Method for removal of surface-active impurities and calcium from conjugated bile salt preparations: comparison with silicic acid chromatography

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Abstract Some commercial preparations of common natural conjugated bile salts contain impurities (e.g., amines, lipids, and calcium) that are likely to affect their physicochemical properties. A method was developed for purifying commercial preparations of sodium salts of glycine- and taurine-conjugated bile acids. The method consists of passage of a dilute aqueous solution of the sodium bile salt through three columns in sequence: graphitized carbon, a hydrophobic bonded octadecylsilane (C18) cartridge, and a calcium-chelating resin. The final solution was extracted with chloroform, and the purified bile salt was then isolated by freeze-drying, with a yield of 65–75%. Each bile salt purified by this method was compared with the corresponding bile salt purified by conventional adsorption chromatography on a silicic acid column, using a mixture of methanol and chloroform as eluant. Purity was assessed by visible spectra, by surface tension measurements (using the maximum bubble-pressure method and a Wilhelmy wire method), by chloroform extractability of impurities in the conjugated bile acid, by liposome solubilization, and by chemical analysis of the calcium content. Both purification methods removed colored and surface-active impurities, but the new method was always as or more effective than silicic acid column chromatography. Calcium ion, present in commercial bile salts in concentrations up to 16 mmol/mol bile salt, was removed completely by the three-column method, but not by silicic acid chromatography. The new method is thus a simple, rapid, and efficient procedure for purification of the sodium salts of glycine- and taurine-conjugated bile acids for physicochemical measurements, in which elimination of surface-active impurities and polyvalent cations is desired.--**Del Vecchio, S., J. D. Ostrow, P. Mukerjee, H-T. Ton-Nu, C. D. Schteingart, A. F. Hofmann, C. Cerrè, and A. Roda.** Method for removal of surface-active impurities and calcium from conjugated bile salt preparations: comparison with silicic acid chromatography. *J. Lipid Res.* 1995. **36:** 2639-2650.

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Bile salt anions are alicyclic, amphipathic steroids that undergo gradual, hydrophobic self-association over a moderately narrow concentration range to form aggregates that are termed "micelles" or multimers (1–3). Above this range of bile salt concentrations, often designated as the critical micellization concentration ("CMC") (2), bile salts solubilize amphiphilic lipids, such as phosphatidylcholine (4) or monoacylglycerols (5) with remarkable efficacy. In vivo, mixed micelles containing conjugated bile salt anions and phosphatidylcholine are present in bile (6) and mixed micelles containing monoacylglycerol and fatty acids are found in intestinal content during fat digestion (7). Bile salt anions, like detergent anions, bind counterions, (8–11), a process that may enhance the absorption of divalent cations such as calcium (12) and iron (12, 13).

Very small quantities of surface-active contaminants in detergents and bile salts can, in principle, markedly reduce the measured "CMC", the surface tension at

Abbreviations: Conjugated bile acids as their sodium salts: C-Tau, cholyltaurine; C-Gly, cholylglycine; CDC-Tau, chenodeoxycholyltaurine; CDC-Gly, chenodeoxycholylglycine; DC-Tau, deoxycholyltaurine; DC-Gly, deoxycholylglycine; UDC-Tau, ursodeoxycholyltaurine; UDC-Gly, ursodeoxycholylglycine; DHC-Tau, dehydrocholyltaurine; 12-Oxo-CDC-Tau, 3α,7α-dihydroxy,12-oxo-cholanoyl taurine. "CMC", apparent critical micellization concentration; PC, phosphatidylcholine; [BS], bile salt concentrations; TLC, thin-layer chromatography; ST, surface tensions.

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"CMC", and the surface area per molecule at the interface (14). The nature and concentrations of counterions also may influence the aggregation and surface activity of bile salts (8). Therefore, measurements of the "CMC", of the capacity of bile salts to solubilize amphiphilic lipids, and of the association of bile salts with other organic molecules or counterions, should be based on experiments using salts of pure bile acids, where purity is defined as both the absence of detectable surface-active contaminants and of undesired cations, as well as the absence of other conjugated or unconjugated bile salts.

In general, such experiments in model systems will be performed with the sodium salt of conjugated bile acids because of the dominance of the sodium ion in extracellular fluids and the essentially complete ionization of conjugated bile acids at physiological pH values (15). The major common natural bile acids in humans, including cholic (CA), deoxycholic (DCA), chenodeoxycholic (CDCA), and ursodeoxycholic (UDCA) acids, are available from commercial sources in unconjugated form, virtually free of contamination with other bile acids. Preparation of the N-acyl amidates of these unconjugated bile acids with glycine or taurine is commonly performed with a coupling agent (such as EEDQ) (16, 17), or via the mixed carbonic anhydride method first described in detail by Norman (18). Purification of the conjugated bile acids from the reaction mixture is known to be difficult, because the sodium salts of most taurine-conjugated bile acids (19), and the glycine conjugate of CDCA (20), are crystallized with great difficulty. Accordingly, it has become customary to attempt to remove impurities (e.g., coupling agent, unconjugated bile acids) from the reaction mixture by liquid/liquid extraction at several pH values, followed by isolation of the sodium salt of the conjugated bile acid by freeze-drying (21). These methods, however, may fail to remove colored and surface-active contaminants, and calcium (which we have found may be present in proportions up to 1.6 mol% in commercial bile salts; see below).

We report here a rapid, simple method to purify, with a high yield, commercial preparations of the sodium salts of the major natural glycine- and taurine-conjugated bile acids. The products appear to be much purer than commercially available preparations of these bile salts, as assessed by absorption spectra, surface tension, and calcium content of the bile salts, as well as their ability to solubilize biliary lipids. The purity of products obtained by the new method was often superior to that obtained by a conventional purification procedure, based on adsorption chromatography on silicic acid columns (22).

MATERIALS AND METHODS

Materials

Grade A sodium salts or free acids of eight natural bile acids, cholyltaurine (C-Tau), cholylglycine (C-Gly), chenodeoxycholyltaurine (CDC-Tau), chenodeoxycholylglycine (CDC-Gly), deoxycholyltaurine (DC-Tau), deoxycholylglycine (DC-Gly), ursodeoxycholyltaurine (UDC-Tau), and ursodeoxycholylglycine (UDC-Gly) were purchased from Behring-Calbiochem Corp., La Jolla, CA. The 3,7,12 tri-oxo-bile salt, sodium dehydrocholyltaurine (DHC-Tau), prepared by oxidation of the three hydroxy groups of cholic acid (23), was also purchased from Calbiochem. The sodium salt of 3α,7α-dihydroxy,12-oxo-cholanoyl taurine (12-Oxo-CDC-Tau) was synthesized at UCSD, as described (20). As judged by thin-layer chromatography (24) and HPLC (25), each bile salt contained less than 1% of other bile acid conjugates and < 0.5% unconjugated bile acids, and each contained the expected amount of 3α-hydroxy groups by the steroid dehydrogenase assay (26).

Cholesterol (chromatography standard grade CH-5), L-α-phosphatidylcholine (type IIIE from frozen egg yolk in hexane solution), calcium chloride, and PIPES were purchased from Sigma Chemicals, St. Louis, MO. Distilled water was freed of ions and organic contaminants by passage through columns containing a mix of ion-exchange resins and charcoal (Millipore Co., Broadview, IL); conductance of the purified water was less than 10^{-6} $(m\Omega/cm)^{-1}$. All other chemicals were reagent grade and solvents were HPLC grade. All glassware, including incubation vials, was prewashed successively with phosphate-free detergent, 3.0 M HCl, and methanol; after each washing step, glassware was rinsed five times with distilled water.

New method for purifying the sodium salts of conjugated bile acids

This method is based on percolating a dilute (5 mM), non-micellar aqueous solution of the conjugated bile salt through three columns connected in series, extracting the eluant with chloroform to remove unconjugated bile salts and any non-polar impurities generated by the procedure, and recovering the pure bile salt from the aqueous phase by freeze drying.

Columns and their preparation. The first column contained graphon, a graphitized, ultrapure form of carbon (Carbo-Pak B, Supelco, Bellefonte, PA), to extract surface active and pigmented impurities. Graphon is usually used for gas chromatography and has been used to study adsorption of surfactant to a relatively homogene-

ous, solid surface (27). The Carbo-Pak, 7.5 g per 10 g of bile salt, was prewashed successively with two column volumes of methanol, chloroform, methanol, acetone, and finally water. The second column was a hydrophobic cartridge containing octadecylsilane bonded to silica gel (Sep-Pak reverse-phase C18, Waters, Milford, MA) to remove less polar, surface-active contaminants. The contents of Sep-Paks, 2.5 g per 10 g bile salt, were prewashed with several column volumes of methanol and then water. The third and final column contained a cation-chelating resin (Chelex 100, sodium form, 200–400 mesh; Bio-Rad, Richmond, CA), to remove calcium and other divalent cations. Chelex, 2.0 g per 10 g of bile salt, was soaked in water overnight, then washed with water until the eluate was no longer alkaline.

Purification procedure. The three prewashed columns were connected in series, and a 5 mM (premicellar) aqueous bile salt solution was loaded on the first column and allowed to percolate through the three column beds, without adding further eluant. The first column volume of effluent was discarded and the remaining effluent was extracted thrice with chloroform, to remove unconjugated bile salts as well as other non-polar constituents that might have been extracted from the columns. The purified bile salt was recovered from the extracted eluate by lyophilization and stored in vacuo at -20°C. The overall yield of pure bile salt was 65 to 75%.

Reference method of bile salt purification: adsorption chromatography on a silicic acid column (23)

A 4.5 × 24 cm flash-chromatographic column was packed with 40 μm silica gel particles (#7024-01; J. T. Baker, Phillipsburg, NJ) in chloroform–methanol 85:15 (v/v). The sodium salt of a taurine-amidated bile salt, or the protonated acid of a glycine-amidated bile acid, adsorbed to twice its mass of silica gel and was then eluted using a linear gradient of chloroform–methanol from 85:15 to 70:30 (v/v). Flow-rate was 10 mL per min, with 50-mL fractions collected. Eluted fractions were examined by TLC (24) and those containing the desired bile acid or bile salt were pooled and evaporated to dryness under vacuum. After the glycine-amidates were converted to their sodium salts by titration to pH 8.0 with 0.2 N NaOH in methanol, the purified bile salts were evaporated to dryness under vacuum, yielding a white powder.

Assessment of purity

Surface tension measurements. The relationship of surface tension to bile salt concentration, [BS], was determined using two different methods at 24–25°C. The first was a dynamic method, using a commercial maximum bubble-pressure device (Sensadyne 6000, Chem-Dyne Research, Milwaukee, WI), equipped with two tubular glass probes of diameter 0.5 and 4.0 mm (28). The bubble frequency was 1 per sec and the instrument was calibrated with double-distilled water and methanol. Bile salts were dissolved in double-distilled water at concentrations from below 0.5 mM to over 100 mM; total sodium concentration was adjusted to 0.15 M with NaCl.

The second was a quasi-equilibrium method that used a Wilhelmy device, consisting of a platinum wire, 0.025 in diameter, suspended from a Cahn Model RTL Electrobalance (Cerritos, CA) (29, 30). Duplicate 0.5-mL samples of each bile salt solution were placed in Teflon wells that had been previously washed with HCl, water, and ethanol and then air-dried. By means of a motor-driven cam, the tip of the wire was slowly dipped into and withdrawn from the ovoid globule of bile salt solution in repeated 15-sec cycles. Before each series of three to four measurements on each duplicate sample, the wire was rinsed with acetone and double-distilled water, and then heated to incandescence in a Bunsen burner flame. Double-distilled water (surface tension 72.23 dyne/cm) was used to calibrate the system and to dissolve the bile salt. Bile salts were studied only at three concentrations, roughly 0.4–0.5, 1.0, and 2.0–5.0 times the published "CMC" values for that bile salt in water (3).

Estimation of apparent "CMC" values. Surface tensions (ST), determined with the maximum bubble pressure device, were plotted against the logarithm of [BS]. The "CMC" was defined as the intersection of the lines extrapolated from the apparently linear portions of the steep (premicellar) and shallow (micellar) portions of the curve straddling the "CMC" range. The slopes of these lines were calculated by least-squares analysis (31) in two ways: in the first, all the data points were used; in the second selected data points were used, with the deviant values at very low and very high [BS] being excluded from the analysis.

Chloroform extraction of conjugated bile acids. An aqueous solution of conjugated bile salt (10 or 50 mM) in sodium phosphate buffer, pH 7.0 (ionic strength = 0.15) was extracted with 4 vol of chloroform by shaking for 10 min at 25°C. Bile acid concentrations were determined enzymatically on the aqueous phase and on the concentrated solution formed by redissolving the dried chloroform extract in a minute volume of the buffer.

Solubilization of biliary lipids. The ability of bile salts to solubilize phosphatidylcholine (PC) or PC/cholesterol mixtures to an isotropic solution was tested by the co-precipitation method (32). For studies of PC solubilization, 1 vol of a methanolic solution of bile acid was added to 4 vol of a chloroform solution of the PC and the mixed solution was incubated for 2 h at 50°C. After

evaporation of the solvent under a stream of nitrogen, the residue was reconstituted with 1.0 mL aqueous Tris buffer, pH 8.0, to give a final bile salt concentration of 50 mM and PC concentrations of 2–10 mM.

For solutions containing cholesterol as well as PC and bile salt, cholesterol in methanol was added to the dried PC and the mixture (15 mM PC + 3 mM cholesterol) was warmed for 1 h at 50°C. The appropriate volume of this PC/cholesterol solution was mixed with 1.0 mL of bile salt (50 mM C-Tau or C-Gly, 20 mM UDC-Tau and 10 mM DC-Tau or DC-Gly) in methanol to yield final molar ratios of bile salt:PC:cholesterol of 50:15:3. After dilution to 2.0 ml with methanol, the solution was sonicated for 1 min at 50°C at 60 Hz and 1 mA (Model B224, Branson, Shelton, CT), and the methanol was then slowly evaporated under a stream of nitrogen at 50°C. The residue was reconstituted to 1.0 mL with aqueous sodium phosphate buffer, pH 7.4, yielding final [BS] as above.

In each study, the buffered solutions were sealed under nitrogen, and shaken at 60 cps at 55°C. After 24 h and 7 days later, the turbidity of the resultant mixtures was assessed visually and by absorbance at 600 nm.

Bile salt concentrations

Bile salt concentrations in each stock solution, and the two phases of the chloroform extraction studies, were measured fluorometrically by the 3α-hydroxysteroid dehydrogenase assay (26); for DHC-Tau, which has a 3-keto group, the reaction was run in the reverse direction (33).

Calcium concentrations

Calcium concentrations of 100 mM bile salt solutions in purified water were determined with a commercial device based on competitive binding of a chelating dye (Calcette, Model 4008, Precision Systems, Sudbury, MA) (9).

Statistics (31)

Linear regression was utilized to determine slopes and intercepts of linear segments of the plots of ST versus log [BS]. Comparisons of surface tensions, measured with the Wilhelmy balance, among samples purified by the two methods and the corresponding commercial bile salt, were assessed by Student's *t*-test with Yates' correction for small sample size.

RESULTS

Colored contaminants

The lots of commercial taurine-amidated bile salts were visibly yellow and showed several overlapping peaks of spectral absorbance between 390 and 490 nm, with the largest at 470 nm. By contrast, bile salts purified by either method were colorless and had no spectral absorbance between 390 and 490 nm. During lyophilization of aqueous solutions of some commercial samples, fractional crystallization occurred, yielding zones that were enriched in yellow pigments, one of which was intensely blue-fluorescent. By TLC, a single yellow, fluorescent contaminant was observed that was soluble in acid, water, and ethanol and insoluble in chloroform, acetone, and ether, and formed a dense translucent, glassy solid on freeze-drying. The commercial glycine-amidated bile salts were not visibly yellow.

Surface tension

Measurements by the maximum bubble pressure method (**Fig. 1**). The surface tension versus log [BS] plots for all bile salts, except DHC-Tau, were triphasic, with a steep linear decline from about 0.1 to 0.5 times the "CMC", a curvilinear portion of decreasing negative slope straddling the "CMC", and a second linear decline with a small negative slope beginning at concentrations about 2–3 times the "CMC". In most cases, data points at very low bile salt concentrations (below 0.1–0.2 times the "CMC") deviated from the linear component in the premicellar range, and points more than 10 times the "CMC" usually deviated from the linear component in the micellar range. For C-Gly and CDC-Gly, the commercial samples were more surface-active than samples purified by the new method, showing lower surface tensions both below and above the "CMC" (Fig. 1, upper panels). For C-Tau, the commercial sample had lower surface tensions only above the "CMC" (Fig. 1, lower left). The curves obtained on commercial samples of CDC-Tau, DC-Tau, DC-Gly, and UDC-Tau were virtually identical to those obtained after purification by the new method (shown for CDC-Tau only, Fig. 1, lower right). The differences between commercial and purified samples were, however, small and not statistically significant.

The ST versus log [BS] curves for C-Tau, CDC-Tau, or UDC-Tau were the same for samples purified by either method. Curves for C-Tau and DC-Tau purified with the Chelex column alone were identical to those obtained with samples purified by the new three-column method. These comparisons were not performed for the other bile salts.

"CMCs" derived from data obtained with the bubble pressure method for the various bile salt samples (**Table 1**) were, except for UDC-Tau, lower than published "CMC" values (3) obtained with the same method. Purification by the new method apparently increased the "CMC" for C-Gly and possibly also for C-Tau and CDC-Tau, compared with the commercial samples. The silicic

acid column increased the "CMC" of C-Tau more than did the new method, but the two purification methods yielded closely similar "CMCs" for CDC-Tau and UDC-Tau.

Measurements by the quasi-equilibrium Wilhelmy method (**Fig. 2** and **Fig. 3**). Measurements of surface tension in water using the Wilhelmy wire procedure were generally higher than values obtained using the maximum bubble pressure method, but the differences were due to the absence of added Na^+ ion in the solutions used for the Wilhelmy procedure (3, 8). Unlike the maximum bubble pressure method, the Wilhelmy method often revealed highly significant differences in surface tensions between purified and commercial preparations; these differences were greatest below and near the "CMC". In this range, the samples of seven of the conjugated bile salts (C-Tau, C-Gly, CDC-Tau, CDC-Gly, DC-Tau, UDC-Gly, and DHC-Tau), purified by the new method, showed 10–24% higher surface tensions than the corresponding commercial samples ($P < 0.0005$ for each). At bile salt concentrations well above the "CMC", by contrast, the surface tension of each of these purified bile salts was never more than 7% greater than the surface tension of the commercial bile salt, but the differences were still highly significant ($P < 0.005$ for each). A smaller, but still significant difference was observed with UDC-Tau at all three concentrations ($P < 0.001$). Except for conjugates of UDC, the increase in surface tension with purification was greater for the taurine-amidates than the corresponding glycine amidates.

There were no differences between surface tensions obtained with the two purification methods for C-Tau, DC-Gly, UDC-Tau, UDC-Gly, or 12-Oxo-CDC-Tau, but surface tensions were at least 5% higher for C-Gly,

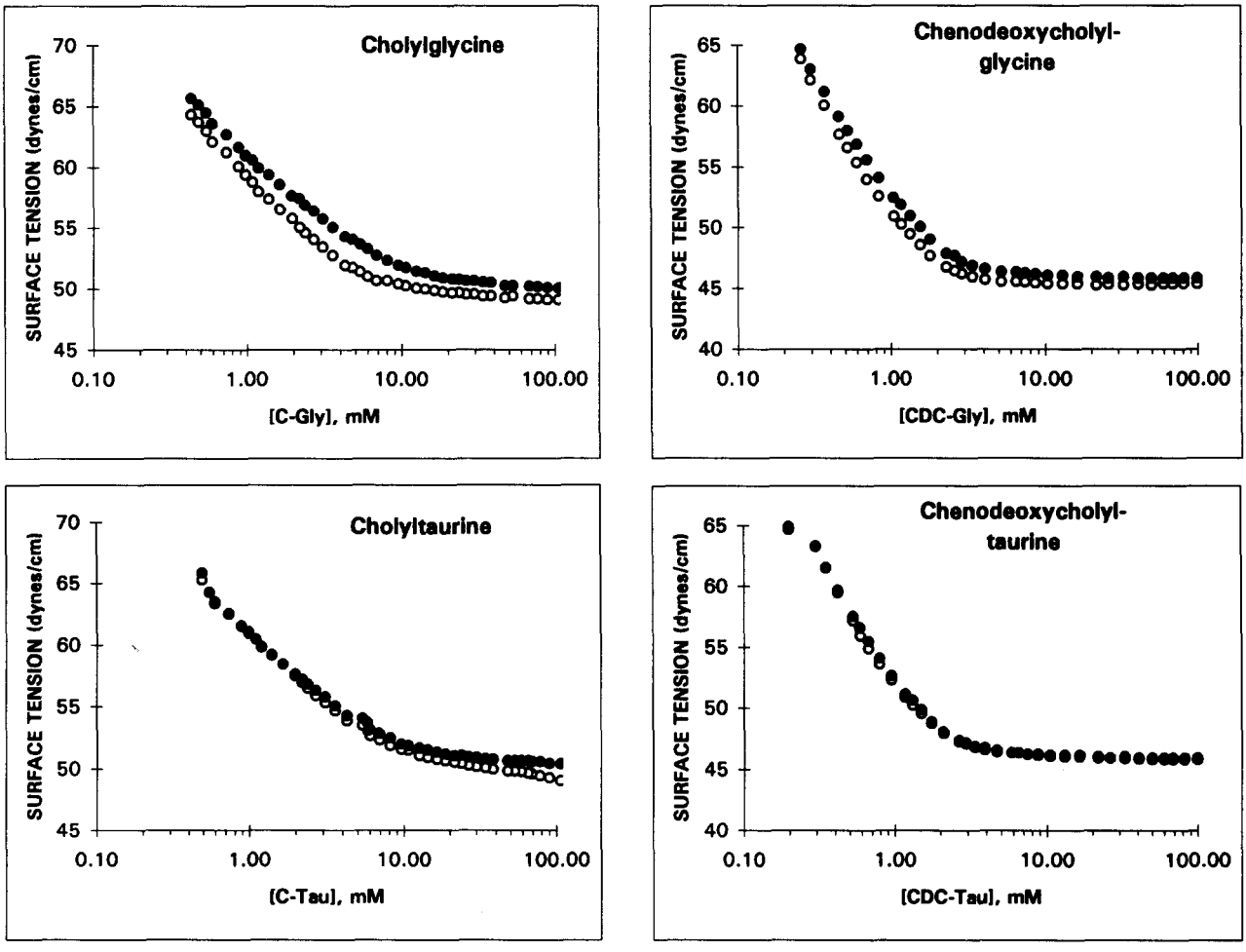

Fig. 1. Effects of aqueous bile salt concentration on surface tension, determined by the maximum bubble pressure method, for commercial preparations (○) of four major conjugated bile salts of human bile compared with the same batches after purification by the new 3-column method (●); temperature 24–25°C, total sodium adjusted to 0.15 M with NaCl. Each point represents the average of 120 measurements, obtained over 2 min that varied by no more than ± 0.2 dynes/cm (± 0.4%), as reported previously (52). The logarithmic scale on the abscissa was necessary to clearly depict bile salt concentrations that spanned three orders of magnitude. Note the triphasic curves, with two pseudolinear arms flanking a curved central segment around the apparent "CMC". Patterns with other conjugated bile salts, not shown, were like that for CDC-Tau (lower right), showing virtually no difference between the commercial and purified samples.

TABLE 1. Apparent "CMC" values of different bile salt preparations in aqueous solution (0.15 M total sodium) using the maximum bubble pressure method for surface tension

Bile Salt	"CMC" Values			
	Current Work			Published (3)
	Comm.	New	ACSA	ACSA
	mmol/L			
C-Tau	6.5	7.3	8.8	6.0
C-Gly	5.3	7.3	nd	11.0
CDC-Tau	1.5	2.0	1.7	2.8
CDC-Gly	1.7	1.7	nd	1.8
DC-Tau	1.7	1.7	nd	2.4
DC-Gly	2.0	2.0	nd	2.0
UDC-Tau	3.8	3.9	3.8	2.2

"CMC" was defined as the intersection of the apparently linear segments of the plot of surface tension versus log bile salt concentration below and above the "CMC" range (see Fig. 1); nd, not determined. Abbreviations of types of preparation: Comm., unpurified commercial bile salt (Calbiochem™); New, purified by the new three-column method; ACSA, adsorption chromatography on silicic acid.

CDC-Tau, CDC-Gly, and DC-Tau prepared by the new method as compared with the silicic acid method ($P < 0.0001$ for each). DHC-Tau purified by silicic acid was not available for comparison.

Bile salt contents

The 3α-hydroxysteroid dehydrogenase assay (26) revealed no significant differences between gravimetrically and enzymatically measured bile salt content in either the purified or commercial preparations. Thus, the increase in surface tension and "CMC" seen with purification of most of the bile salts was not due to a misleadingly low bile salt content in the commercial preparations.

Extractability into chloroform

When aqueous micellar solutions of the commercial samples were extracted with chloroform, an emulsion was formed, whereas this did not occur after purification by either method. Over 0.1% (by weight) of each

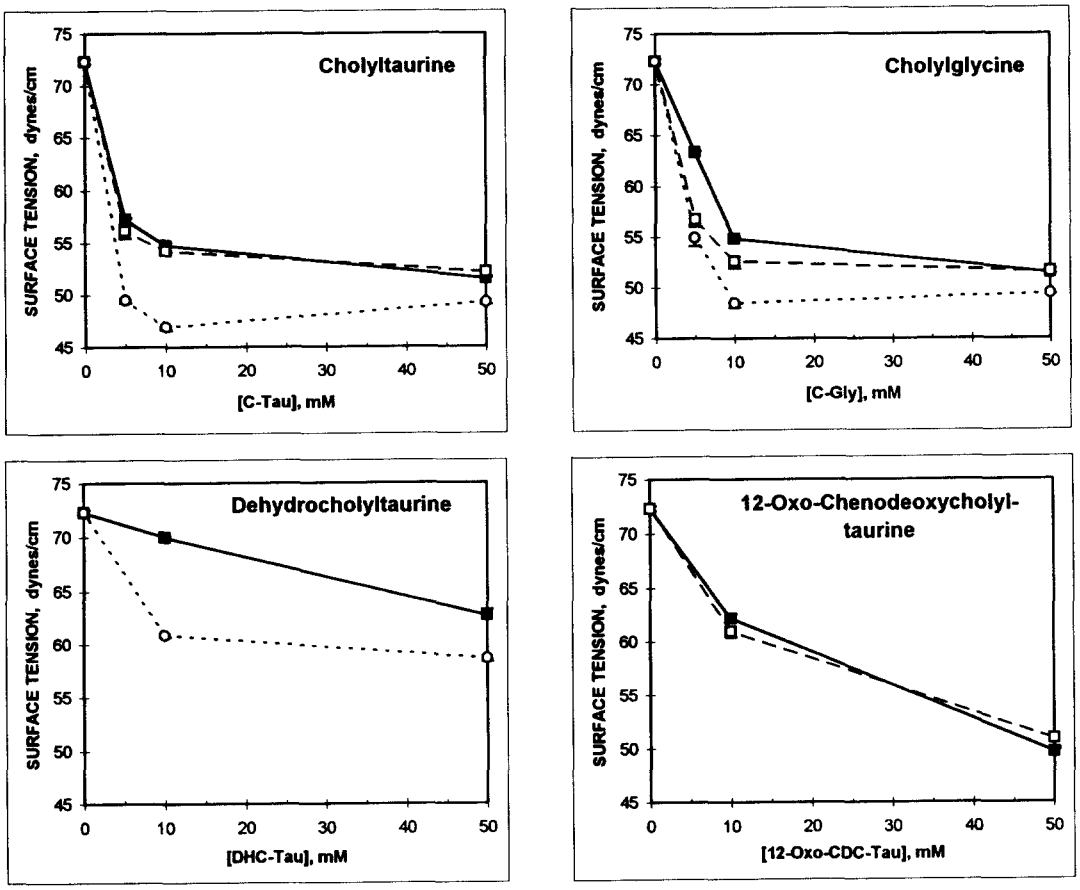

Fig. 2. Surface tensions (mean ± SD), determined by Wilhelmy wire method, for various samples of two conjugated trihydroxy bile salts, one per-oxo, and one hydroxy-oxo bile salt. Commercial samples from Calbiochem™(○) are compared with corresponding bile salts after purification by the new 3-column method (■) and the reference silicic acid column method (□). Each point is the average of 4–8 determinations, performed at 23–25°C with the bile salts dissolved in double-distilled water at approximately 0.5 ×, 1.0 ×, and 2–5 × the expected "CMC". Many of the error bars were too small in range to be shown.

commercial bile salt was extracted into the chloroform phase, whereas, after purification, less than 0.01% of the bile salt was extracted.

Solubilization of biliary lipids

When coprecipitated mixtures of conjugated bile salts with phosphatidylcholine (PC) were reconstituted in Tris buffer, those prepared with commercial bile salts were optically clear at a ratio of 10 mmol of PC to 50 mmol of bile salt. By contrast, comparable samples prepared with bile salts purified by the new method were turbid; clear solutions were obtained only with PC/bile salt ratios of 5/50 or lower. In the studies of solubilization of PC plus cholesterol, turbidity was observed when using C-Tau, C-Gly, and DC-Gly purified by the new method, but not with the corresponding samples obtained commercially or purified by the silicic acid

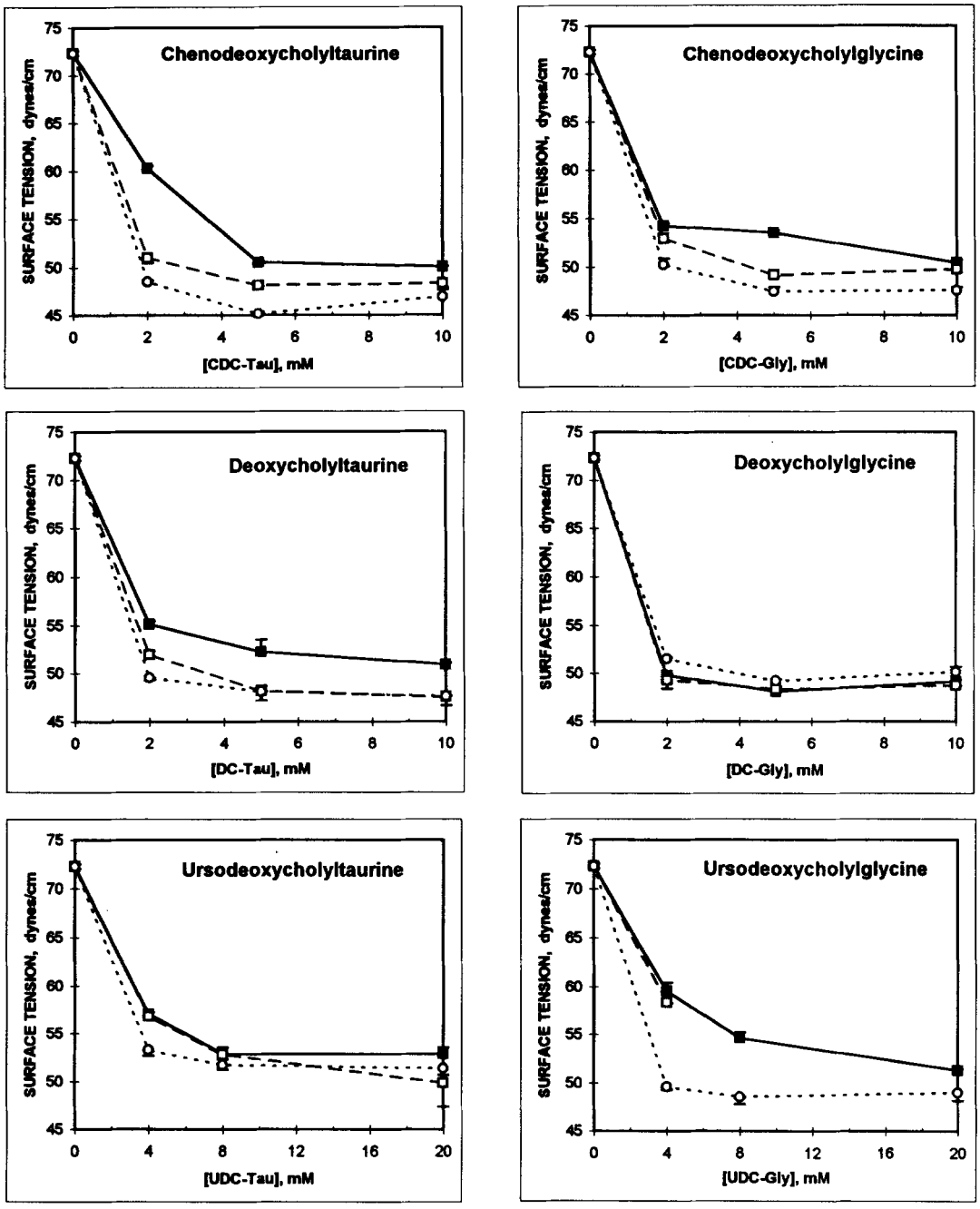

Fig. 3. Surface tensions, determined by Wilhelmy balance assay, comparing the taurine- and glycine-conjugates of three dihydroxy-bile acids. Conditions, plots, symbols, and footnotes as in Fig. 2. Surface tensions of UDC-Gly purified by the silicic acid column method could not be measured at 8 and 20 mM, due to incomplete dissolution of the sodium salt.

column. By contrast, all DC-Tau-based systems yielded clear solutions while all the UDC-Tau-based systems were turbid. Visual turbidity was confirmed by absorbance ($O.D._{600\ nm}$) ranging from 0.03 to 0.08, whereas all systems with $O.D._{600\ nm}$ below 0.02 appeared optically clear. Systems that were turbid at 24 h did not clear with more prolonged incubation; rather, they became more turbid and somewhat yellow in color over the succeeding week. All solutions that were clear at 24 h remained clear 1 week later.

Calcium contents of commercial bile salts ranged from 3 to 16 mmol per mol bile salt, and were even higher (up to 30 mmol per mol) in bile salts purified by silicic acid chromatography. By contrast, no calcium was detectable in bile salts purified by the new method.

DISCUSSION

Pure bile acids do not exist by themselves in native bile. However, to determine the properties of bile acids per se or the roles of individual bile components in model bile systems, it is necessary to use pure compounds. One can then compare model systems with native bile, and then determine which other components account for the observed differences. For proper interpretation of such studies, unknown contaminants must be removed as much as possible, whether they are derived from the source bile used to isolate the bile acids and lipids or are added during the synthesis and subsequent processing of the components. As shown in our work, these unknown contaminants in bile salt preparations may have potent physicochemical effects and may unpredictably affect the interactions of bile salts with phosphatidylcholine and cholesterol, and thus the properties of model bile.

Methodologic considerations

Hartley, in his monograph on micellar solutions, noted that "a substance, which is itself a powerful cleaning agent, is, ipso facto, difficult to clean" (34). Nonetheless, the new method described here appears to remove colored and surface-active organic impurities, as well as calcium, from dilute solutions of the sodium salts of the major glycine- and taurine-conjugated bile salts found in human bile, as well as from two conjugated keto-bile salts. The method is simple and rapid. Although the method reported here was applied to bile acids from only one commercial source, we believe it should be applicable to preparations from other firms.

The new method appears to remove organic impurities from conjugated bile salt solutions, but avoids contaminating these solutions with organic impurities present in the adsorbent columns that were used (graphitized carbon, and octadecylsilane bonded to silica). This success is attributed to the thorough prewashing of the already very pure adsorbents with various solvents and percolating the bile salts through the columns at submicellar concentrations, at which the bile salts have relatively less avidity for surface-active and amphipathic impurities as compared to concentrations above the "CMC" (35). At submicellar concentrations, therefore, the bile salts do not compete effectively for hydrophobic impurities adsorbing to the column. In addition, the initial 10% of effluent bile salt was discarded to eliminate most of the impurities that would bind to the eluted bile salt. Finally, the third, chelating column removes any calcium (and presumably other polyvalent cations) that might be present in the first two adsorbents. Any surface-active impurities, as well as contaminating unconjugated bile acids, remaining in the eluate from the columns, are then removed by chloroform extraction.

Graphon, with its graphitized surface, was expected to have a stronger affinity for pigments than for bile salts. This was the case, as the major portion of the bile salts emerged from the charcoal column, whereas the pigmented impurities were retained.

The octadecylsilane-silica, in a cartridge, is known to adsorb bile salts from aqueous solutions (36). Thus, any residual impurities removed by this column had to have greater affinity for the hydrophobic adsorbent than did the hydrophobic face of the bile salt molecule. Octadecylsilane has been used previously by others to decrease the amount of surface-active impurities in sodium dodecyl sulfate (SDS) (14, 37). Virtually complete removal of surface active impurities can be achieved if the octadecylsilane step is followed by prolonged foam fractionation, which leaches surface-active impurities into the foam (37). The foam-fractionation technique, however, requires many hours to be successful and has yields below 10%. By contrast, the new method reported here is faster, requires no special equipment, and yields are 65 to 75%.

Purification of conjugated bile salts/acids by silicic acid chromatography in the reference method is based on a different principle. The column is initially washed with less polar mixtures of methanol and chloroform, eluting the less polar impurities present in the bile salt/acid. When the conjugated bile salt/acid is later eluted, the more polar impurities remain on the column. Unfortunately, some of the calcium in the silica elutes with the bile salts, which have affinity for divalent cations (9–11). In the new method, any calcium present in the commercial sample or eluted from the charcoal and octadecylsilane columns is removed by the chelating column at the end of the three-column train. When using the reference method, therefore, it is suggested

that a Chelex step should be added after silicic acid chromatography and sodium salt formation.

It was not feasible to extract the unconjugated bile salts from the commercial bile salt conjugates before purification because troublesome emulsions occurred. Therefore, chloroform extraction was performed on the final eluate from the three columns, which had a slightly acidic pH and contained bile salts at concentrations below, or close to, their "CMC" in water. Though an even more acidic pH is optimal for eliminating unconjugated bile acids, which are in part ionized at pH 6.0 (15), the submicellar [BS] prevents micellar solubilization of unconjugated bile acids and salts (38).

Bile salt purity

The new method will not eliminate contamination of a given conjugated bile salt by closely related conjugated bile salts. Obviously, to synthesize a pure conjugated bile salt, one must begin with an unconjugated bile acid that is free of contamination by other unconjugated bile acids. Manufacturers usually certify the purity of a commercial bile salt by TLC or HPLC, showing, as we did, that each bile salt preparation contains < 1–2% of other bile salts; this system, however, is not designed to detect the presence of the other contaminants that we detected.

Impurities are generated by the chemical methods used to prepare bile salt conjugates (16, 17). During conjugation with glycine, glycyl-glycine conjugates (39) and bile acid dimers may be formed in which the carboxyl group of the bile acid is esterified to a hydroxyl group of a second bile acid (C. D. Schteingart and H-T. Ton-Nu, unpublished observations). The amine used in preparing conjugates by the mixed anhydride method may oxidize to form colored contaminants, which are presumably azo-amines (16, 17). Both purification methods removed these impurities, but may not remove taurine-conjugated bile salts with chromatographic mobilities similar to the desired conjugated bile salt. If overlapping fractions containing detectable proportions of these other conjugated bile salts are discarded, yields of the desired conjugate may be quite low.

We found that the commercially available bile salts used in these studies often contained varying amounts of calcium, as well as pigmented and surface-active contaminants that rendered them less than ideal for physicochemical measurements. The new purification procedure described here removed these contaminants as follows. *1)* Commercial samples of taurine-conjugated bile salts contained yellow, yellow-fluorescent, polar, basic contaminants, whereas purified samples were colorless. *2)* Most of the commercial samples contained surface-active impurities, as revealed by low surface tension values obtained with the Wilhelmy technique, as well as by the formation of emulsions when shaken with chloroform, whereas the purified samples had higher surface tensions and did not form emulsions with chloroform. *3)* Purified samples solubilized biliary lipids into larger vesicles than commercial samples, as revealed by the turbidity obtained when the purified samples were shaken with PC ± cholesterol. *4)* Compared with the purified samples, commercial samples contained an impurity that promoted extraction of the conjugated bile salt into chloroform, presumably an amine that formed a chloroform-soluble ion pair with the bile salt anion (40). *5)* Commercial samples contained up to 16 mmol calcium per mol bile salt, whereas there was no detectable calcium in the samples purified by the new method.

Surface-active impurities

For most bile salts, removal of impurities increased surface tension measurements much more with the Wilhelmy balance than the maximum bubble pressure method, which usually gave similar results for the commercial and purified samples. This probably reflects the non-equilibrium nature of the bubble-pressure method, which was in fact designed to minimize the influence of traces of surface-active impurities (28, 41). When performed at rapid bubble intervals (e.g., 1/sec, as used in this study), this technique measures only the predominant surface-active agent (28). As the bubble interval is slowed, there is increasing time for the impurities to accumulate at the air/water interface, and the surface tension progressively decreases (37, 41).

In view of the insensitivity of the bubble-pressure method in detecting the removal of surface-active impurities by the three-column purification method, we did not perform a direct comparison of samples purified by the three-column method with those purified by the silicic-acid method for all bile salts. The "CMCs" of silicic acid-purified bile salts, determined by the bubble pressure method, have been published (ref. 3 and Table 1).

The 20% lower surface tensions of the commercial samples measured with the Wilhelmy technique indicate that these samples contained surface-active impurities. The effects of purification on surface tension were generally more marked for the conjugates of trihydroxy- than of dihydroxy-bile salts. We suspect that this reflects the much greater surface activity of the more hydrophobic dihydroxy-bile salts, diminishing the relative influence of the surface-active impurities. Alternatively, the commercial dihydroxy-bile salts may have contained less surface-active impurities.

All the above effects may be explained by enhancement of self-association and hetero-association (e.g.,

lipid solubilization) of bile salts in the presence of the surface-active impurities. Our studies did not directly determine whether the small amounts of contaminating calcium contributed also. The similar behavior of many bile salts purified by the new method, which removed calcium, with their counterparts purified by the silicic acid column, which apparently added calcium, suggests that these low proportions of calcium have little influence on bile salt self-aggregation, especially in the presence of 0.15 M Na^+, in agreement with several reports (42, 43).

Bile salts undergo hydrophobic self-association in aqueous solution (1–3, 7), and it has become conventional to describe the self-association of bile salts using the model of classical ionic detergents (e.g., SDS) that show extensive cooperativity in self-association and form large micelles at or above a sharply defined critical micellization concentration ("CMC") (1, 2). Unlike classical detergents, however, bile salts progressively self-associate into much smaller aggregates (1). Bile salts often exhibit a range of concentrations over which effects of self-association become important, as assessed, e.g., by self-diffusion (44), surface tension (1, 3), or solubilization of hydrophobic molecules (1, 3, 45, 46). Poorly defined apparent "CMC" values are often determined by extrapolation of the pseudolinear pre- and postmicellar segments of various plots reflecting the surface activity of, or solubilization by, varied concentrations of bile salts. As noted elsewhere (45), this approach yields a spectrum of "CMC" values, depending on which sets of data points are selected (2), that may account in part for the differences in the present and published values in Table 1. An additional difficulty arises from the fact that the maximum bubble pressure method may yield surface tension values that are higher than equilibrium values, and these differences may depend upon bile salt concentration. The main point, however, is that when the same procedure was used to determine the "CMC", many of the samples of purified bile salts yielded higher apparent "CMC" values than their commercial counterparts, in keeping with the removal of the surface-active impurities.

Effects of purification on solubilization of biliary lipids

Despite their minimal contribution to the weight of the commercial bile salt samples, removal of the surface-active impurities had major effects on the ability of the bile salt preparations to solubilize biliary lipids. Solubilization of either PC or PC plus cholesterol, using the customary coprecipitation method, often yielded turbid solutions with systems containing bile salts purified by the new method, in contrast to clear solutions obtained using commercial or silicic acid-purified bile salts. The turbidity persisted despite sonication and prolonged shaking and warming, and probably resulted from the formation of large, multilamellar vesicles.

The surface tension assays suggested that no surface-active impurities were generated by the three-column purification method. It appears that the formation of optically clear model biles by the coprecipitation method (32) results from surface-active contaminants present in less pure bile salt preparations. Presumably, such impurities in the commercial bile salt samples caused large multilamellar vesicles, present in the cloudy systems obtained with our purer bile salts, to convert to an optically clear mixed micellar phase (47). Studies are needed to determine which additional bile components are necessary to transform the lamellar phase into a mixed micellar phase. Free fatty acids and bile pigments are possible candidates, as these amphipathic molecules are present in gallbladder bile in low millimolar concentrations (48–51).

In summary, we have described a rapid simple method for purification of commercial samples of conjugated bile salts. We did not determine whether this method is as thorough in removal of surface-active impurities as the "gold standard" foam fractionation method (1, 28, 46), but yields were in the range of 65 to 75% as compared with yields of less than 5% for the laborious foam fractionation procedure. With several assays, we have demonstrated changes in the self-aggregation of the bile salts and their ability to solubilize biliary lipids as a result of removal of residual surface-active impurities. These findings suggest that prior physicochemical studies with less purified bile salts may require reinterpretation quantitatively if not qualitatively. The method provides a highly satisfactory compromise between the need for high purity and the need for simplicity and high yield in the purification of conjugated bile salts suitable for use in physicochemical studies.

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