notes on methodology

A facile hydrolysis–solvolysis procedure for conjugated bile acid sulfates

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Summary Methods for hydrolyzing and solvolyzing conjugated bile acid sulfates were compared on reference mixtures of conjugated and unconjugated bile acid sulfates using gas–liquid chromatography to assess recovery, and thin-layer chromatography and zonal scanning to define the products occurring after hydrolysis. Conventional methods in which solvolysis preceded vigorous alkaline saponification gave incomplete recoveries. However, essentially complete recovery of primary and secondary bile acid sulfates was obtained with a mild alkaline saponification procedure followed by acidification and extraction into ether, in which complete solvolysis was shown to occur within 12 hours. Based on these findings, we developed and validated a simple hydrolysis–solvolysis procedure; the method features mild alkaline hydrolysis, acidification to pH 1, and extraction with ether followed by a 1-hour incubation.

Supplementary key words: solvolysis · conjugated bile acids · lithocholic acid · sulfated bile acids

Bile acids present in body fluids are often identified and quantitated by gas–liquid chromatography (GLC). Since bile acids are present in bile and serum as the *N*-acyl glycine or taurine conjugates, a hydrolysis procedure has been an essential first step in the sequence of reactions entailed in the conversion of bile acids to volatile derivatives. Commonly, this is achieved by saponification in strong alkali (1), although enzymatic deconjugation with cholylglycine amidase has been used with apparent success by several groups recently (2, 3). Acid hydrolysis has never been employed because of supposed destruction of the bile acid molecule. Palmer (4) showed that the secondary bile acid, lithocholate, is present in bile as the sulfate, and it is now considered likely that the majority of lithocholate present in bile and serum is present as the 3-sulfated *N*-acyl glycine or taurine conjugate (5). More recently it has been shown that the primary bile acids are extensively sulfated in patients with obstructive hepatobiliary disease and, indeed, the majority of urinary bile acids may be sulfated in such patients (6, 7).

Palmer (1) suggested that conventional alkaline hydrolysis procedures destroy bile acid sulfates, and he proposed that a solvolysis step should precede the conventional alkaline hydrolysis deconjugation procedure. In several recent studies we found that the observed proportion of lithocholate in biliary bile acids was similar whether or not a solvolysis procedure preceded deconjugation by alkaline hydrolysis. In these studies, we also found essentially complete recovery of added taurolithocholate sulfate from model mixtures whether or not solvolysis was included in our analytical procedure. Stimulated by these puzzling observations, we have undertaken a systematic study of the efficacy of several conventional solvolysis–hydrolysis procedures and have not only clarified why good recovery was obtained in our previous studies, but also developed an improved method for solvolysis that appears to have sufficient utility to justify its description here.

Experimental design

In the first set of studies, we used GLC to compare the recovery of sulfolithocholyltaurine from several conventional methods for hydrolysis and solvolysis. In the second set of studies, using thin-layer chromatography and zonal scanning, we determined the products produced after hydrolysis or solvolysis of labeled sulfolithocholyltaurine or sulfolithocholate. In the third set of studies, we examined a new method for hydrosolvolysis of lithocholate sulfates, using gas–liquid chromatography, and then applied the method to a number of bile acid sulfates to show that the method had general application.

Materials and methods

Chemicals. [24-^{14}C]Lithocholate (sp act 4.2 mCi/mmole) was obtained from New England Nuclear, Boston, MA, or Mallinckrodt Chemical Corporation, St. Louis, MO. [11,12-^{3}H]Lithocholate (sp act 1500 mCi/mmole) was prepared by reduction of Δ^{11}-litho-

Abbreviation: GLC, gas–liquid chromatography.

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TABLE 1. Mean percent recovery (±SEM) of lithocholic acid from sulfolithocholyltaurine using GLC[a]

	Solvolysis (Palmer & Bolt) (1)			Solvolysis (Burstein & Lieberman) (15)			No Solvolysis		
	%			%			%		
Sulfolithocholyltaurine, mg/ml	0.5	1.0	2.5	0.5	1.0	2.5	0.5	1.0	2.5
Strong alkaline hydrolysis (Palmer & Bolt) (1)	70.0 (3.0)	71.1 (5.2)	72.0 (3.0)	66.0 (4.3)	77.5 (5.6)	72.3 (2.1)	53.0 (4.0)	69.5 (5.1)	54.0 (3.0)
Mild alkaline hydrolysis[b]	66.0 (4.1)	82.6 (4.9)	79.1 (3.0)	62.0 (5.1)	73.6 (4.8)	79.3 (2.9)	79.0 (4.0)	81.2 (5.1)	81.9 (2.9)

[a] The sulfolithocholyltaurine was added to samples of a human bile pool of known bile acid composition.
[b] As discussed in the text, this expression "mild alkaline hydrolysis" is used for convenience only, since the method, as carried out, features acidification and extraction into ether, where solvolysis occurs.

cholenic acid as described (8). Lithocholic acid (Mann Research Labs, New York, NY) was conjugated with taurine according to Norman (9), and its 3α sulfate was prepared according to Fieser (10). Both were recrystallized from methanol or ethanol as the diammonium salts. Using TLC with the system of Cass et al. (11), the compounds ran as single spots. [11,12-^{3}H]Sulfolithocholate and its taurine conjugate were prepared from [11,12-^{3}H]lithocholate and [11,12-^{3}H]lithocholyltaurine, and [24-^{14}C]sulfolithocholate was prepared from [24-^{14}C]lithocholate as described by Palmer and Bolt (1). The sodium salts of 3α-sulfochenodeoxycholate and 3α-sulfodeoxycholate were a generous gift from Professor G. A. D. Haslewood.

Solvolysis. Solvolysis was carried out using modifications of the method for steroid hydrogen sulfates (12). We used two different modifications. In Method A (13), 0.4 ml of bile in 1.6 ml of ethanol was mixed with 8 ml of equilibrated ethyl acetate (prepared by adding 50 ml of ethyl acetate to 5 ml of 20% NaCl and adjusting the pH to 1 with 50% H_2SO_4). The solution was mixed and incubated for 24 hr, then neutralized with ammonium hydroxide and evaporated to dryness.

In Method B (1), 0.4 ml of bile was dissolved in 2 ml of ethanol and acidified to pH 1 or less with 2 N HCl. This was diluted with nine volumes of acetone, and the mixture was incubated at room temperature for 48 hr. The sample was evaporated to dryness and refluxed with 5 ml of methanolic 5% KOH for 2 hr. The solution was neutralized with Dowex 50X-W, filtered, and evaporated.

Hydrolysis. In Method I, a vigorous alkaline hydrolysis (1), the sample was dissolved in methanol, transferred to a nickel bomb (Parr Instrument Co., Moline, IL), and the methanol was then evaporated away. Four ml of methanol and 4 ml of 5 N NaOH were added, as well as nordeoxycholic acid as internal standard. The solution was well mixed and kept at 120°C for 19 hr. After cooling, the mixture was transferred to large extraction tubes with multiple rinses of deionized water, acidified to pH 2 with 5 N HCl, extracted three times with three volumes of ether, and evaporated to dryness. An external standard of bile acids containing cholylglycine, chenodeoxycholylglycine, deoxycholylglycine, and lithocholylglycine was used for correction of loss during hydrolysis.

In Method II, a mild alkaline hydrolysis, 0.2 ml of bile in 1.8 ml of ethanol was pipetted into nickel bombs, 200 μl (1 mg/ml) of nordeoxycholic acid (0.2 ml of a solution of 1.0 mg/ml) solution was added as internal standard, and the mixture was evaporated to dryness. A mixture of 4 ml of 2 N NaOH and 4 ml of 50% methanol in water 1:1 (v/v) was added. After thorough mixing, the sample was hydrolyzed for 4 hr at 115°C and then transferred to extraction tubes with washes of deionized water. After acidification with 5 N HCl to pH 1, the bile acids were extracted with ether (3 × 36 ml) and evaporated to dryness.

Methylation. The sample was dissolved in 2 ml of chloroform–methanol 2:1 (v/v); excess ethereal diazomethane (prepared from *N*-nitroso methyl urea and 20% methanolic KOH) was added; the solution was kept at room temperature for 15 min. It was then evaporated to dryness.

Acetylation. An acetylation reagent (14) (10 ml of acetic anhydride, 14 ml of glacial acetic acid, and 0.1 ml of 70% perchloric acid) was prepared and cooled to 0°C. Two ml were added to each sample, and the solution was kept at 0°C for 1.5 hr. The reaction was stopped by the addition of 4 ml of 20% NaCl and the bile acids were extracted with ether (3 × 10 ml). Phase separation was achieved by centrifugation (14). The pooled supernatant phases were evaporated to dryness.

GLC. Gas–liquid chromatography was carried out

TABLE 2. Effect of subsequent treatment on mean percent solvolysis (±SEM) after mild alkaline hydrolysis of sulfolithocholyltaurine, as determined by TLC–zonal scanning

	Ether Extraction after Acidification with HCl				Dowex 50 (H^+) Neutralization; No Extraction
	Incubation Time				Incubation Time
	1 hr	2 hr	3 hr	12 hr	12 hr
	%				%
Initial label[a]					
sul-lit-tau					
Products[a]					
sul-lit-tau	0.8 (0.1)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	11.0 (1.2)
sul-lit	70.0 (1.2)	65.2 (1.3)	67.3 (4.6)	8.9 (0.8)	79.7 (3.6)
lit	28.1 (1.1)	34.1 (0.9)	32.2 (3.9)	90.4 (1.1)	2.8 (0.3)
Initial label[a]					
sul-lit					
Products[a]					
sul-lit	66.1 (1.4)	64.5 (1.7)	65.8 (5.6)	12.7 (0.7)	93.0 (3.7)
lit	28.4 (0.5)	34.7 (0.9)	32.1 (4.3)	86.6 (0.8)	4.3 (0.6)

[a] Abbreviations: sul-lit-tau, sulfolithocholyltaurine; sul-lit, sulfolithocholate; lit, lithocholate.

using a cyano–silicone stationary phase (AN 600) as described previously (15).

Results and discussion

Comparison of procedures. In these experiments, increasing amounts of sulfolithocholyltaurine were added to bile containing a defined bile acid composition. Solvolysis was carried out by Method A or Method B or was omitted. After each of these methods, deconjugation was carried out by strong alkaline hydrolysis (Method I) or mile alkaline hydrolysis (Method II). Results (**Table 1**) indicated that recovery was incomplete with all methods, but that greatest recovery was obtained with a combination of no solvolysis and mild alkaline hydrolysis.

These experiments suggested that neither solvolysis nor vigorous alkaline hydrolysis was essential. They raised the possibility that the mild alkaline hydrolysis procedure was achieving both solvolysis and hydrolysis, and we speculated that the methanol present in the saponification mixture might be inducing solvolysis.

To gain further insight into the events occurring during the mild alkaline procedure, we repeated the above studies varying the concentration of base or alcohol. The results, assessed by GLC, indicated a highly variable recovery, so we could conclude that the efficient solvolysis observed in the first set of experiments could not be explained by solvolysis occurring during the deconjugation procedure. We then proceeded to test the possibility that solvolysis was occurring after hydrolysis when samples were extracted into ether. Since HCl was used for acidification of the aqueous saponification mixture, a con-

TABLE 3. Effect of subsequent treatment after alkaline hydrolysis on mean percent solvolysis (±SEM) of chenodeoxycholate-3-sulfate and deoxycholate-3-sulfate[a]

	Neutralization with HCl; Addition of Aqueous HCl (1 M) (10 ml to 30 ml ether)			Extraction into Ether: Acidification with HCl						Dowex 50 (H^+) Neutralization; No Extraction
				Addition of Aqueous HCl (12.5 M) (5 ml to 30 ml ether)			Addition of HCl Gas (>5 M HCl in ether)			
	Incubation Time			Incubation Time			Incubation Time			Incubation Time
Sample	2 hr	4 hr	12 hr	20 min	40 min	60 min	20 min	40 min	60 min	12 hr
		%			%			%		%
3α-Sulfocheno-deoxycholate	87.7 (0.4)	85.5 (3.7)	91.7 (0.8)	101.6 (±1.2)	100.9 (±0.7)	98.9 (±0.7)	98.2 (0.6)	99.7 (0.3)	100.3 (0.7)	12.6 (0.2)
3α-Sulfodeoxy-cholate	71.6 (0.8)	68.8 (1.7)	90.3 (0.5)	100.3 (±0.7)	99.6 (0.8)	99.3 (0.4)	97.6 (0.4)	98.7 (0.5)	98.9 (0.6)	12.2 (0.2)

[a] Determination by gas–liquid chromatography of the reaction mixture.

TABLE 4. Proposed method for complete recovery of bile acid sulfates in bile

Step	Comment
1. Dilute bile samples with nine volumes isopropanol (distilled); centrifuge; take supernatant. Store in brown serum bottles with Teflon-faced silicone rubber liners fastened by crimped closures.	Isopropanol precipitates protein and is excellent solvent for cholesterol. Biliary lipids are stable for at least 1 year if samples are stored at −15°C.
2. Transfer aliquots of isopropanol supernatant to Parr bombs. Add internal standard. Carry out mild alkaline hydrolysis in 8 ml of 1.7 N NaOH–methanol 3:1, 4 hr at 115°C.	Nordeoxycholic, hyocholic, or 12 ketochenodeoxycholic acid have been used by many workers. Ideally, four internal standards should be used. One should be conjugated with glycine; a second with taurine; a third conjugated with glycine and sulfated; and a fourth conjugated with taurine and sulfated. The procedure hydrolyzes bile acid conjugates to unconjugated bile acids without destruction of bile acid structure.
3. Transfer sponification mixture to tube in ice bath. Acidify to pH 1 with 5 ml concentrated HCl (12.5 M); add 3 volumes of ether; shake thoroughly. Incubate for 20 min.	Bile acid sulfates are completely hydrolyzed and no destruction of bile acids will occur, even if incubation is prolonged to 1 hr.
4. Extract two additional times with three volumes of ether.	Bile acids are completely extracted.
5. Evaporate to dryness. Methylate with freshly prepared diazomethane and acetylate (14). Dissolve in acetone or ethyl acetate.	Bile acid methyl ester acetates are formed, which are stable derivatives suitable for GLC analysis. Samples may be saved for subsequent mass spectrometry.
6. GLC analysis using AN 600, SP 525 or SP 1000 as stationary phases.	3-Methoxy derivatives may be formed in trace amounts with diazomethane procedure for esterification. Quantitative analysis is obtained if internal standard has been added.
7. Mass spectrometry.	Permits identification of unusual bile acid derivatives (19).

siderable fraction of the HCl might be extracted into the wet ether phase.

Determinants of solvolysis. To show that solvolysis did not occur during the deconjugation procedure but did occur after extraction into ether, [^{3}H]sulfolithocholyltaurine and [24-^{14}C]sulfolithocholate were added to the bile sample. The nature of the products after alkaline hydrolysis was determined using thin-layer chromatography and zonal scanning. The alkaline hydrolysis reaction mixture was then acidified to pH 1 with HCl and extracted with ether, or was neutralized with a cation exchange resin in the H^+ form (Dowex 50, Dow Chemical Co., Midland, MI) and not extracted. In ether, continuing solvolysis occurred, and was complete after 12 hr. The sample neutralized with the cation exchange resin was incubated for 12 hr, but no solvolysis occurred (**Table 2**).

Solvolysis of other bile acid sulfates. In these experiments, the completeness of solvolysis of sulfochenodeoxycholate and sulfodeoxycholate was assessed using the mild alkaline hydrolysis procedure followed either by acidification to pH 1 with HCl and subsequent extraction with ether, or by neutralization with a cation exchange resin (and no extraction). Again, complete solvolysis occurred after a 12-hr incubation in acidified ether, whereas no significant hydrolysis occurred when the sample was merely neutralized with an ion exchange resin and not extracted.

Revised procedure for solvolysis. Complete solvolysis was obtained by using ether saturated with HCl. To prepare this, HCl gas was bubbled through ether kept at 0°C. Based on change in weight, complete saturation of the ether with HCl occurred by 20 min. The concentration of the HCl was 6.8 M. Using this solution, complete solvolysis occurred within 20 min. When concentrated aqueous HCl (5 ml of 12.5 M HCl) was added to 30 ml of ether, complete solvolysis also was induced within 20 min (**Table 3**).

In **Table 4**, we propose a scheme for bile acid analysis that, in our hands, will give good (greater than 90%) recovery of conjugated bile acids including sulfolithocholyl conjugates in bile. The method has not been validated for sulfates of primary bile acid conjugates, but such validation is beyond the scope of this paper.

Comment

These results indicate that the solvolysis of bile acid sulfates is easily achieved, after deconjugation by mild alkaline hydrolysis, by a brief incubation in ether containing HCl. Similar methods have been applied for the solvolysis of other steroid sulfates (16), and there is good evidence for rapid solvolysis of steroids occurring in ether to which acid has been added (17). In our hands, recovery was superior to that achieved by several other solvolysis procedures traditionally carried out prior to the alkaline

saponification step. We also found that a mild alkaline hydrolysis procedure gave good (greater than 90%) recovery of the bile acids employed in our study.

Our results appear to provide an explanation for the failure of Carey and Williams (18) to find bile acid sulfates in bile after intravenous administration of labeled lithocholate to two bile fistula subjects. They carried out a conventional alkaline hydrolysis procedure, and then extracted their samples into *n*-butanol with acidification. It is likely that solvolysis occurred at this time.

One limitation of our study is that we have not tested our method with glycine or taurine conjugates of sulfated primary bile acids. However, we would anticipate that these should be deconjugated readily during the mild alkaline procedure. Our results also suggest that it may be possible to determine selectively the nonsulfated bile acids present in a sample by neutralizing the saponification reaction mixture with Dowex 50, rather than with HCl. A second limitation of our study is that we have not defined in detail the mildest conditions for alkaline hydrolysis that achieve complete deconjugation of sulfolithocholylglycine and sulfolithocholyltaurine. The simple and rapid solvolysis procedure reported here should facilitate detailed studies on alkaline and enzymatic deconjugation of bile acid sulfates.

Problems remain in the analysis of bile acids. Enzymatic deconjugation may not hydrolyze sulfated bile acids, and conjugated bile acids cannot be analyzed by GLC. Thus, alkaline hydrolysis continues to be required for complete bile acid recovery. What is needed is a sulfocholylglycine amidase or, alternatively, a nonaqueous method for bile acid deconjugation that is applicable to both sulfated and nonsulfated bile acids.

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