Fatty acid bile acid conjugates (FABACs)—New molecules for the prevention of cholesterol crystallisation in bile

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Abstract

***Background*—Cholesterol gall stones are a frequent disease for which at present surgery is the usual therapy. Despite the importance of bile acids it has become evident that phospholipids are the main cholesterol solubilisers in bile. Even phospholipid components, such as fatty acids, have anticrystallising activity.**

***Aim*—To synthesise fatty acid bile acid conjugates (FABACs) and study their effects on cholesterol crystallisation in bile in vitro and in vivo.**

***Methods*—FABACs were prepared by conjugation of cholic acid at position 3 with saturated fatty acids of variable chain length using an amide bond. Cholesterol crystallisation and its kinetics (crystal observation time, crystal mass) were studied in model bile, pooled enriched human bile, and fresh human bile using FABACs with saturated fatty acids of varying chain length (C-6 to C-22). Absorption of FABACs into blood and bile was tested in hamsters. Prevention of biliary cholesterol crystallisation in vivo was tested in hamsters and inbred mice.**

***Results*—FABACs strongly inhibited cholesterol crystallisation in model as well as native bile. The FABACs with longer acyl chains (C-16 to C-22) were more effective. At a concentration of 5 mM, FABACs almost completely inhibited cholesterol crystallisation in fresh human bile for 21 days. FABACs were absorbed and found in both portal and heart blood of hamsters. Levels in bile were 2–3 times higher than in blood, indicating active secretion. Appreciable levels were found in the systemic circulation 24–48 hours after a single administration. Ingested FABACs completely prevented the formation of cholesterol crystals in the gall bladders of hamsters and mice fed a lithogenic diet.**

***Conclusions*—FABACs are potent inhibitors of cholesterol crystallisation in bile. They are absorbed and secreted into bile and prevent the earliest step of cholesterol gall stone formation in animals. These compounds may be of potential use in cholesterol gall stone disease in humans.**

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Bile is the only significant pathway for cholesterol excretion from the body and most people have bile supersaturated with cholesterol. Approximately 15% of the population have gall stones, mostly cholesterol gall stones. The residence time of bile in the biliary tract is measured in hours. Prevention of cholesterol crystallisation during this interval may prevent the formation of gall stones. For existing symptomatic gall stones, the current therapy is cholecystectomy. It has been shown that cholesterol gall stones can be dissolved by oral bile salt therapy[1 2] but this was found to be of low efficacy.

In the past decade it became apparent that phospholipids, and not bile salts, are the main cholesterol solubilisers in bile.[3 4] Increasing the proportion of phospholipids in bile or modulating their molecular composition prevents cholesterol crystallisation.[4–6] Even parts of the phospholipid molecule such as saturated fatty acids have anticrystallising activity.[7 8] Modulation of biliary phospholipids in vivo is, however, presently not feasible.

Conjugation with bile acids is a known technique to effect the hepatic uptake and biliary secretion of various drugs.[9 10] In the present study we have synthesised fatty acid bile acid conjugates (FABACs) and tested their effects on cholesterol crystallisation kinetics in model and human bile. We have also investigated their absorption, biliary secretion, and effectiveness in preventing biliary cholesterol crystallisation in vivo.

Materials and methods

Egg yolk phosphatidylcholine (PC), 99% pure, was purchased from Avanti Polar Lipids, Inc. (Alabaster, Alabama, USA), and free fatty acids (FFA), cholesterol, sodium taurocholate (TC), and cholic acid (CA), >98% pure, from Sigma Chemical Co. (St Louis, Missouri, USA). Cholesterol, TC, and CA were recrystallised prior to use. All other chemicals and solvents were ACS or reagent grade. The glassware was acid washed prior to use.

PREPARATION OF CA AMIDES (FABACS)

3β Azido 7α, 12α dihydroxy, 5β cholan 24 oic acid methyl ester[11] was reacted with triphenyl phosphine and a series of saturated fatty acids in warm toluene. This reaction resulted in

Abbreviations used in this paper: FABACs, fatty acid bile acid conjugates; PC, phosphatidylcholine; TC, taurocholate; CA, cholic acid; C-16, C-18…, saturated fatty acids of chain length 16, 18, etc; COT, crystal observation time.

methyl esters of CA substituted at the 3β position with alkyl amido groups. The methyl ester function was then hydrolysed to give 3β-alkylamido-cholic acids. The saturated fatty acids used were: caproic (C-6), caprylic (C-8), capric (C-10), lauric (C12), myristic (C-14), palmitic (C-16), stearic (C-18), arachidic (C-20), and behenic (C-22) acids. The products obtained were purified by silica gel chromatography and characterised by their ^{1}H nuclear magnetic resonance and mass spectrometry.

BILE

(A) Model bile

The model bile was prepared from a mixture of cholesterol (in chloroform), PC (in chloroform), and TC (in methanol) in concentrations of 15, 30, and 150 mM, respectively, as previously described.[5] The final model bile solution was incubated under argon at 37°C, and aliquots were taken for analysis at predetermined time points throughout the crystallisation process.

(B) Human bile

Human gall bladder bile was obtained from cholesterol gall stone patients at cholecystectomy. Informed consent was obtained according to a protocol approved by the local institutional human subjects committee. Bile was ultracentrifuged (200 000 *g*, 45 minutes, 25°C) prior to testing to remove crystals and cell debris. All crystallisation experiments were performed at 37°C.

(C) Pooled enriched bile

Several gall bladder bile samples were pooled and mixed with a concentrated lipid solution (cholesterol:PC:TC 180:360:1200 mM) 19:1 (vol:vol) to provide a supersaturated rapidly nucleating bile solution.

The test compounds were added to human bile at various concentrations (1–10 mM). In model bile the test compounds (5–30 mM) were either added or substituted for equimolar amounts of the original TC by coprecipitation with the three biliary lipids during the initial drying process.

EVALUATION OF CHOLESTEROL CRYSTAL FORMATION AND GROWTH

(A) Crystal observation time (COT)

COT was determined as described by Holan and colleagues.[12] COT was defined as the initial time of detection of at least three cholesterol monohydrate crystals by polarised light microscopy per field at 100-fold magnification.

(B) Measurement of crystal mass

Chemical analyses of cholesterol were performed on each sample on the last day of the experiment, as previously described.[5] In brief, bile samples were ultracentrifuged, and the amount of cholesterol in the pellets was calculated (per ml of bile) by subtracting the amounts in the supernatant solutions from the total. The crystalline character of the pellet was confirmed by polarised light microscopy.

ANIMAL EXPERIMENTS

Male Golden Syrian hamsters, four weeks old (90–100 g), were fed a lithogenic diet[13] enriched with cholesterol (1%), palmitic acid (1.2%), and corn oil (2%) (w/w). Male inbred mice (C57J/L), six weeks old (20 g), were fed a lithogenic diet[14] enriched with butter fat (15%), cholesterol (1%), CA (0.5%), and corn oil (2%) (w/w). Control animals were fed a regular rodent chow. FABACs mixed with saline or water were administered to the animals intragastrically (via a syringe and tube). At predetermined time points the animals were anaesthetised (after an overnight fast), and blood was drawn by cardiac puncture and catheterisation of the portal vein. Gall bladder bile was removed with a syringe for microscopic examination (performed in a blinded manner) and aliquoted for chemical analysis. The gall bladder, and samples of the liver, kidneys, heart, and small and large intestines were taken for histological examination.

ANALYTICAL METHODS

Biliary lipids were extracted by chloroform: methanol (2:1, vol:vol) and quantitated as previously described.[15] FABAC concentrations were determined by high pressure liquid chromatography (Kontron) using a Phenomenex Luna reversed phase C-18 column. Samples were applied dissolved in methanol. The running phase was methanol 100% at a flow rate of 0.9 ml/min. FABACs were detected at 206 nm.

Results

All FABACs used in this study were conjugates of CA (at position 3), with saturated fatty acids of variable chain length. The conjugation was in the beta configuration using an amide bond (fig 1). The structure was confirmed by nuclear magnetic resonance and mass spectrometry for each new batch. The purified powdered FABACs, particularly those with long chain fatty acids, were almost insoluble in water. They dissolved completely in human bile at concentrations of 30 mM. Dissolution in model bile was less complete.

IN VITRO EXPERIMENTS

Figure 2A and B shows a representative series of 59 tests performed in model solutions with various FABACs. The effects of replacement of 20% of bile salts (30 mM) on COT (fig 2A) and crystal mass (fig 2B) in a model bile solution by equimolar amounts of various FABACs are shown. The FABACs had fatty acids of different chain length from 14 to 20 carbon

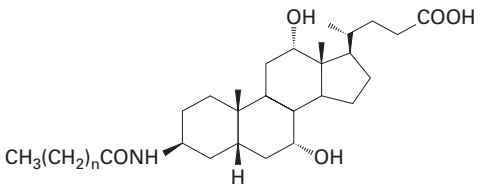

Figure 1 General structure of the fatty acid bile acid conjugates (FABACs). Cholic acid is conjugated using an amide bond (at position 3) with saturated fatty acids of variable chain length.

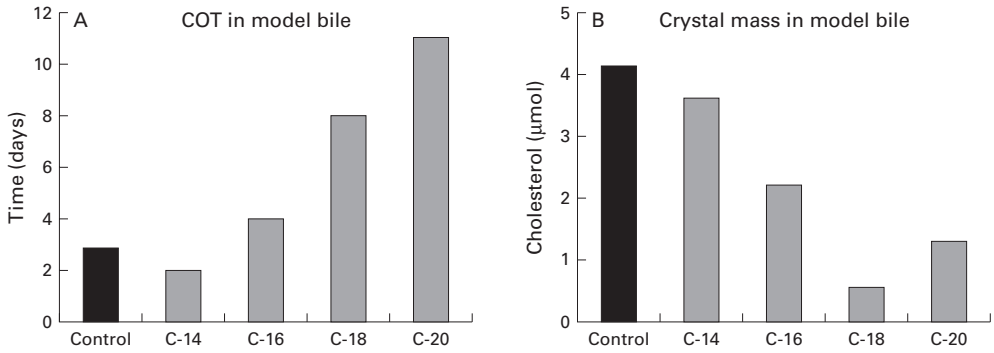

Figure 2 Effects of fatty acid bile acid conjugates (FABACs) on cholesterol crystallisation in model bile. (A) Crystal observation time (COT) and (B) total crystal mass after 14 days of incubation in the control model bile (cholesterol 15 mM, egg yolk PC 30 mM, sodium taurocholate 150 mM) and test biles in which 20 mol% of bile salts (30 mM) were replaced by equimolar amounts of FABACs (C-14, C-16, C-18, and C-20).

atoms. The FABAC with a C-14 saturated fatty acid had little activity compared with the control model bile. All other compounds prolonged COT (up to 400% of control for C-20). They reduced the cholesterol crystal mass to 14% of control (for C-18). FABACs with shorter chain fatty acids (C-6 to C-12) had little activity in terms of cholesterol crystallisation (data not shown).

EX VIVO EXPERIMENTS

Figure 3 shows representative examples of a total of 36 tests performed in human bile with various FABACs. Figure 3A shows the effects of addition of C-16, C-18, and C-20 FABACs at 5 mM to pooled enriched human bile on the crystal mass after 22 days of incubation. The controls were pooled bile with and without CA added at 5 mM. All three FABACs markedly reduced the final crystal mass, while equimolar amounts of CA had no effect.

Figure 3B shows the effects of addition of 5 mM of C-20 and C-22 FABACs to fresh human gall bladder bile on crystal mass after 21 days of incubation. Addition of each of the two FABACs reduced the crystal mass to minute amounts (1.9% and 5.7% of control, respectively). Some FABACs (for example, C-20 and C-22) reduced cholesterol crystallisation in human bile at concentrations of 3–5 mM (data not shown).

A saturated human bile (cholesterol 12.6 mM, phospholipids 29.2 mM, bile salts 91.2 mM, CSI 137) was incubated at 37°C for 21 days. Crystals were observed from day 8. They multiplied and reached a plateau. On day 21, 30 mM of a C-20 FABAC were added and microscopic observation was continued. Three days after addition of the FABAC, crystals were still seen. On day 10 after addition of the FABAC, no cholesterol crystals were present on repeated observations, indicating their dissolution.

IN VIVO EXPERIMENTS

Figure 4 shows blood and bile levels of C-16 and C-20 FABACs in hamsters after single intragastric administration of 30 mg of the compound at 0 time. The animals were kept fasted and were sacrificed at 1–4 hours. Animals tested at 6–48 hours were allowed food but were fasted for three hours prior to sacrifice in order to have a larger gall bladder volume. The compounds were found in the heart and portal blood, and in bile. Levels in bile were higher, indicating active secretion against a concentration gradient. Biliary levels of the C-16 conjugate peaked earlier (at two hours) while levels of the more hydrophobic C-20 peaked at four hours. There were small amounts of the C-20 conjugate in bile at 24 and 48 hours, suggesting a degree of enterohepatic circulation. The compound persisted in blood, at even higher concentrations than in bile, for 24–48 hours.

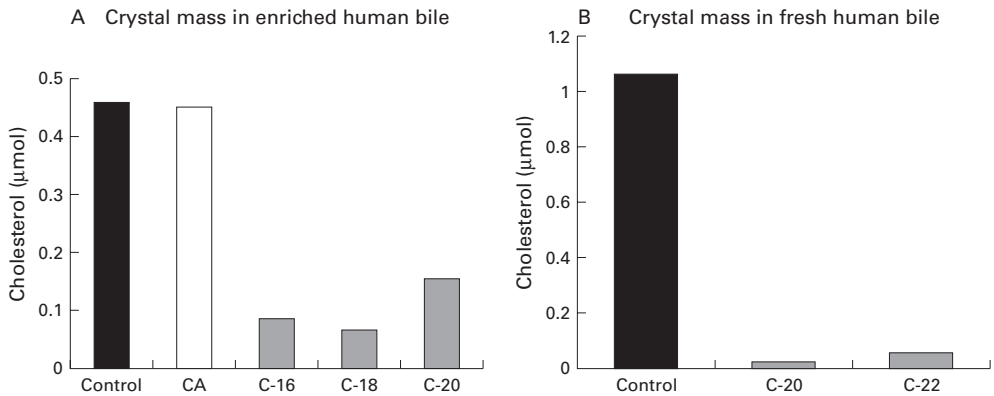

Figure 3 Effects of fatty acid bile acid conjugates (FABACs) on cholesterol crystallisation in human bile. (A) Cholesterol crystal mass after 22 days of incubation in pooled lipid enriched human bile, without (control) and with 5 mM of cholic acid (CA) or FABACs C-16, C-18, and C-20. (B) Cholesterol crystal mass after 21 days of incubation of fresh human gall bladder bile without (control) and with 5 mM of FABACs C-20 and C-22.

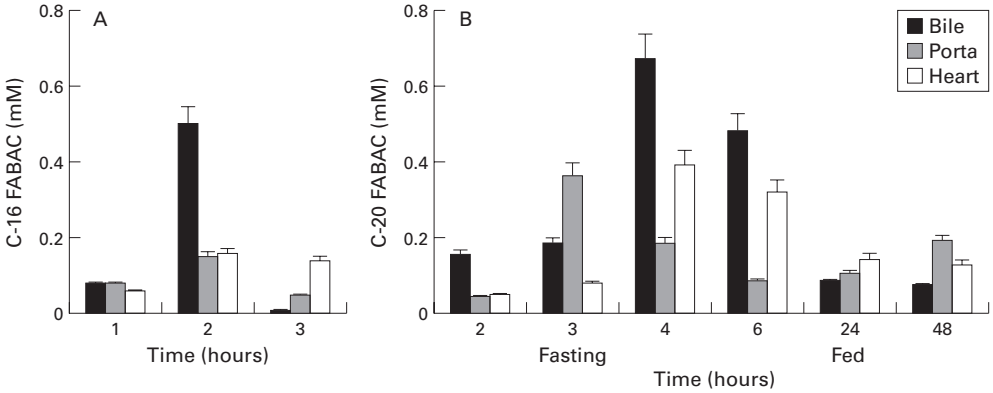

Figure 4 Absorption and secretion of C-16 (A) and C-20 (B) fatty acid bile acid conjugates (FABACs) in hamsters 1–48 hours after single intragastric administration of 30 mg (4.6 and 4.3 mmol, respectively) of the compounds. Blood (heart and portal) and bile concentrations of C-16 and C-20 FABACs are shown.

Twelve hamsters were fed a lithogenic diet for 10 weeks. Six hamsters were fed a regular diet. At 5.5 weeks, six on the lithogenic diet and three on the normal diet were also given a C-20 FABAC, 15 mg per animal per day. All were sacrificed at the end of 10 weeks. None of the hamsters on the normal diet had cholesterol crystals in bile. All animals on the lithogenic diet had cholesterol crystals in gall bladder bile. None of the animals whose lithogenic diet was supplemented with a C-20 FABAC had cholesterol crystals in gall bladder bile. FABAC concentrations in gall bladder bile were up to 0.65 mM. Blood levels of alanine aminotransferase, aspartate aminotransferase, creatinine, albumin, and alkaline phosphatase were within normal limits and not significantly different between the four groups. Blood cholesterol levels were 266 (35) mg/dl in animals on the lithogenic diet only, 221 (60) mg/dl in animals supplemented with C-20 FABAC ($p=0.6$), and ranged between 76 and 99 mg/dl in hamsters on the normal diet. Triglyceride levels were 190 (34) mg/dl, 223 (25) mg/dl ($p=0.11$), and 139–144 mg/dl, respectively. Histological examination of the liver, kidney, heart, and small and large bowel showed only microvesicular fat in the liver of all animals receiving the lithogenic diet.

Eighteen inbred C57J/L mice were fed a lithogenic diet. Nine mice were also given 3 mg of a C-20 FABAC daily, administered intragastrically suspended in 0.15 ml of water. The animals were sacrificed on day 14. All animals on the lithogenic diet had abundant cholesterol crystals in gall bladder bile. None of the animals supplemented with the FABAC had cholesterol crystals in bile. FABAC concentrations in gall bladder bile were up to 0.4 mM.

Discussion

FABACs are a new family of synthetic molecules. In the present study we have shown that they prevent cholesterol crystallisation in supersaturated model solutions and human bile, probably by markedly increasing cholesterol solubility. Moreover, they can dissolve existing cholesterol crystals. Since they are absorbed and secreted into bile, and prevent cholesterol crystallisation in vivo, they are potential new candidates for the medical prevention and/or dissolution of cholesterol gall stones in humans.

Several aspects of the metabolism, mechanism of action, and effects of FABACs remain to be studied. The ideal FABAC should have strong anticrystallising activity, be effectively absorbed and secreted into bile, have a significant enterohepatic recirculation, a long biological half life, and be devoid of significant side effects. An ideal compound fulfilling all of these requirements is not yet available and our study reports on the effects of several potentially useful FABACs.

We have studied mainly conjugates of CA with saturated long chain fatty acids. Conjugates with unsaturated fatty acids and ursodeoxycholic acid conjugates are being screened. Some FABACs exhibited anticrystallising effects in human bile at concentrations as low as 3–5 mM. In model solutions they were effective only at concentrations of 30 mM. At concentrations of 5 mM at which they are similarly soluble in both solutions, they showed an effect only in bile. The mechanism of this higher efficacy in human bile compared with model solutions is being studied. Holzbach *et al* previously observed that cholesterol precipitated much faster from model solutions than from human bile at identical lipid concentrations.[16] In vivo, in two animal species, FABACs prevented the formation of cholesterol crystals at biliary concentrations of 0.4–0.65 mM. This is lower than the concentrations required in vitro.

Long chain saturated free fatty acids have cholesterol solubilising activity.[7] FABACs can be used as both a means of introducing these fatty acids into bile and in addition they seem to be much more potent cholesterol solubilisers and have different properties from free fatty acids.

An unexpected finding was the high concentration and prolonged circulation of FABACs in the systemic circulation. This is unlike bile salts which are transported via the portal vein to the liver and bile, with only minimal amounts reaching the systemic circulation. FABACs are much more hydrophobic than bile salts and it is possible that they are at least partly absorbed via the lymph and thus go directly to the systemic circulation. The prolonged circulation in the vascular tree of

these compounds with a proven cholesterol solubilising activity inevitably raises the question of a potential effect in arteriosclerosis.

FABACs were previously found in faeces as a result of bacterial metabolism.[17] Kritchevsky's group synthesised FABACs of urso and chenodeoxycholic acids in an attempt to slow down the catabolism of these compounds by the bacterial flora.[18] They did not study any effects on cholesterol crystallisation. However, these and other reports in the literature relate to esters of fatty acids with bile acids. They lack the amide group contained in our FABACs. The amide group is essential as it confers stability to these compounds and prevents rapid breakdown by digestive and bacterial enzymes in the gut.

Prior to human studies, toxicity (which has not been noted to date) will have to be carefully assessed. Activity in the target organ, human gall bladder bile, has already been demonstrated. There is no a priori reason to assume that absorption and biliary secretion in humans will be different but this remains to be tested. After the safety of FABACs is established, absorption and biliary secretion in humans can be rapidly evaluated, for example, in patients with a T tube or prior to cholecystectomy.

In our animal studies we used high doses of FABACs (approximately 10-fold the estimated human dose) to screen for effects as well as toxicity. A dose ranging study is now in progress and preliminary data indicate that FABACs are effective at doses used in bile salt therapy in humans.

Inevitably the main question will relate to potential efficacy. Will these new molecules be more effective than the bile salts urso and chenodeoxycholate? Will they be useful for gall stone prevention? dissolution? or both? These issues remain to be evaluated. A potential effect in arteriosclerosis is being investigated.

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1 Danzinger RG, Hofmann AF, Schoenfield LJ, *et al.* Dissolution of cholesterol gallstones by chenodeoxycholic acid. *N Engl J Med* 1972;**286**:1–8.
2 Nakagawa S, Makino I, Ishizaki T. Dissolution of cholesterol gallstones by ursodeoxycholic acid. *Lancet* 1977;**2**:367–9.
3 Jungst D, Lang T, Huber P, *et al.* Effect of phospholipids and bile acids on cholesterol nucleation time and vesicular/micellar cholesterol in gallbladder bile of patients with cholesterol stones. *J Lipid Res* 1993;**34**:1457–64.
4 Halpern Z, Moshkowitz M, Laufer H, *et al.* Effect of phospholipids and their molecular species on cholesterol solubility and nucleation in human and model biles. *Gut* 1993;**34**:110–15.
5 Ringel Y, Somjen GJ, Konikoff FM, *et al.* The effects of phospholipid molecular species on cholesterol crystallization in model biles: the influence of phospholipid head groups. *J Hepatol* 1998;**28**:1008–14.
6 Ringel Y, Somjen GJ, Konikoff FM, *et al.* Increased saturation of the fatty acids in the sn-2 position of phospholipids reduces cholesterol crystallization in model biles. *Biochim Biophys Acta* 1998;**1390**:293–300.
7 Zijlstra AIM, van Overveld M, de Bruijn MA, *et al.* The influence of free fatty acids on biliary equilibria and their impact on cholesterol crystallization. *Gastroenterology* 1996; **110**:A952
8 Gilat T, Konikoff FM, Somjen GJ. Cholesterol crystallization—Effects of phospholipid species and derivatives. *3rd International Conference on Gallstones,* October 10–14, 1999, Tiberias, Israel: 27.
9 Kramer W, Wess G, Schubert G, *et al.* Liver-specific drug targeting by coupling to bile acids. *J Biol Chem* 1992;**267**: 18598–604.
10 Kramer W, Wess G. Bile acid transport systems as pharmaceutical targets. *Eur J Clin Invest* 1996;**26**:715–32.
11 Kramer W, Kurz G. Photolabile derivatives of bile salts. Synthesis and suitability for photoaffinity labeling. *J Lipid Res* 1983;**24**:910–23.
12 Holan KR, Holzbach RT, Hermann RE, *et al.* Nucleation time: a key factor in the pathogenesis of cholesterol gallstone disease. *Gastroenterology* 1979;**77**:611–17.
13 Ayyad N, Cohen BI, Mosbach EH, *et al.* Palmitic acid enhances cholesterol gallstone incidence in Sasco hamsters fed cholesterol enriched diets. *Lipids* 1992; **27**:993–8.
14 Khanuja B, Cheah YC, Hunt M, *et al.* Lith1, a major gene affecting cholesterol gallstone formation among inbred strains of mice. *Proc Natl Acad Sci USA* 1995;**92**:7729–33.
15 Konikoff FM, Laufer H, Messer G, *et al.* Monitoring cholesterol crystallization from lithogenic model bile by time-lapse density gradient ultracentrifugation. *J Hepatol* 1997;**26**:703–10.
16 Holzbach RT, Kibe A, Thiel E, *et al.* Biliary proteins. Unique inhibitors of cholesterol crystal nucleation in human gallbladder bile. *J Clin Invest* 1984;**73**:35–45.
17 Kelsey MI, Molina JE, Huang SK, *et al.* The identification of microbial metabolites of sulfolithocholic acid. *J Lipid Res* 1980;**21**:751–9.
18 Kritchevsky D, Poli G, Scolastico C, *et al.* Novel derivatives of 3 alpha,7 alpha-dihydroxy-5 beta-cholan-24-oic acid (chenodeoxycholic acid) and 3 alpha,7 beta-dihydroxy-5 beta-cholan- 24-oic acid (ursodeoxycholic acid). *Steroids* 1986;**47**:41–8.