholesteryl oleate	136	0	0
Cholesteryl linoleate	339	0	0
Cholesteryl sodium sulfate	12	0	0
Lithocholic acid	15	96	0
Nordeoxycholic acid	15	98	0
Deoxycholic acid	15	94	0
Chenodeoxycholic acid	15	92	0
Cholic acid	15	93	0
Hyocholic acid	15	95	0
3α,7α-Dihydroxy-12-ketocholanic acid	15	92	1
3α-Hydroxy-7,12-diketocholanic acid	15	88	1
Dehydrocholic acid	15	82	3
Glycolithocholic acid	20	91	0
Glycochenodeoxycholic acid	20	89	0
Glycocholic acid	20	92	0
Taurolithocholic acid	20	89	0
Taurochenodeoxycholic acid	20	87	0
Taurocholic acid	20	83	1
Lithocholic acid 3-sulfate	5	4	89
Nordeoxycholic acid 3-sulfate	5	0	90
Chenodeoxycholic acid 3-sulfate	10	3	89
Chenodeoxycholic acid 7-sulfate	10	0	90
Glycolithocholic acid 3-sulfate	7	3	87
Glycodeoxycholic acid 3-sulfate	7	0	92
Glycochenodeoxycholic acid 3-sulfate	14	1	88
Glycochenodeoxycholic acid 7-sulfate	14	0	86
Glycocholic acid 3-sulfate	7	0	89
Taurolithocholic acid 3-sulfate	7	0	83
Taurochenodeoxycholic acid 3-sulfate	14	0	81
Taurochenodeoxycholic acid 7-sulfate	14	0	86

[a] The recoveries take into account the amount of non-sulfated bile acids (80 ng chenodeoxycholic acid, 66 ng cholic acid) found in the 200 μl of pooled fasting serum added to each standard. The pooled serum had no detectable sulfated bile acids.

extraction, it was felt desirable to develop a new technique to separate unconjugated NSBA from unconjugated SBA after enzymatic hydrolysis. The pK'_A of cholic acid, which represents the most polar unconjugated NSBA, and the pK'_A of lithocholic acid sulfate, one of the less polar SBA, were found to be 4.97 and 5.42, respectively, in the range of molarities expected in serum.[1] This meant that the pH had to be maintained below 4.97 to insure that all NSBA were in their protonated form and extractable from water. After many trials, pH 4.6 was found to be optimal for extraction of 100% cholic acid into the ether phase. It is worth pointing out that vigorous shaking and 10 min of standing previous to every centrifugation are critical for good recoveries. At pH 4.6, lithocholic acid sulfate remained in the water phase because the presence of a sulfate group increases the aqueous solubility of bile acids considerably. However, when a more polar organic solvent such as ethyl acetate was used, up to 40% lithocholic acid sulfate was lost in the NSBA fraction at pH 4.6. Henegouwen et al. (35) claimed that an important fraction of sulfated forms undergo solvolysis in ether at pH 1. However with twelve different bile acid sulfates, there was no evidence by TLC and GLC analyses that significant solvolysis had taken place at pH 4.6 (Tables 2 and 3). Solvolysis seemed to occur only in the presence of an excess of HCl.

Solvolysis

In our hands, the solvolysis procedure of Parmentier and Eyssen (14) led to complete desulfation of unconjugated bile acid sulfates without the formation of degradation products.

Thirty-two unlabeled bile acids and sterols were run individually through the entire procedure. Yields higher than 81% were obtained (**Table 4**). Reproducibility of the method was verified by running seven samples from the same aqueous mixture of 3α,7α-dihydroxy, 12-ketocholanic acid and nordeoxycholic acid 3-sulfate. Recoveries for these two internal standards were 92.1 ± 1.4% and 89.7 ± 3.1%, respectively. There was no apparent overlap and this was further supported by the fact that [24-^{14}C]taurocholic acid gave recoveries of 86.2 ± 5.1% for the NSBA fraction and 0.6 ± 0.9% for the SBA fraction in these same seven samples.

We have tested so far a limited number of fasting and postprandial sera from normal controls, from infants with neonatal cholestasis, and from a group of children with cirrhosis. Fig. 2 shows the GLC pattern derived from a mixture of bile acids and cholesterol standards and those of the non-sulfated and of the sulfated fraction from a 2-month-old infant with a well described cholestatic syndrome called hepatic ductular hypoplasia (36).

The technique described in this paper overcomes the problems associated with columns and TLC separation. It has significant advantages in terms of accuracy and reproducibility for the separation and the analysis of sulfated and of unsulfated serum bile acids. Furthermore, it requires only small amounts of serum. It appears particularly attractive for the study of cholestatic syndromes, especially since recent experimental work suggests that sulfation does not protect against the cholestatic potential of bile acids, particularly those conjugated with glycine (8).

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[1] Lepage, G., C. C. Roy, and A. M. Weber. Unpublished data.

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