papers and notes on methodology

Quantification of mucin in human gallbladder bile: a fast, specific, and reproducible method[1]

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Abstract It is generally accepted that gallbladder mucin (GBM) plays an important role in the pathogenesis of cholesterol gallstone (ChG) disease. However, it remains unclear whether ChG patients have higher GBM concentrations than controls. Discrepant findings regarding biliary mucin concentrations may be due to methodological problems with the assays commonly used. The methods currently used to quantitate mucin in bile have not been systematically evaluated. To establish a reliable method for mucin quantification in bile, we evaluated three mucin assays: the classic Pearson-PAS (periodic acid Schiff) assay, a direct fluorometric assay, and a new PAS or fluorometric assay with the following modifications of the Pearson assay: preincubation of bile samples with TBS containing KSCN and sodium taurocholate and micellation of biliary lipids during gel chromatographic fractionation using 25 mM sodium taurocholate in the elution buffer. SDS-PAGE and monoclonal anti-human-GBM (GBM59) were used to identify mucin. Highly specific and reproducible mucin isolation was achieved with the modified method. We found considerable loss of mucin during the different purification steps in the Pearson method. The direct fluorometric assay showed unspecific fluorometric signal with low molecular constituents of bile. Our experiments showed that human-GBM can be accurately measured after a simple modified chromatographic fractionation followed by a PAS or fluorometric assay.—**Miquel, J. F., A. K. Groen, M. J. A. van Wijland, R. del Pozo, M. I. Eder, and C. von Ritter.** Quantification of mucin in human gallbladder bile: a fast, specific, and reproducible method. *J. Lipid Res.* 1995. **36:** 2450–2458.

Supplementary key words human gallbladder mucin • cholesterol gallstone formation • cholesterol nucleation • glycoprotein

For many years gallbladder mucin has been implicated in an important role in the pathogenesis of cholesterol gallstone disease. Mucin-type glycoproteins are the most abundant proteins in bile, and mucins are found in the nuclei of both cholesterol and pigment gallstones (1, 2). Studies in animal models of cholesterol gallstone disease have shown an increased mucin secretion in response to a lithogenic diet. Moreover, mucin hypersecretion is regularly found before cholesterol crystal formation and cholesterol stone appearance (3–5). Mucin is also a well-known cholesterol pronucleating protein and stimulates cholesterol crystal growth in supersaturated model bile (6–10). Furthermore, the highly viscous mucus gel on the luminal side of the gallbladder epithelium and the soluble mucin in bile are believed to enhance the residence time of lithogenic bile and cholesterol crystals in the gallbladder, allowing the growth of crystals and, thereby, serving as a nidus for gallstone formation (2, 11).

Despite this evidence, the role of mucin in the pathogenesis of gallstone disease is controversial. It remains unclear whether cholesterol gallstone patients have higher gallbladder mucin concentration than patients with pigment stones or controls (12–15). Bile is a highly complex biological fluid with lipids in different molecular-associated forms (i.e., micelles, vesicles, lamellae), proteins, and pigment. Major efforts have been undertaken by different research groups to validate methods for total protein quantification in bile (16–18). These assays are hampered by the presence of high concentrations of lipid and pigment. So far, methods to determine mucin have not been validated rigorously. The quantification in bile of this high molecular weight and highly O-glycosylated protein offers additional difficulties.

Abbreviations: H-GBM, human gallbladder mucin; BSM, bovine submaxillar mucin; PGM, pig gastric mucin; CsCl-d.g.c., cesium chloride-density gradient ultracentrifugation; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TBS, Tris-buffered saline; PBS, phosphate-buffered saline; STC, sodium taurocholate; PEG, polyethyleneglycol; KSCN, potassium thiocyanate; PAS, periodic acid Schiff; CNA, cyanoacetamide.

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Quantification of soluble gallbladder mucin has been mainly carried out using the method of Pearson et al. (19). This assay uses the high molecular weight and buoyant properties of mucin for its separation from other biliary constituents by gel chromatography and CsCl-density gradient centrifugation, respectively. These steps are necessary to finally apply the PAS assay without the interference of other glycoproteins, pigment, and lipids. More recently, other assays for gallbladder mucin quantification have been used by different research groups (14, 15, 20) without formal validation assays. The mean gallbladder mucin concentrations published by different research groups vary widely (0.2–10 mg/ml), (8, 11, 13–15, 19, 21). High viscosity of bile samples, poor chromatographic separation, loss of mucin during the different purification steps and interference of other biliary components are possible explanations for these contradictory results.

The purpose of this study was *1)* to evaluate the specificity and reproducibility of mucin quantification in human gallbladder bile with the classic Pearson method and a direct fluorometric assay, and *2)* to develop and validate a more simple, faster, and specific method. Our results suggest that soluble gallbladder mucin can be measured accurately with the PAS or fluorometric assay after dilution of bile samples and chromatographic fractionation with 25 mM STC in the elution buffer.

MATERIAL AND METHODS

Chemicals and reagents

Sepharose 4B-Cl was obtained from Pharmacia AB (Uppsala, Sweden). Cesium chloride (CsCl) was purchased from Beckman Instruments Inc. (Palo Alto, CA). Monoclonal anti-H-GBM antibody (GBM59) was raised as described previously (15). This mouse monoclonal antibody belongs to the IgM class. It reacts specifically with human gallbladder mucin with no cross-reactivity to either pig gastric or bovine submaxillar mucin or with concanavalin-A binding human biliary glycoproteins (15, 22). Alkaline phosphatase-conjugated sheep anti-mouse Ig-antibody was purchased from Boehringer Mannheim Biochemica (Mannheim, Germany). Pig gastric (PGM) and bovine submaxillar mucins (BSM) were purchased from Sigma Chemical Co. (St. Louis, MO). All chemicals were of analytical grade and obtained from either Sigma Chemical Co. or Merck (Darmstadt, Germany).

Bile samples

Fresh gallbladder bile was obtained from gallstone patients undergoing elective laparoscopic cholecystectomy. The study was approved by the Ethics Committee of the Ludwig-Maximilians-Universität, München, Germany, and informed consent was given by the individual patients. At the beginning of the operation, before any surgical manipulation of the gallbladder and under visual control, gallbladder bile was completely aspirated into a sterile syringe by transcutaneous puncture with a 14-gauge needle. The samples were immediately mixed with proteinase inhibitors phenylmethylsulfonyl fluoride, EDTA, N-ethylmaleimide, and sodium azide (NaN_3) as antimicrobial agent at final concentrations of 1 mM, 5 mM, 10 mM, and 0.02%, respectively. Aliquots for biochemical determinations were flushed with N_2 and stored at -30°C.

Human gallbladder mucin purification

Human gallbladder mucin (H-GBM) was purified from two "with bile" samples exactly as previously described (12). These colorless bilirrubin-free samples with low total lipid content (< 1 g/dl) provided an excellent source of gallbladder mucin. These purified mucins were free of low molecular weight proteins as judged by 5–12% gradient SDS-PAGE under reducing conditions. The purified mucin sample contained less than 0.5% lipid by weight as determined by thin-layer chromatography (data not shown). We have compared PAS-reactivity for PGM, BSM, and for this purified H-GBM. Most studies used PGM as standard for GBM quantification in human bile (12, 13, 19). As shown in **Fig. 1,** standard curves with different slopes were observed with the different mucins tested. This observation was originally described by Mantle and Allen (23). Although it is clear that the sensitivity per mg of material depends on the degree of oxidation by periodate, the structural basis for the observed differences is not entirely clear. Clearly,

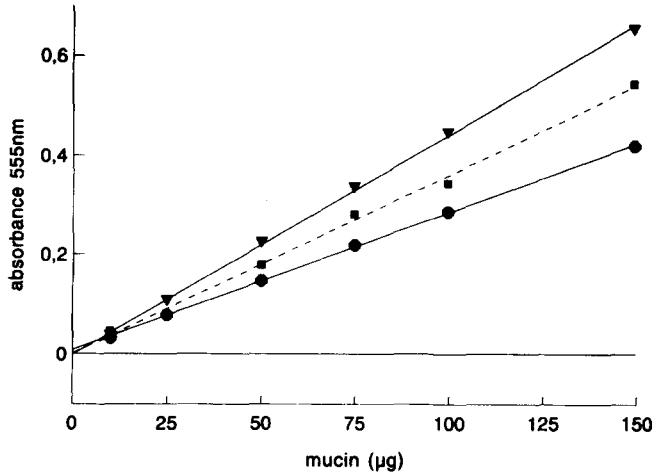

Fig. 1. Standard curves for the periodic acid/Schiff assay with pig gastric mucin (▼); bovine submaxilar mucin (■); and purified human gallbladder mucin (●).

the use of pig gastric mucin as standard for H-GBM quantification could underestimate the real mucin content in human gallbladder biles. BSM most closely resembled the H-GBM reactivity with the PAS assay. In this study, we used purified H-GBM as standard.

Pearson method

In six bile samples, the concentration of biliary mucin was determined as described by Pearson et al. (19) and modified by Harvey et al. (12). Briefly, fresh bile samples (5–7 ml) were first dialyzed (mol wt cutoff 10,000) at 4°C for 72 h against distilled water containing 0.02% NaN_3. Dialyzed bile was centrifuged at 30,000 *g* for 30 min and the pellet was discarded. The supernatant was fractionated on a Sepharose 4B-Cl chromatograph (80 × 2.6 cm column, Pharmacia AB, Uppsala, Sweden) with PBS containing 0.02% NaN_3 as eluant, at a constant flow rate of 0.5 ml/min. Fractions of 5 ml were collected and analyzed for glycoprotein, protein, pigment, and cholesterol. The excluded fractions (F I) containing the mucin-type glycoproteins were pooled and concentrated by reverse dialysis against 60% PEG (w/v) to approximately 10 ml. Further purification of mucin was performed by CsCl-d.g.c. as described elsewhere (22). Mucin content was measured in the colorless high density fractions (density 1.45–1.55 g/ml) by the periodic acid Schiff (PAS) reaction (23).

Modified method

Dilution and solubilization of samples. To reduce the viscosity and ensure homogeneity of the samples, 0.6–1 ml fresh gallbladder bile was diluted 1:1 (v/v) with 0.1 M Tris-HCl buffer (pH 7.5) containing potassium thiocyanate (KSCN) and NaN_3 to reach final concentrations of 0.22 M and 0.02%, respectively. To avoid the formation of lipid aggregates in vesicular form, sodium taurocholic acid (STC) was added to this buffer to a final concentration of 12.5 mM. The mixture was gently shaken for 12 h and centrifuged at 12,000 *g* for 10 min to remove insoluble material. All steps were carried out at 4°C.

Gel filtration chromatography. After centrifugation of the mixture, 1 ml of the supernatant was fractionated on a small Sepharose 4B-Cl gel-chromatography (35 × 1 cm column; Pharmacia AB, Uppsala, Sweden) at a constant flow rate of 0.5 ml/min. Fractions of 1 ml were collected. To avoid coelution of biliary lipids in vesicular form (i.e., cholesterol/phospholipid vesicles) with high molecular weight glycoproteins, 25 mM of STC in PBS (10 mM phosphate buffer, 0.2 M NaCl, 0.02% NaN_3, pH 7.4) was used as elution buffer. Lower concentrations of STC in the elution buffer yielded turbid fractions in the void volume (24, 25). The column was calibrated with standard molecular mass markers (range from 17 to 670 kDa; Sigma, St. Louis, MO) and pure H-GBM. The void and total volumes of the column were estimated with Dextran 2000 and cyanocobolamine, respectively. The eluted fractions were analyzed for protein, glycoprotein, cholesterol, bile salts, and pigment as described below. Purified H-GBM was recovered in the void volume in fractions 9 to 14 (data not shown). This fraction was named F I. The included fractions were pooled in two separated fractions according to the apparent molecular mass (F II < Vo > 200 kDa; F III < 200 kDa), dialyzed (mol wt cutoff 10,000) for 12 h against distilled water, concentrated by reverse dialysis against 60% PEG (w/v) to approximately 5 ml, and stored at -30°C. Recovery of mucin was estimated by adding different concentrations of purified H-GBM (0.3, 0.6, 1.2, and 2.4 mg/ml; n = 2 for each concentration) to bile samples depleted of mucin as previously described (8); in brief, bile samples were centrifuged at 150,000 *g* for 2 h and subsequently the supernatant was filtered (0.2 µm Millipore filter). This procedure reduced the mucin content to less than 0.05 mg/ml whereas no significant loss of lipids occurred.

Quantification of biliary mucin. The excluded fractions (F I) were pooled and dialyzed for 24 h against 5 L distilled water containing 0.02% NaN_3 at 4°C (3 changes) to remove bile acids. Mucin concentrations were measured in F I with either the PAS (n = 22) or the fluorometric (n = 7) assay. Briefly, for the PAS assay 500 µl of the pooled F I was brought to 1 ml with distilled water and the PAS method was applied exactly as described by Mantle and Allen (23). For the fluorometric assay, 250 µl of alkaline reagent was added to 200 µl of the pooled F I samples and the mucin content was measured by derivatization with 2-cyanoacetamide, as described below.

Direct fluorometric assay

In 19 bile samples, mucin was also quantified with a fluorometric assay for O-glycosylated glycoproteins as described by Crowther and Wetmore (26). Before the fluorometric assay, bile was delipidated using the method of Wessel and Flügge (27). Briefly, 50 µl of bile was diluted 1:1 (v/v) in PBS buffer and 400 µl methanol was added. After a short centrifugation (9000 *g* for 1 min), 200 µl of chloroform and 300 µl of distilled water were added, thoroughly mixed, and centrifuged once more. The upper phase was discarded and 300 µl of methanol was added. After a further centrifugation (9000 *g* for 2 min), lipid-free proteins were recovered in the pellet. For the fluorometric mucin determination, the pellet was resuspended in 200 µl of PBS, 250 µl of alkaline reagent (1 ml 0.15 N NaOH and 200 µl of 0.6 M 2-cyano-acetamide) was added and the mixture was incubated at 100°C for 30 min. Subsequently, 2 ml of 0.6

M borate buffer (pH 8) was added and the fluorescence was measured at 383 nm (excitation 336 nm).

Analytical methods

CsCl-density gradient centrifugation (CsCl-d.g.c.). CsCl-d.g.c. was carried out as previously described (22). After dialysis of the F I-fractions, CsCl was directly dissolved in these fractions to a final concentration of approximately 60% (w/v), sufficient to achieve a starting density of 1.42 g/ml. Centrifugation was carried out in 6-ml polyallomer tubes at 4°C at 300,000 *g* for 6 h in a TV-865 Sorvall vertical rotor (DuPont Instrument, Bad Homburg, Germany). Six fractions (0.8 to 1 ml) were collected by puncturing the tube at the bottom (gradient fractionator; Hoefer Scientific Instr., San Francisco, CA). Analysis of proteins and glycoproteins was performed as described below. The densities were determined by weighing the samples. In a parallel set of four F I samples, no differences were observed comparing this mode of CsCl-d.g.c. with CsCl-d.g.c. at 300,000 *g* for 24 h in a 70.1 Ti fixed-angle rotor (Beckman Instrument) as described by Smith and LaMont (28). This rapid centrifugation step was further validated by comparing both methods with purified H-GBM solubilized in PBS at concentrations from 0.3, 0.6, 1.2, and 2.4 mg/ml (n = 2 for each concentration); over 90% of mucin was recovered at its typical density (1.45–1.55 g/ml) (data not shown).

Electrophoresis and dot blots. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out under reducing conditions in 5–12% gradient separating gels with 3% stacking gel according to the method of Laemmli (29). Samples were run at a constant voltage of 50 mA in a Mighty Small 117CM vertical slab unit (Hoefer Scientific Instrument, San Francisco, CA). Aliquots of native bile and pooled elution fractions (F I to F III) were brought to 1 ml vol with distilled water, precipitated with 7% (vol/vol) trichloroacetic acid, delipidated with ice-cold diethyl ether–ethanol 3:1 (vol/vol), and resuspended with Laemmli's sample buffer. Gels were stained for proteins with silver (30) and for glycoproteins with the PAS method (31). Dot-blot analysis was performed by direct loading of a 4-µl sample onto nitrocellulose sheets. After drying for 10 min at 37°C, the sheets were blocked by incubation with TBS-Tween 0.1% (50 mM Tris-HCl, 0.1% Tween 20, 0.2 M NaCl, pH 7.5) (32). For mucin detection, the membranes were incubated for 2 h with the monoclonal GBM59 in TBS-Tween 0.05% at a dilution of 1:3000. The bound antibodies were detected by incubation with alkaline phosphatase-labeled sheep anti-mouse immunoglobulins and the chromogenic substrates for alkaline phosphatase (5-bromo-4 chloro-3-indolyl phosphate and nitroblue tetrazolium). The sensitivity of these dot blots assays was 15–20 ng. We have previously checked by Western blot analysis that this GBM59 antibody specifically reacts with the high molecular weight glycoproteins that did not completely enter a 5–12% gradient SDS-PAGE under reduced conditions, with no cross-reactivity to either concanavalin-A-binding human biliary glycoproteins or with other low molecular weight biliary proteins (22).

Chemical analysis

Cholesterol, total bile acids, and phospholipids in native biles were determined by standard techniques as previously described (33). The cholesterol saturation index was determined using Carey's critical tables. Biliary proteins were measured after TCA precipitation and delipidation by the Lowry method modified by Jüngst et al. (18) using human serum albumin as standard. In the eluted chromatographic and CsCl-d.g.c. fractions, protein was estimated by the Bradford assay and cholesterol by a commercially available kit (Boehringer-Mannheim, Germany); bile pigment was measured by absorption spectrophotometry at 420 nm and glycoprotein by the modified PAS assay according to Mantel and Allen (23).

Statistics

All results are expressed as mean ± SD. A two-tailed paired Student's *t*-test was used to compare differences among groups. Associations between variables were searched with scatter plots and confirmed by linear regression analysis. The significance level was set at $P < 0.05$.

RESULTS

Evaluation of the Pearson method

Bile obtained from the gallbladders of six patients was fractionated on a Sepharose 4B-Cl column as described by Pearson et al. (19). Packing and occlusion of the column occurred with two other samples. The time required for the fractionation of one sample was over 10 h. **Figure 2A** shows a representative chromatographic profile of a bile sample. As expected, a strongly PAS-positive glycoprotein fraction was excluded from the Sepharose 4B column (void volume; F I). A considerable amount of lipid in vesicular form coeluted in the void volume; this was confirmed by the turbidity of the samples and by cholesterol determination. The included fractions were pooled in F II (< Vo > 200 kDa) and F III (< 200 kDa). Proteins and glycoproteins of lower molecular mass coeluted with lipids and pigment. Dot blots of these fractions showed a strongly positive immunoreactivity with specific monoclonal anti-H-GBM antibody

(GBM59) not only in F I but also in F II (Fig. 2B). Gradient SDS-PAGE with silver stain and counterstaining with PAS showed that high molecular mass glycoproteins (mucin) were the main constituents of F I; some

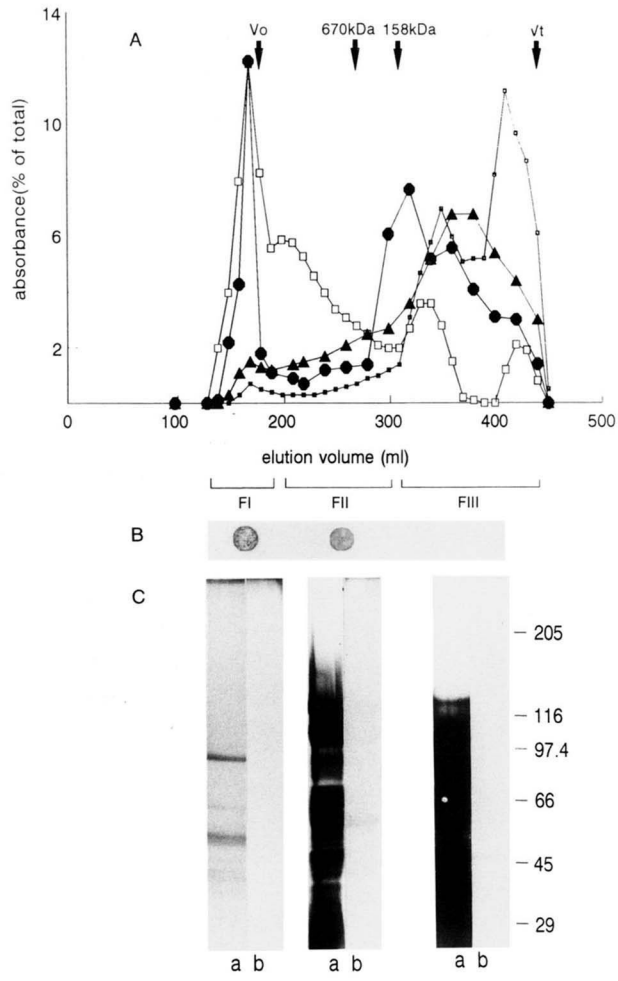

Fig. 2. A: Sepharose 4B-Cl chromatography of a representative postdialyzed bile sample (No. 1) according to the Pearson method (19). Five ml of bile was applied to the column (80 × 2.6 cm) and eluted at a constant flow rate of 0.5 ml/min at 4°C. Fractions of 5 ml were collected and screened for glycoprotein (●); protein (▲); cholesterol (□); and bile pigment (■). Results are expressed on the ordinate as percent of total absorbance. Excluded fractions were rich in high molecular weight glycoproteins and lipids, with cloudy appearance (FI); the included fractions were separately pooled according to their mol wt in FII (< Vo > 200 kDa) and FIII (< 200 kDa). Arrows indicate molecular mass standards. B: Reactivity of the different pooled fractions with monoclonal antibody GBM59 was tested by dot-blot analysis. H-GBM was detected both in the excluded fractions (FI) and in the included fractions (FII). C: Five to 12% gradient SDS-PAGE of biliary proteins. Proteins from the different pooled fractions of Sepharose 4B-Cl chromatography were precipitated with 7% TCA followed by ether/ethanol delipidation. Fifty μg of protein (FII and III) or glycoprotein (FI) was applied to each line. Gels were first stained for protein with silver (a) and counterstained thereafter with PAS reagent (b). Migration of molecular mass markers as indicated (kDa). High molecular weight glycoproteins that did not completely enter the gel are identified in both FI and FII.

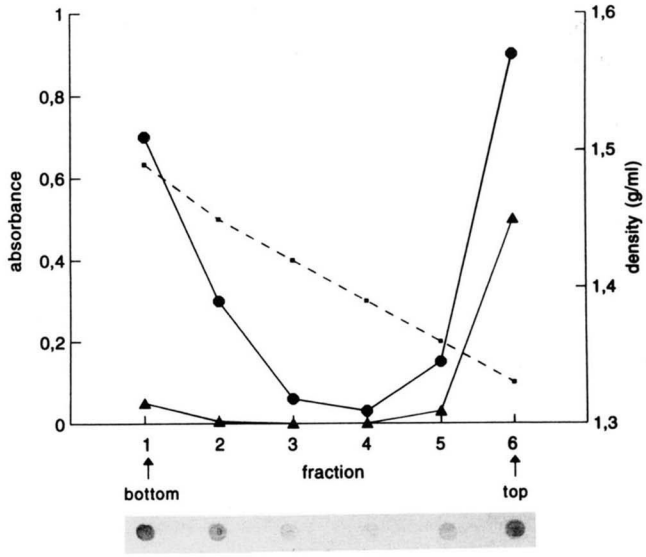

Fig. 3. CsCl-density gradient centrifugation and dot-blots of the pooled glycoprotein-rich V_o fractions obtained by the Pearson method. FI of the Sepharose 4B chromatography (Fig. 2A) was centrifuged in a vertical rotor at 300,000 *g* for 6 h at a starting density of 1.42 g/ml (dotted line). After centrifugation, the tubes were fractionated into six equal fractions by puncturing the bottom. Fractions were analyzed for glycoprotein (●) by the PAS assay and for protein (▲) by the Bradford assay. The reactivity of the different fractions with monoclonal antibody GBM59 was tested by dot-blotting. H-GBM was detected in both the high density fractions 1 and 2 and in the cloudy low density fractions 5 and 6.

low molecular mass proteins coeluted in this fraction (Fig. 2C). As expected, several polypeptide bands visualized with silver and/or PAS stain were found in F II and F III. High molecular mass glycoproteins were also seen in F II, confirming that mucin also eluted in this fraction. The excluded fraction (F I) was further purified by CsCl-d.g.c. as described by Pearson et al. (19). Because of the particular buoyant properties of mucin, this procedure separates highly glycosylated proteins (high density fractions 1 and 2) from noncovalently bound proteins and lipids (lower density fractions 5–6) (**Fig. 3**). According to Pearson et al. (19), only the PAS positive signal recovered in the high density fractions is considered to be mucin. However, in our experiments only 41 ± 22% (range 19–84%) of the total PAS positive reaction present in F I was recovered in the high density fractions characteristic for mucin (density 1.45–1.55 g/ml). To trace mucin, dot blot analyses were performed. Unexpectedly, the low density fractions showed a strongly positive immunoreactivity with monoclonal GBM59, indicating that mucin was also present in these fractions in association with biliary lipids in vesicular form. With the Pearson method, a high interassay variation was observed (36 ± 10%; range 20–50%, n = 6).

Modified method for gallbladder mucin determination

In the same six bile samples described above and in all the subsequent samples (total n = 22), a new method was applied for mucin separation and quantification as described in Material and Methods. Compared to the Pearson method, faster and more reproducible elution profiles were obtained and packing of the column never occurred. **Figure 4A** shows a characteristic elution profile of a bile sample. High molecular mass glycoproteins were recovered in the void volume (F I) together with a very small amount of low molecular weight proteins. F I was colorless or slightly pigmented and the lipid content was negligible (< 1% of total cholesterol). All lipids eluted in the included volume together with lower molecular mass proteins (F II and F III). Dot blot analysis and SDS-PAGE confirmed that mucin only eluted in the void volume (F I) (Figs. 4B and 4C). **Figure 5** shows a CsCl-d.g.c. of F I and dot blot analysis of the fractions recovered. A mean of 81 ± 14% (n = 15) of the total PAS reaction was recovered in the high density fractions (density 1.45–1.55 g/ml). Dot blot analysis with GBM59 confirmed that only these fractions contained mucin. After gel chromatography, recovery of purified H-GBM added to mucin-depleted native biles in concentrations between 0.3 and 2.4 mg/ml was 80 ± 5% (range 73–87%). After CsCl-d.g.c., 93 ± 1% (range 90–95%) of added mucin was recovered in the high density fractions. Mucin concentration in 22 consecutive bile samples was 0.86 ± 1.5 mg/ml. The interassay and intraassay coefficient of variations were 7.7 ± 5% and < 5%, respectively.

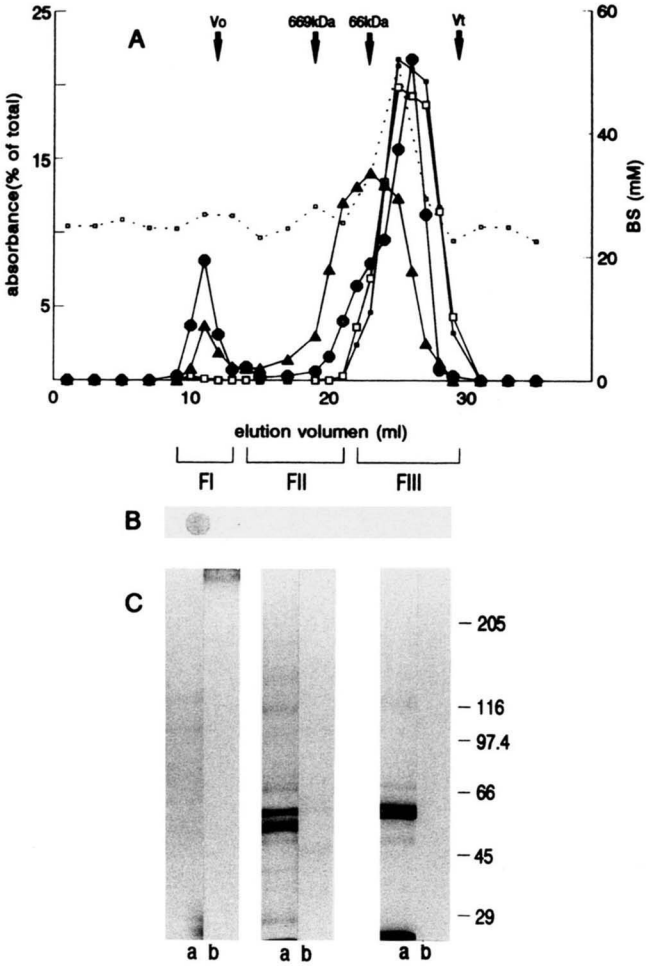

Fig. 4. A: Sepharose 4B-Cl chromatography of a bile sample (No. 1) after dilution (1:1) and incubation for 12 h with TBS-KCSN (0.22 mM)-STC (12.5 mM) as described under Modified Method. Diluted bile was centrifuged and 1 ml of the supernatant was applied to the column (35 × 1 cm) and eluted at a constant flow rate of 0.5 ml/min at 4°C with PBS-STC (25 mM) as eluant. Fractions of 1 ml were collected and screened for glycoprotein (●), protein (▲), cholesterol (□), bile pigment (tiny solid square), and bile salts (tiny open square). Results are expressed on the ordinates as percent of total absorbance and bile salt (BS) concentration in mM. Excluded fractions were colorless and rich in high molecular weight glycoproteins without lipids or pigment (FI). The included fractions were pooled in FII and FIII according to their molecular weight as in Fig. 2. B: Reactivity of the different pooled fractions with monoclonal antibody GBM59 tested by dot-blotting showed that H-GBM was exclusively detected in the excluded fractions. C: Five to 12% gradient SDS-PAGE of biliary proteins from the different pooled fractions obtained after fractionation. After TCA precipitation and ether/ethanol delipidation, 50 µg of protein (FII and III) or glycoprotein (FI) was applied to each line. Gel was first stained for protein with silver (a) and counterstained thereafter with PAS reagent (b). Molecular mass markers as indicated (kDa). High molecular weight glycoprotein was exclusively identified in void volume fractions (FI).

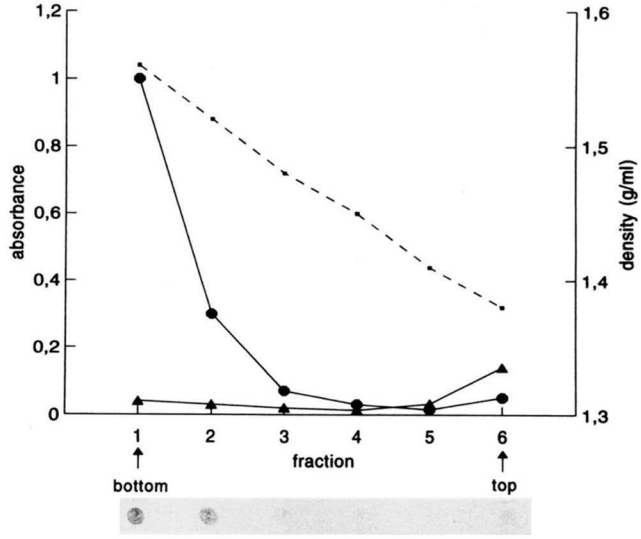

Fig. 5. CsCl-density gradient centrifugation and dot-blots of the pooled glycoprotein rich V_o fractions (FI) obtained as described under Modified method. FI of the Sepharose 4B chromatography (Fig. 4A) was centrifuged in a vertical rotor at 300,000 *g* for 6 h at a starting density of 1.42 g/ml (dotted line). After centrifugation, the tubes were fractionated into six equal fractions by puncturing the bottom. Fractions were analyzed for glycoprotein (●) by the PAS assay and for protein (▲) by the Bradford assay. Reactivity of fractions with monoclonal antibody GBM59 was tested by dot-blotting. H-GBM was detected only in the high density fractions 1 and 2.

TABLE 1. Analysis of biliary lipids, cholesterol saturation index, total protein, and soluble mucin content by the Pearson and modified PAS assays

Sample	TLC	CSI	Total Protein	Mucin Content	
				Pearson Assay	Modified-PAS Assay
	g/dl		*mg/ml*	*mg/ml*	
1	10.2	1.5	13.3	0.39	0.92
2	20.4	1	31.2	0.19	0.76
3	3.7	2	5.3	0.23	0.30
4	17.6	0.7	8.8	3.0	5.20
5	8.9	0.9	11.9	0.21	0.20
6	21.4	1	26.0	0.57	2.30
7	0.1	nd	1.6	nd	0.51
8	12.1	0.9	33.5	nd	1.30
9	13.7	1.2	9.6	nd	0.61
10	5.8	1.7	9.8	nd	0.52
11	20.1	1.1	22.8	nd	1.97
12	2.1	1.3	1.3	nd	0.49
13	8.3	1	6.1	nd	1.27
14	nd	nd	nd	nd	0.41
15	nd	nd	nd	nd	0.11
16	6.1	1.3	2.4	nd	0.31
17	6.7	1.4	2.1	nd	0.20
18	16.4	1.5	11.1	nd	0.34
19	6.6	1.2	13.0	nd	0.18
20	9.0	4.0	9.5	nd	0.37
21	8.8	0.9	2.6	nd	0.21
22	12.5	0.5	5.0	nd	0.43

While a good correlation was observed between the Pearson method and the modified method in the first six samples ($r = 0.96$; $P < 0.01$), mucin concentrations measured by the modified methods were about double compared to the Pearson method (1.6 ± 1.9 and 0.76 ± 1 mg/ml, respectively; $P = 0.07$; n = 6, **Table 1**).

Fluorometric assay

The concentration of soluble gallbladder mucin was also measured with a sensitive fluorometric assay described by Crowther and Wetmore (26). This assay specifically estimates the content of O-glycosylated glycoproteins, like mucins, by derivatization with 2-cyanoacetamide. This method has been used to quantitate mucin in bile directly obviating the need to prepurify the glycoprotein (15). To test whether this assay indeed accurately monitors mucin content we compared the results of the direct fluorometric assay with mucin determinations after purification, i.e., Sepharose 4B-Cl gel chromatography, with either the PAS or fluorometric tests. The mucin content of 19 samples was determined as described by van Wijland et al. (15). The interassay and intraassay variation of the assay were < 5%. A good linear correlation was observed between the direct fluorometric assay and the quantification of mucin after purification with either the PAS ($r = 0.84$; $P < 0.01$, n = 19) or the fluorometric assay ($r = 0.82$; $P < 0.01$, n = 11). However, the mean value obtained with the direct fluorometric assay was 2-fold higher than the value obtained after purification and quantification with either PAS (1.5 ± 1.2 vs. 0.69 ± 0.6 mg/ml, $P < 0.01$, n = 19) or fluorometry (1.5 ± 1.2 vs. 0.4 ± 0.2 mg/ml, $P < 0.01$, n = 11). In contrast, a good correlation with similar mean value was obtained when the mucin content was measured with either the PAS or fluorometric assay after chromatographic purification ($r = 0.96$, $P < 0.01$; 0.56 ± 0.38 vs. 0.50 ± 0.24 mg/ml, respectively; P = n.s., n = 6).

Additional experiments were carried out to clarify whether the higher values obtained by the direct fluorometric assay were due to nonspecific reactivity of the CNA with other biliary components. Bile samples were fractionated on Sepharose 4B-Cl according to our modified method and the fluorometric signal in the eluted fractions was compared with the reactivity towards GBM59 on dot blots. We found fluorometric signals in the included fractions with no reactivity towards GBM59 (data not shown). This shows that the fluorometric assay lacks specificity for mucin in gallbladder bile and also reacts with low molecular weight substrates other than mucin.

DISCUSSION

There is convincing evidence that gallbladder mucin plays an important role in the formation of cholesterol gallstones in the gallbladder (1–10). One of the mechanisms by which soluble gallbladder mucin may promote cholesterol gallstone formation is the pronucleating activity of gallbladder mucin (7–10). Measurements of concentrations of mucin in bile are essential to test pronucleating activity in physiological concentrations. Furthermore, an important criterion to establish the credibility of a putative promoter is that the concentration of the candidate promoter compared to that found in control samples should be increased in bile samples acquired from patients with cholesterol gallstone disease (34). This criterion is particularly important for mucin, as mucins from different sources seem to have a similar cholesterol crystal-promoting activity (7, 10). For instance, mucin from control patients was found as effective as that from patients with gallstones in promoting cholesterol crystal formation (7, 8). In recent years, conflicting data have been generated regarding the concentrations of mucin in the gallbladder bile of patients with cholesterol gallstones and control patients. No significant differences have been found in two major studies (12, 15). In contrast, other groups were able to find higher mucin levels in cholesterol gallstone patients as compared to controls (11, 13, 14).

Most investigators used the method of Pearson et al.

(19) to quantify mucin levels in human bile. To our knowledge, no data are available on the reproducibility of this very time-consuming method. We, therefore, performed spiking experiments to re-evaluate and validate this method. Our results demonstrate considerable interassay variability, up to 50% in the concentrations of biliary mucin. In addition, major losses of mucin occurred during both the chromatographic fractionation (Fig. 2) and the CsCl-d.g.c. (Fig. 3). The losses during gel chromatographic fractionation were probably due to poor separation as a consequence of the high viscosity of native bile samples. Separation of mucin from lipids with CsCl-d.g.c was incomplete due to mucin–lipid interaction (9, 10, 28) that lead to a shift of mucin from the high to the low density fractions. Dialysis of bile samples during the Pearson procedure removes bile salts which generate biliary cholesterol-phospholipid vesicles (35). Biliary vesicles are large lipid structures (550–1000 Å) that coelute with mucin after gel chromatography. This coelution may induce mucin–lipid interaction (9, 10) leading to the loss of mucin into the low density fractions after CsCl-d.g.c. Indeed, PAS-reactivity in the low density fractions after a gradient centrifugation has been observed previously (19, 28). However, this observation has not been further analyzed.

To overcome the shortcomings of the currently used method, we modified the procedure. The modifications proposed in this study include two major experimental steps: to ensure homogeneity and to lower viscosity of samples, biles were diluted with buffer containing the chaotropic ion KSCN. This principle has been previously used for the solubilization of sputum specimens from patients to purify bronchial mucins (36). In addition, we added STC to the running buffer of gel chromatography in sufficient concentrations (25 mM) to allow complete micellation of biliary lipids and to avoid vesicle formation (35, 37). These two modifications led to a marked improvement in the quality of the gel chromatographic fractionation step with adequate separation of mucin from other biliary constituents like lipids, pigment, and other glycoproteins (Fig. 4). In contrast to the Pearson method, void volume fractions now exclusively contained mucins and no lipids. In addition, and perhaps most importantly, micellation of biliary lipids prevented the interaction between mucin and lipids in vesicular form and, therefore, after CsCl-d.g.c, mucin was exclusively found in the high density fractions (Fig. 5). As the chromatographic fractionation step was now remarkably uniform and the void volume fractions were not contaminated with lipids, pigments, and other glycoproteins that may interfere with the PAS or fluorometric assays, we were able to quantify mucins directly in the F I fractions without further purification steps such as CsCl-d.g.c. This not only shortened the time needed for a determination from 7 days to about 24 h but also considerably lowered the costs involved in the procedure.

A fluorometric assay has been described to specifically quantify O-glycosylated glycoproteins like mucins (26) and was recently applied to quantify mucin in human bile (15). We further evaluated the validity of this direct fluorometric method for mucin determination in human gallbladder biles. Although a good correlation was found between the direct fluorometric assay and mucin quantification with either the PAS or the fluorometric assays after chromatographic purification, very different mean values were obtained. A close correlation and similar mean values were found with all assays after chromatographic purification. Additional experiments showed that CNA also reacted with low molecular biliary components other than mucin, confirming the lack of specificity of the direct fluorometric mucin quantification in human gallbladder bile. It is well conceivable that problems similar to the direct fluorometric mucin quantification in bile exist in other assays recently used for mucin determination in bile. A complex hexosamine method (14) and a direct dot blot assay with PAS staining (20) has been applied without previous validation assays. Indeed, the values of biliary mucin content communicated by these authors are remarkably low (14) or high (20).

Taken together, our experiments clearly indicate that human gallbladder mucins can be accurately measured after a simple chromatographic fractionation followed by PAS or fluorometric assay.

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