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Quantitation of cholesterol-carrying particles in human gallbladder bile

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Abstract The inter-mixed micellar/vesicular (non-phospholipid-associated) bile salt concentration (IMC) can be rapidly measured in model biles by centrifugal ultrafiltration, thus allowing reliable separation of vesicular and micellar cholesterol carriers by gel filtration with an elution buffer containing bile salts at the correct IMC (Donovan, J. M., and A. A. Jackson. 1993. *J. Lipid Res.* **34:** 1121–1129). We adapted this method to the more complex human gallbladder bile and examined the relationship between cholesterol solubilization and crystallization in gallbladder biles from 10 cholesterol gallstone patients. The IMC (mean ± SEM) was 9.67 ± 1.97 (range 3.56 – 35.02) mm with significant enrichment with hydrophilic bile salt species. Upon gel filtration of these biles with an eluant buffer containing 10 major bile salts at concentrations according to their IMC, cholesterol was found to be solubilized mainly in mixed micelles. Vesicles were detected in all 10 biles after separation by KBr density gradient ultracentrifugation but in only 5 of these biles with the IMC method. Biles without vesicles had a lower CSI (1.15 ± 0.12 vs. 1.90 ± 0.28, $P < 0.05$), a higher total lipid concentration (11.9 ± 2.3 vs. 5.9 ± 1.1, $P < 0.05$), and a higher bile salt/(bile salt + phospholipid) ratio (0.83 ± 0.01 vs. 0.74 ± 0.04, $P = 0.07$). For both IMC and ultracentrifugation methods, vesicular cholesterol concentration showed a negative correlation with crystal observation time and a positive correlation with cumulative crystal score during 21 days. Our data indicate that methods such as density gradient ultracentrifugation overestimate vesicular cholesterol solubilization in human biles. —**Eckhardt, E. R. M., B. J. M. van de Heijning, K. J. van Erpecum, W. Renooij, and G. P. VanBerge-Henegouwen.** Quantitation of cholesterol-carrying particles in human gallbladder bile. *J. Lipid Res.* 1998. **39:** 594–603.

Supplementary key words bile • bile salts • cholesterol • crystallization • gallstones • IMC • micelles • vesicles

Crystallization of cholesterol from supersaturated bile is a prerequisite for gallstone formation (1, 2). The sterol molecule is poorly soluble in an aqueous environment, and is solubilized in bile in mixed micelles together with bile salts and phospholipids (mainly phosphatidylcholine). In the case of cholesterol supersaturation, the sterol may also be solubilized in vesicles together with phospholipids (3–8). Although, in addition to mixed micelles and vesicles, lamellar structures have recently been described in model and human biles (9, 10), their existence remains controversial (11). By using video-enhanced microscopy and transmission electron microscopy, several authors have shown that cholesterol monohydrate crystals may originate from vesicles after their aggregation and fusion, both in model and human biles (12–14).

Until now, studies on lipid compositions and relative contributions to cholesterol solubilization by vesicular and micellar carriers have been hampered by lack of a reliable method for their separation from bile. In model systems, phospholipid distribution between vesicles and micelles can be approached by non-invasive techniques such as nuclear magnetic resonance (NMR) spectroscopy, but this is not feasible for cholesterol distribution among carriers (15).[2] Two methods have been used for separation of various cholesterol carriers in bile: gel filtration, generally with a constant concentration (often 7.5 or 10 mm) of one bile salt species (usually taurocholate or cholate) in the elution buffer, and density gradient ultracentrifugation. A major disadvantage of both methods is dilution of the bile sample during the procedure with a solution that may have physical–chemical properties different from the origi-

Abbreviations: CSI, cholesterol saturation index; DLS, dynamic light scattering; EYPC, egg yolk phosphatidylcholine; GC, glycocholate; GCDC, glycochenodeoxycholate; GDC, glycodeoxycholate; GLC, glycolithocholate; GUDC, glycoursodeoxycholate; HPLC, high-performance liquid chromatography; IMC, inter-mixed micellar/vesicular bile salt concentration; TC, taurocholate; TCDC, taurochenodeoxycholate; TDC, taurodeoxycholate; TLC, taurolithocholate; TLCo, total lipid concentration; TUDC, tauroursodeoxycholate.

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[2] The abstract of reference 15 can be found on the Internet at URL: http://www.ncbi.nlm.nih.gov/htbin-post/Entrez/query?uid=7496888&form=6&db=m&Dopt=b

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nal aqueous environment, with potential change of composition of the cholesterol carriers (16).

Apart from mixed micelles, bile contains non-phospholipid-associated bile salts, either as monomers or, above their critical micellar concentration, associated in small simple micelles. The monomeric plus simple micellar bile salt concentration is referred to as "intermixed micellar/vesicular (non-phospholipid-associated) bile salt concentration," usually abbreviated as IMC (17). The IMC can be measured by equilibrium dialysis with frequent exchange of the dialysant (17), but this method is cumbersome and time-consuming. Rapid (<2 h) centrifugal ultrafiltration (18) seems to be more suitable, in particular for non-equilibrium systems such as cholesterol-supersaturated biles obtained from cholesterol gallstone patients. Although IMC measurement by rapid centrifugal ultrafiltration has been validated in model systems, this technique has not been applied for separation of vesicular and micellar cholesterol carriers in human gallbladder biles. The procedure is more complicated in human biles, because of the unknown bile salt compositions and concentrations. In the present study, we adapted the rapid centrifugal ultrafiltration method to human gallbladder biles and we separated vesicular and micellar cholesterol carriers by gel filtration, using the appropriate bile salt composition and correct IMC in the eluant buffer. Results obtained by the IMC method have been compared with data from KBr density gradient ultracentrifugation of the same bile sample. We have also related these data to rate and extent of cholesterol crystallization.

MATERIALS AND METHODS

Materials

Gallbladder biles were obtained at elective cholecystectomy from 17 gallstone patients (3 male/14 female, mean age 47.5 years). There were no cases of acute cholecystitis or bile duct obstruction. All patients had multiple cholesterol stones (defined as a cholesterol content > 70% of dry weight) and well concentrated biles.

All chemicals were of the highest purity available. Ultrafilters with a molecular weight cut-off of 10 kDa were purchased from Sartorius (Göttingen, Germany: Centrisart I) and from Spectrum (Houston, TX, Centri/Por). Microfilters with a pore size of 5 μm and 0.22 μm were from Millipore Corporation (Molsheim, France). Sephacryl S400 and S1000 column materials were from Pharmacia LKB Biotechnology AB (Uppsala, Sweden), and polystyrene beads with a mean diameter of 0.46 μm were from Sigma Chemical Co. (St. Louis, MO). The enzymatic cholesterol assay kit was obtained from Boehringer (Mannheim, Germany), and the enzymatic phospholipid kit was from Sopar Biochem (Brussels, Belgium). 3α-Hydroxysteroid dehydrogenase for the enzymatic measurement of bile salt concentration (19) and a colorimetric chloride-kit were purchased from Sigma. The bile salts tauroursodeoxycholate (TUDC), glycocholate (GC), taurochenodeoxycholate (TCDC), glycochenodeoxycholate (GCDC), glycodeoxycholate (GDC), taurolithocholate (TLC) and glycolithocholate (GLC) were purchased from Sigma, taurocholate (TC) and taurodeoxycholate (TDC) from Fluka Chemicals (Buchs, Switzerland). Glycoursodeoxycholate (GUDC) was a generous gift from Tramedico (Weesp, Netherlands). All bile salts were of the highest purity available and yielded single peaks with HPLC. Egg-yolk phosphatidylcholine (EYPC) and cholesterol were obtained from Sigma.

The HPLC C18 column was from Supelco (Supelcosil LC-18-DB, Supelco, Bellefonte, PA). C18 cartridges (Sep-pak®) for solid-phase bile salt extraction were obtained from Waters (Milford, MA).

Model bile preparation

Model biles were prepared according to Kibe et al. (20). Sodium taurocholate in methanol was mixed with EYPC and cholesterol in chloroform. The mixture was dried at 45°C under a mild stream of nitrogen and subsequently during 24 h under reduced pressure. The lipid film was reconstituted in 10 mm Tris/150 mm NaCl/3 mm NaN_3, incubated for 16 h at 56°C, and subsequently stored at 37°C in the dark under nitrogen.

Rapid centrifugal ultrafiltration of bile

A 10 kDa Centrisart ultrafilter was rinsed with H_2O and centrifuged for 5 min at 500 *g* in order to remove glycerol remnants from the membrane. All water was removed carefully from both sides of the membrane with a syringe. The filter was preincubated at 37°C during 30 min before usage. Immediately after cholecystectomy, 2.3 mL of bile was put (in duplicate) into the filter and centrifuged at 500 *g* for 5 min in a pre-warmed (37°C) centrifuge. The filtrate was carefully collected with a syringe. Filtration was repeated four times, adjusting centrifugal speed so as to obtain constant filtrate volumes of approximately 50 μL. Bile salt and chloride concentrations reached stable values in the third filtrate. The lower concentrations in the first and second filtrates resulted from small amounts of water remaining in the membrane after rinsing the ultrafilter (18). We considered the third filtrate to represent the simple micellar + monometric fraction, and therefore

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decided to take this filtrate to measure IMC and bile salt composition. Besides bile salts, no other lipids were detectable in the filtrates (detection limit of the cholesterol assay: 0.125 mm, of the phospholipid assay: 0.048 mm), indicating that no mixed micelles or vesicles had passed through the filter. During ultrafiltration, Gibbs-Donnan effects occurred as a result of uneven distribution across the membrane of non-filterable particles with a highly negative charge (in particular mixed micelles), thus leading to an overestimation of the concentrations of negatively charged monomeric and simple micellar bile salts in the filtrate (17, 18). We corrected the concentrations of bile salts measured in the filtrate (see below) for Gibbs-Donnan effects by multiplying the bile salt concentration in the filtrate by the ratio of chloride concentrations in bile and filtrate (17, 18).

Determination of bile salt species and concentrations in the inter-mixed micellar/vesicular fraction and in corresponding whole bile

After rapid centrifugal ultrafiltration, intermicellar bile salt species composition and the concentration of individual bile salt species were determined in the ultrafiltrate by isocratic HPLC analysis, with phosphate-buffered 70% methanol, pH 5.25, as the eluant (21, 22). An external standard containing known quantities of each of ten major conjugated bile salts was run prior to each analysis.

For HPLC analysis of bile salts in corresponding whole biles, the bile salts were first purified by solid-phase extraction: a C18 Sep-Pak® cartridge was rinsed with 10 mL methanol and 10 mL water and subsequently, 100 μL of bile, dissolved in 2 mL of phosphate-buffered saline, was applied. Thereafter, the cartridge was rinsed with 10 mL water, 3 mL 10% acetone, and 10 mL water. Finally, bile salts were eluted with 3 mL methanol. After evaporation of methanol under a mild stream of nitrogen at 45°C, the residue was dissolved in 1 mL of HPLC buffer and the bile salt species were analyzed. The total bile salt concentrations in whole biles were also determined using the 3 α-hydroxysteroid dehydrogenase method (19). The cumulative bile salt hydrophobicity index was calculated according to Heuman (23).

Separation of vesicular and micellar cholesterol carriers

An LKB-Pharmacia column (gel bed 75 cm, internal diameter 1.6 cm), equipped with a thermostated water jacket, was packed with Sephacryl S1000. Void volume of the column (V_o, 55 mL) was determined with 460 nm polystyrene beads in 10 mm Tris/150 mm NaCl/5 mm sodium dodecyl sulfate at pH 7.5, while total volume (V_t, 160 mL) was determined by applying 4 mL H_2O to the column after equilibration with the eluant buffer and monitoring chloride concentrations in the eluate. Before each bile fractionation, the column was first equilibrated at 60 mL/h with two column volumes of Tris-buffered saline (10 mm Tris/150 mm NaCl/3 mm NaN_3, pH 7.5) containing the 10 major conjugated bile salts as present in the previously measured IMC. Thereafter, 1–4 mL bile was filtered through a 5-μm microfilter and fractionated on the column at a flow rate of 12 mL/h. The eluate was collected in 80 fractions of 2 mL each and cholesterol, phospholipid, and bile salt concentrations were determined in each fraction. The recovery of cholesterol and phospholipids was over 95%. During separation, temperature of column and applied buffers was carefully kept at 37°C. Simultaneously, a sample of the same bile was subjected to KBr density-gradient ultracentrifugation: 2 mL of microfiltered (5 μm) bile was mixed with 0.94 g KBr and 50 mg sucrose. On top of this mixture the following KBr solutions were layered: 1 mL with a density of 1.260 g/mL, 2.5 mL with a density of 1.225 g/mL and 2.5 mL with a density of 1.100 g/mL. A final layer of water was added to completely fill the tube which then was centrifuged for 18 h at 100,000 *g* at 37°C in an SW 40 Ti rotor (Beckman, Palo Alto, CA). Subsequently, the contents of the tubes were divided into 24 fractions of 0.5 mL each. Cholesterol, phospholipid, and bile salt concentrations were determined in each fraction. Recovery of cholesterol, phospholipids, and bile salts was over 95%. Vesicular cholesterol concentration was calculated by multiplying total biliary cholesterol concentration with the fraction of cholesterol dissolved in vesicles.

Dynamic light-scattering experiments

The hydrodynamic radius (R_h) of particles in the vesicular fraction was determined by means of dynamic light-scattering using a Malvern 4700 system and a 25 mW He-Ne laser (NEC Corp., Tokyo, Japan) and the Automeasure version 3.2 software. Data are the means of three measurements.

Assessment of cholesterol crystallization

Bile was filtered immediately after cholecystectomy through a 0.22-μm ultrafilter and stored under N_2 at 37°C in the dark. A 5-μl drop was observed daily during the next 21 days by polarizing microscopy. Cholesterol monohydrate crystals were scored using a semi-quantitative scale (100 × magnification; means of 5 counted fields): 0 = no crystals, 1 + = 1 crystal per field, 2 + = 2–5 crystals, 3 + = 6–10 crystals, 4 + = >10 crystals. Cumulative crystal score for the 21-day observation period was also obtained (maximal score = 22 × 4 = 88). Crystal observation time was defined as the earliest day

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on which crystals were observed. When no crystals were observed within 21 days, the observation time was set at 22 days.

Statistics

Statistical analysis was performed using SPSS software, version 7.0. Unless otherwise mentioned, data are expressed as means ± SEM and compared with Student's *t*-tests. Correlations were calculated with Pearson's tests or, if values were not normally distributed, with Spearman's rank test. A *P* value <0.05 was considered to be statistically significant.

RESULTS

Validation of the IMC gel filtration method

Vesicles isolated by the IMC gel filtration method from supersaturated model bile (TLCo 5.7 g/dL, CSI 1.4, TC/(TC + EYPC) = 0.7) did not transform into micelles after direct reapplication on the same column with the same eluant buffer. Isolated micelles did not transform into vesicles upon rechromatography. Micelles are the only lipid-containing phase expected to be present in unsaturated model bile (TLCo 10 g/dL, CSI 0.7, TC/(TC + EYPC) = 0.7). This was confirmed by gel filtration according to the IMC method, and these micelles did not transform into vesicles upon repeated chromatography after their isolation. Similarly, mixed micelles or vesicles isolated from human biles with the aid of the IMC method did not transform into other phases when re-applied to a Pharmacia S400 column (gel bed 25 cm, internal diameter 0.8 cm, thermostated water jacket at 37°C) which was equilibrated in the same buffer (results not shown).

We hypothesized that after gel filtration with the correct IMC, the bile salt concentration should be identical in all column fractions, except for the fractions containing the mixed micelles. Preliminary experiments had shown that simple micelles from a concentrated (100 mm) standard bile salt mixture, applied on the column and eluted with bile salt-free buffer, eluted as a single peak in fractions right after the point where mixed micelles from a bile sample would elute. This is in line with the particle size of simple micelles, which are appreciably smaller than mixed micelles (24). We therefore assumed that this post-micellar region represents the IMC. Underestimation of the IMC led to an excess of bile salts in this region, as well as high amounts of vesicles (**Fig. 1A**). In contrast, overestimation of the IMC led to a bile salt dip in the post-micellar region, and absence of vesicles (Fig. 1B). As further corrobation that the post-micellar region represents the IMC, there is no difference in bile salt species composition between the elution buffers and the postmicellar zone (**Fig. 2**). Both are enriched in more hydrophilic bile salts. In contrast, the mixed micellar peak is highly enriched in more hydrophobic bile salts (n = 6). Gel filtration experiments included in the present report always revealed stable bile salt concentrations without dips or peaks in the post-micellar regions, indicating correct IMCs and bile salt compositions in the eluant buffers (**Fig. 3A**).

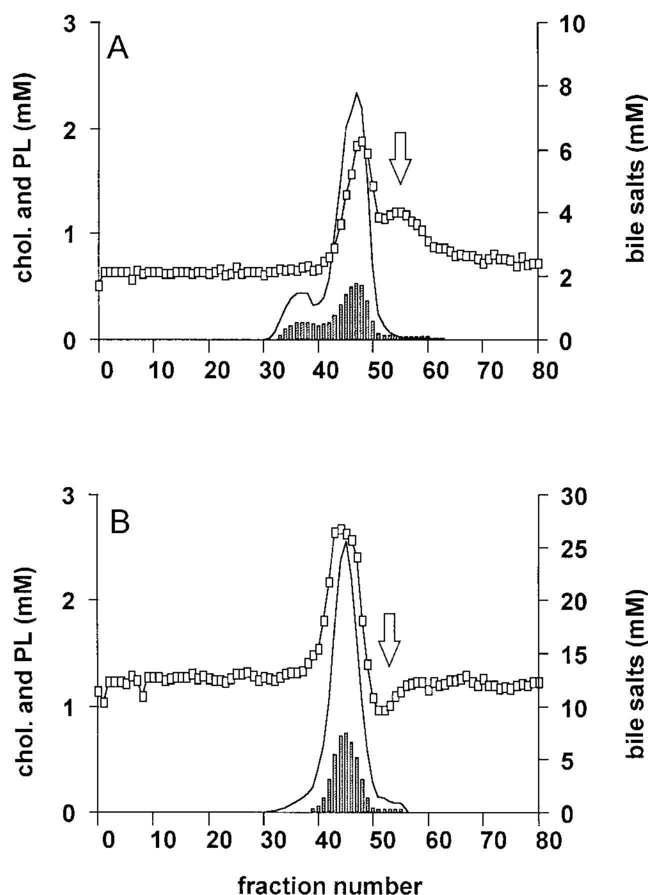

Fig. 1. Fractionation of bile samples by gel filtration with bile salt concentrations in the eluant buffers below (A) and above (B) the IMC. Underestimation of the IMC (A) leads to a bile salt peak in the "post-micellar" elution zone (indicated by an arrow), whereas overestimation (B) leads to a dip in the "post-micellar" zone. Moreover, vesicles are abundant in Fig. 1A but absent in Fig. 1B. Continuous line, phospholipids; bars, cholesterol; open squares, bile salts.

Intermicellar bile salt concentration and species composition

The IMC was determined in gallbladder biles from 17 gallstone patients. There was a wide range (3.56–35.02 mm) of IMCs in human gallbladder biles, with a mean of

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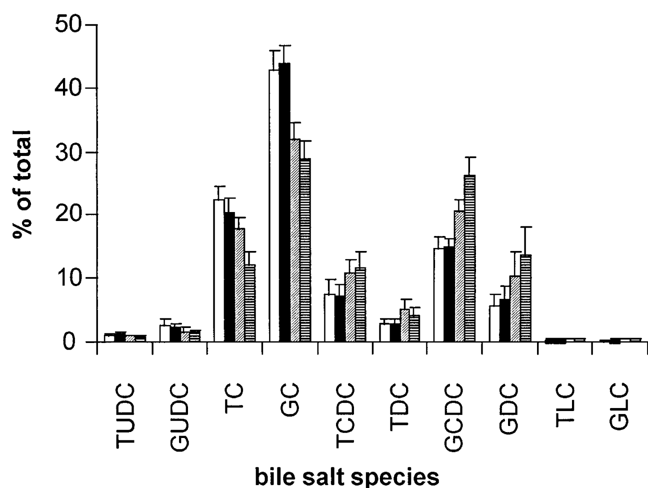

Fig. 2. Bile salt species composition (means ± SEM) in the elution buffer (according to the measured IMC; open bars), in the post-micellar region (solid bars), in the original bile (diagonally hatched bars), and in mixed micelles (horizontally hatched bars). Compositions in the elution buffer and the post-micellar regions are identical and significantly enriched in hydrophilic bile salt species (n = 6).

9.67 ± 1.97 mm. The mean correction factor for Gibbs-Donnan equilibrium effects during ultrafiltration was 0.77 ± 0.12 (range 0.56–0.98). The IMC correlated significantly with total biliary bile salt concentration (r = 0.62, $P < 0.01$) and with total lipid concentration (r = 0.56, $P < 0.02$), but not with mole % cholesterol or CSI.

The bile salt species composition in the ultrafiltrates differed in all 17 cases from the bile salt composition in the original biles: ultrafiltrates were significantly enriched in more hydrophilic species (in particular TC and GC), whereas the more hydrophobic species TCDC, TDC, GCDC, and GDC were relatively less represented in the ultrafiltrates as compared with corresponding whole biles (**Fig. 4**: $P < 0.00001$).

Separation of cholesterol carriers in bile

Biliary lipid composition and crystallization parameters for all 17 patients are shown in **Table 1**. Biles from the first 10 patients listed in Table 1 were fractionated both by gel filtration according to the IMC method and by KBr density gradient ultracentrifugation. From the other patients the amount of bile was too low for analysis of cholesterol solubilization. As can be seen for a representative experiment in Fig. 3A, gel filtration revealed predominant solubilization of cholesterol and phospholipids in mixed micelles, whereas ultracentrifugation of the same bile showed higher amounts of these lipids solubilized in vesicles (Fig. 3B). The IMC gel filtration method showed that only in 5 out of 10 biles were cholesterol and phospholipids partly solubilized in vesicles, whereas according to the ultracentrifugation method, in all of these biles relatively large amounts of these lipids could be found in vesicles.

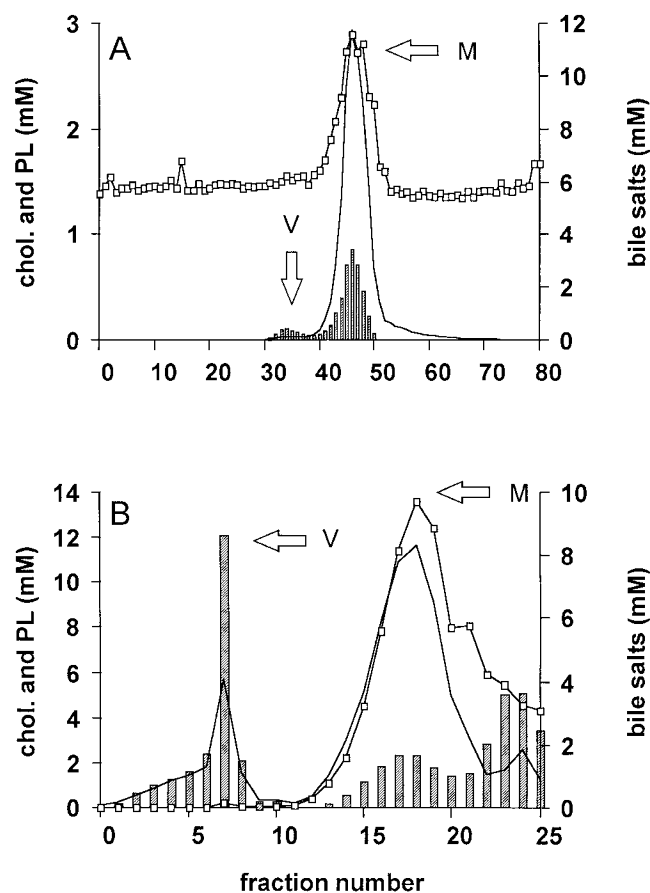

Fig. 3. Cholesterol (bars) and phospholipid (line) solubilization in gallbladder bile (CSI 1.64, TLCo 6.6 g/dl) according to the IMC gelfiltration method (A) and, simultaneously, according to the KBr-density gradient ultracentrifugation method (B). Bile salts are indicated with open squares. Vesicles are indicated with V, mixed micelles with M. The ultracentrifugation method leads to considerable overestimation of vesicular cholesterol solubilization (57.8% of total biliary cholesterol vs. 12.9% according to the IMC gel filtration method) and underestimation of vesicular cholesterol to phospholipid ratio (1.81 vs. 2.36).

As shown in **Table 2**, with the IMC gel filtration method a lower percentage of cholesterol and phospholipids was found solubilized in vesicles compared to the data from ultracentrifugation. Vesicles isolated by gel filtration has smaller hydrodynamic radii (R_h) and higher cholesterol/phospholipid ratios than vesicles from ultracentrifugation.

Biliary lipid composition, vesicular cholesterol content, and crystallization

As shown in **Table 3**, biles with vesicles according to the IMC method had a higher CSI (1.90 ± 0.28 vs. 1.15 ± 0.12; P = 0.04) and higher mole % biliary cho-

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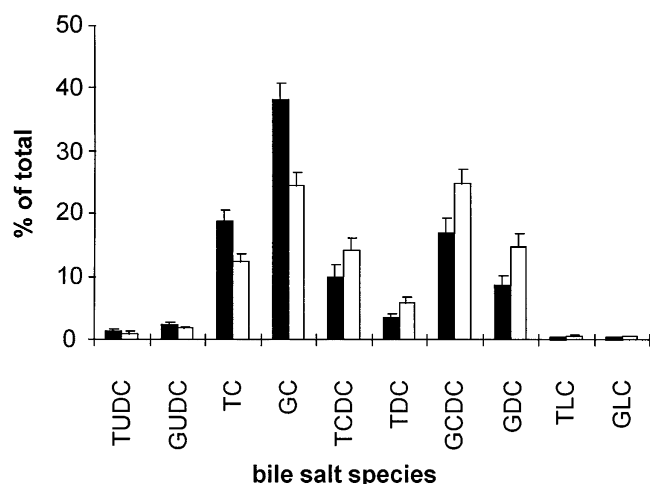

Fig. 4. Mean bile salt species composition (±SEM) in 17 intermicellar (simple micellar + monomeric) fractions (solid bars) and corresponding gallbladder biles (open bars). The intermicellar fraction is significantly enriched in hydrophilic bile salt species.

lesterol than biles without vesicles. Most biles without vesicles were slightly supersaturated or near saturation (samples 1–4 and 10 in Table 1). Total lipid concentrations and bile salt/(bile salt + phospholipid) ratios were lower in biles with vesicles, but the cumulative bile salt hydrophobicity index was not different.

With both IMC gel filtration and KBr density gradient ultracentrifugation methods, vesicular cholesterol concentration showed a positive correlation with CSI ($r = 0.89$, $P = 0.001$ and $r = 0.73$, $P = 0.02$, respectively) and a negative correlation with BS/(BS + PL) ratio ($r = -0.70$, $P = 0.02$ and $r = -0.72$, $P = 0.03$, respectively). With the IMC method only, a negative correlation existed between vesicular cholesterol concentration and total lipid concentration ($r = -0.72$, $P < 0.02$).

As shown in Table 3, biles with vesicles according to the IMC gel filtration method tended to have shorter crystal observation times and a higher cumulative crystallization score than biles without vesicles, but differences were not significant, probably due to small numbers. Although IMC gel filtration yielded lower vesicular cholesterol concentrations than KBr density gradient ultracentrifugation, vesicular cholesterol concentrations according to both methods correlated positively with cumulative crystallization and negatively with crystal observation time (**Fig. 5**).

DISCUSSION

Reliable separation of vesicular and micellar cholesterol carriers from gallbladder bile may provide important information on the mechanism of cholesterol crystallization and pathogenesis of gallstone formation. Although gel filtration (4–8, 25), ultracentrifugation (26), and microscopic (13) studies on human biles

TABLE 1. Patient characteristics, biliary lipid composition, and crystallization parameters

Patient	Sex	Age	Stones	Bile Salts		Phospholipid		Cholesterol		IMC	TLCo	CSI	BS/(BS+PL)	CryOT	CryCS
				mm	%	mm	%	mm	%						
		yr	*n*							*mm*	*g/dL*			*days*	*21 days*
1	F	26	>10	261.2	73.3	65.7	18.5	29.3	8.2	20.6	19.1	1.14	0.80	9	39
2	M	67	>10	97.2	77.9	19.7	15.8	7.8	6.2	4.7	6.6	1.18	0.83	17	16
3	F	74	>30	111.7	78.3	22.9	16.1	8.0	5.6	11.6	7.6	1.03	0.83	5	31
4	F	44	>100	176.3	78.4	29.9	13.3	18.6	8.3	11.7	11.7	1.55	0.85	22	0
5	F	51	23	125.3	68.8	35.9	19.7	21.0	11.5	4.7	9.8	1.68	0.78	11	25
6	F	36	15	77.6	64.2	29.0	24.0	14.3	11.8	6.6	6.6	1.64	0.73	1	61
7	M	40	2	37.1	47.8	25.4	32.7	15.1	19.5	11.0	4.4	2.76	0.59	2	62
8	F	75	>20	75.2	75.6	13.5	13.6	10.8	10.9	7.4	5.2	2.29	0.85	1	81
9	F	75	2	43.6	69.8	14.6	23.4	4.3	6.9	3.6	3.4	1.12	0.75	9	25
10	F	36	2	209.4	77.4	46.4	17.2	14.6	5.4	16.9	14.5	0.84	0.82	2	69
11	F	46	11	160.7	68.7	50.6	21.6	22.7	9.7	4.3	12.7	1.29	0.76	1	34
12	F	58	2	98.7	65.8	30.7	20.5	20.6	13.74	3.6	8.0	1.99	0.76	na	na
13	F	32	2	246.2	78.9	49.0	15.7	16.8	5.4	35.0	16.5	0.87	0.83	22	0
14	F	26	2	171.5	74.3	45.6	19.8	13.6	5.9	7.3	12.5	0.85	0.79	4	na
15	M	54	2	161.4	69.9	46.9	20.3	22.6	9.8	4.0	12.4	1.35	0.77	na	na
16	F	41	10	143.9	72.8	40.5	20.5	13.3	6.7	6.2	10.7	0.97	0.78	3	na
17	F	25	5	192.7	72.1	51.0	19.1	23.4	8.8	5.3	14.3	1.24	0.79	1	76
Mean		47		140.6	71.4	36.3	19.5	16.3	9.1	9.7	10.4	1.40	0.78	7.3	40
SEM		4.20		15.96	1.84	3.56	1.11	1.59	0.89	1.97	1.86	0.13	0.01	1.95	7.63

Abbreviations: CryOT, crystal observation time; CryCS, cumulative crystallization after 21 days; na, not available. Patients 1–10 were included in the study comparing gel filtration and KBr density gradient ultracentrifugation. In sample numbers 1, 2, 3, 4, and 10 gel filtration revealed no vesicles.

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TABLE 2. Cholesterol solubilization in 10 gallbladder biles from cholesterol gallstone patients according to IMC gel filtration and KBr density gradient ultracentrifugation method

Variables	Gel filtration	Ultracentrifugation	*P* Value
Biles with vesicles	5/10	10/10	
% Cholesterol in vesicles	8.0 ± 3.2	29.3 ± 8.1	<0.01
% PL in vesicles	2.5 ± 1.5	17.6 ± 7.2	<0.01
Cholesterol concentration in vesicles (mm)	1.0 ± 0.4	4.2 ± 1.2	<0.02
PL concentration in vesicles (mm)	0.49 ± 0.25	3.72 ± 1.13	<0.01
Vesicular C/P ratio[a]	2.25 ± 0.60	1.17 ± 0.19	0.18
Micellar C/P ratio[a]	0.29 ± 0.02	0.23 ± 0.01	0.09
Vesicular R_h (Å)[a]	340 ± 46	657 ± 61	0.07

Data are represented as means ± SEM.
[a] Only calculated for biles with vesicles.

have indicated the existence of vesicular modes of cholesterol transport, data from these reports should be considered in qualitative rather than quantitative terms; due to dilution of the bile sample during the procedure with solutions that may have physical chemical properties different from the original aqueous environment, potential changes of and interconversions between various lipid carriers may have occurred. For example, gel filtration studies were generally performed with fixed concentrations (often 7.5 or 10 mm cholate or taurocholate) of bile salts in the eluant buffer. Although Donovan et al. (16–18) have separated cholesterol carriers from model bile systems by applying bile salts at correct IMCs and compositions in the eluant buffers, this has not yet been done in human biles. Therefore, in the present study, we separated vesicles and micelles from gallbladder biles of cholesterol gallstone patients by using gel filtration at the correct IMCs and bile salt compositions. Apart from the confirmation of correct separation by absence of phase transitions when vesicles or micelles were reapplied on the column under identical conditions, we also observed that the bile salt concentrations in the post-mixed micellar zone provided important information in this respect. If simple micellar bile salts are applied on the column, they elute in this region, in line with their particle sizes (24). Similarly, our finding of hydrophilic bile salt compositions, identical to the IMC, in this zone would suggest that it is representative of the IMC. The enrichment of the IMC with more hydrophilic bile salt species is in line with observations made on model bile systems containing multiple bile salt species (27). We also found that eluant buffer bile salt concentrations above the IMC led to a dip of bile salt concentrations in the postmicellar zone, whereas eluant buffer bile salt concentrations below the IMC caused a postmicellar bile salt peak. At the correct IMC, stable bile salt concentrations without postmicellar peaks or dips should be expected. This method may be used in future gel filtration studies to get an impression of reliability of separation of various lipid carriers.

In agreement with previous data from model bile systems (16–18), we found a strong positive correlation of the IMC with total lipid concentration but not with CSI. In model systems it has been shown that increases in cholesterol content that induce a phase transition from a one-phase micellar system to a two-phase system of micelles and vesicles do not alter IMC values (17). Although we could not demonstrate an effect of bile salt/(bile salt + phospholipid) ratio on IMC (data not shown), this may relate to the relatively narrow range of this parameter in human gallbladder biles (Table 1).

We found smaller amounts of lipids solubilized in vesicles upon separation of cholesterol carriers by gel

TABLE 3. Biliary lipid composition and crystallization parameters in biles with and without vesicles as revealed by the IMC gel filtration method

Variables	Vesicles	No Vesicles	*P* Value
CSI	1.90 ± 0.28	1.15 ± 0.12	0.04
Mole % cholesterol	12.1 ± 2.0	6.8 ± 0.6	0.04
TLCo (g/dL)	5.9 ± 1.1	11.9 ± 2.3	0.04
BS/(BS+PL) ratio	0.74 ± 0.04	0.83 ± 0.01	0.07
CryOT[a] (days)	4.8 ± 2.2	11.0 ± 3.7	0.19
CryCS[b] (after 21 days)	50.8 ± 11.1	31.0 ± 11.6	0.25
Cumulative bile salt hydrophobicity index	0.35 ± 0.01	0.31 ± 0.02	0.20

[a] Crystal observation time.
[b] Cumulative crystallization score during 21 days.

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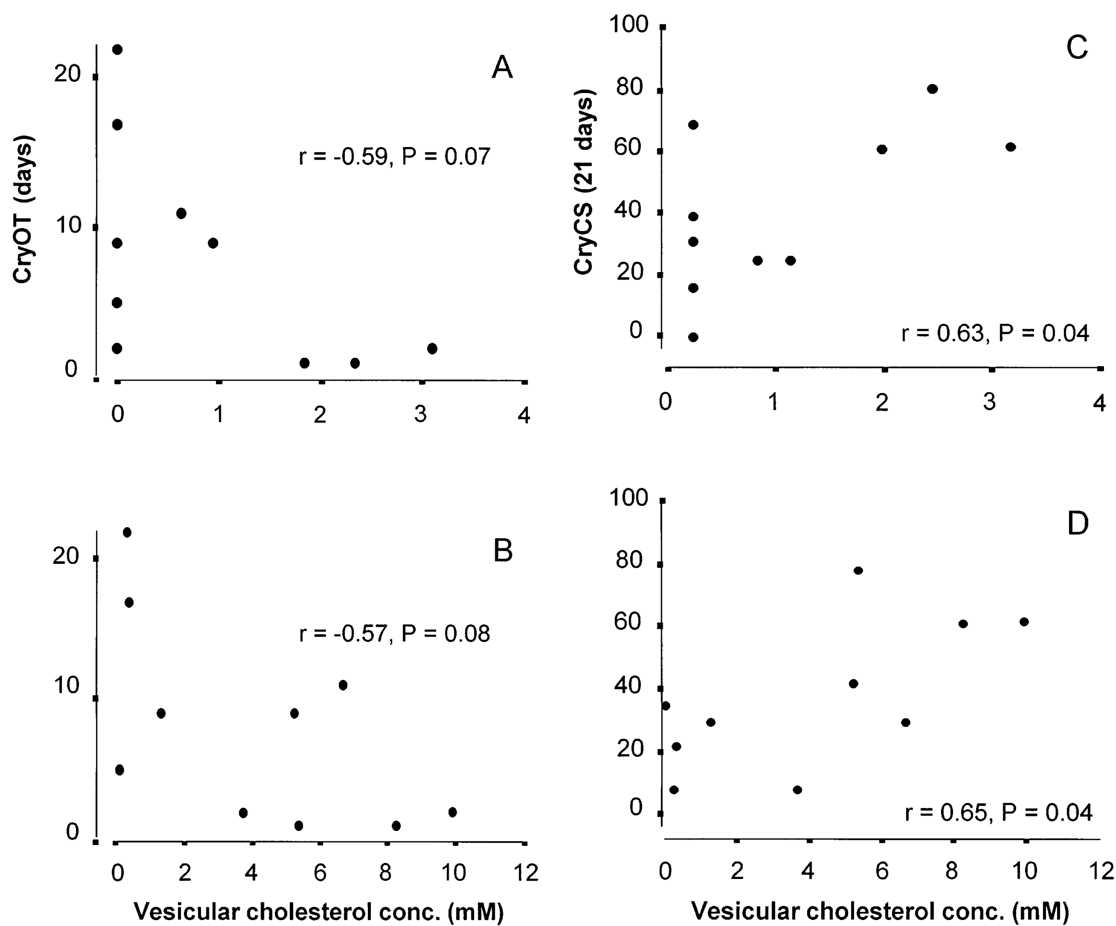

Fig. 5. Negative correlation between vesicular cholesterol concentration and crystal observation time (CryOT), both with the IMC gel filtration (A) and KBr density gradient ultracentrifugation (B) methods. Positive correlation between vesicular cholesterol concentration and cumulative crystal score (CryCS) with IMC (C) and ultracentrifugation (D).

filtration according to the IMC method than by density gradient ultracentrifugation, which is in agreement with previous data in model biles (28).

Vesicles could be detected by gel filtration in only five of ten gallbladder biles, but were detected in all ten biles with the aid of density gradient ultracentrifugation. Our findings of lower total lipid concentrations (5.9 vs. 11.9 g/dL) and (not significantly) lower bile salt/(bile salt + phospholipid) ratios (0.74 vs. 0.83) in biles with vesicles than in biles without vesicles (according to the IMC-gel filtration method) were not unexpected, as micellar solubilities are decreased at lower total lipid concentrations, and bile systems may shift from a three-phase (containing vesicles, micelles, and solid cholesterol crystals) zone to a two-phase (containing micelles and solid cholesterol crystals) zone upon increasing bile salt/(bile salt + phospholipid) ratio with leftward shift in the ternary (cholesterol–phospholipid–bile salt) phase diagram (29). Similarly, higher CSIs (1.9 vs. 1.2) in biles with vesicles are readily explained, as in these cases more cholesterol has to be solubilized in non-micellar carriers.

The absence of vesicles in a significant number of our cholesterol gallstone patients deserves further comment. Because most biles without vesicles were only slightly supersaturated or near saturation, the amounts of vesicles may have been low and under the detection limit of the IMC–gel filtration method. Nevertheless, our data indicate that most cholesterol is solubilized in micelles. One might also argue that the absence of vesicles in a considerable number of our cholesterol gallstone patients could be explained by the non-equilibrium state of the sample during the separation procedure (in which cholesterol may be transiently solubilized in supersaturated mixed micelles (30)) with potential vesicle formation if more time would have elapsed.

Another intriguing possibility would be that, apart from the well-known vesicular mode of cholesterol crystallization (12), non-vesicular pathways of cholesterol

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crystallization may occur in human biles, which would have potential implications for the mechanism of gallstone formation.

A recent study by Wang and Carey (31) would also be in line with the existence of non-vesicular pathways of cholesterol crystallization in human biles, based on data complementary to our findings. Approximately 50% of cholesterol gallstone patients included in that study had gallbladder biles plotting in the left two-phase zone of the phase diagram (containing micelles and solid cholesterol crystals) and exhibited the crystallization pathway typical of that zone: plate-like cholesterol monohydrate crystals appeared before anhydrous cholesterol crystals (i.e., arcs, helices, tubules). The remaining gallbladder biles in that study plotted in the middle three-phase zone (containing micelles, vesicles, and solid cholesterol crystals) of the phase diagram with crystallization sequences characteristic of that zone (vesicles appearing first, subsequently plate-like cholesterol monohydrate crystals, followed by anhydrous cholesterol crystals). Although no detailed information on crystallization sequences is available for biles included in the present report, bile salt/(bile salt + phospholipid) ratios calculated from the data provided by Wang and Carey (31) ranged from 0.76 to 0.86, remarkably similar to the present report. Recent studies in the prairie dog, a well known model for cholesterol gallstone formation when fed a lithogenic diet, also point to a non-vesicular pathway of cholesterol crystallization in vivo (32). Nevertheless, as Wang and Carey provided evidence in their model bile studies (Fig. 1 of ref. 29) that small unilamellar vesicles, which are not visible on light microscopy, may occur at least transiently, before equilibrium is reached, in the left two-phase zone of the phase diagram, the existence of non-vesicular crystallization pathways in human biles should be considered speculative at this moment.

In summary, the present study indicates that density gradient ultracentrifugation systematically overestimates amounts of cholesterol and phospholipids solubilized in vesicles in human gallbladder biles. The absence of vesicles in a significant proportion of gallbladder biles of cholesterol gallstone patients should stimulate further research into the existence of non-vesicular pathways of cholesterol crystallization during cholesterol gallstone formation.

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