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Journal of Chromatography B, 653 (1994) 1–7

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Simultaneous microanalysis of biliary cholesterol, bile acids and fatty acids in lecithin using capillary column gas chromatography: an advantage to assess bile lithogenecity

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(First received July 14th, 1993; revised manuscript received November 26th, 1993)

Abstract

Simultaneous determination of biliary lipids was performed by alkaline hydrolysis, the formation of the methyl ester derivatives of fatty acids that are constituents of phospholipids and of the acetylated methyl ester derivatives of bile acids, and subsequent analysis by capillary column gas chromatography. Complete separation and satisfactory recovery of cholesterol, bile acids, and fatty acids were achieved. Also, the accuracy of the calculation of the bile cholesterol saturation index was enhanced by computation. Since the degree of acyl chain unsaturation affects the cholesterol-holding capacity in vesicles, this method provides a unique insight into bile metastability by the quantitative assessment of fatty acids in lecithin.

1. Introduction

Cholesterol is solubilized in bile predominantly in the form of mixed micelles of bile salts and phospholipids, and the capacity of bile to solubilize cholesterol is regulated by its concentration relative to that of the bile salts and phospholipids. Thus, the formation of insoluble cholesterol in bile can be the result of an increase in the amount of cholesterol, a decrease in the amount of bile salts or phospholipids, or a combination of both factors. It is now clearly established that excess cholesterol relative to bile salts and phospholipids in gall bladder bile, *i.e.* supersaturated bile, is important in the formation of cholesterol gallstones. In 1968, Admirand and Small [1] calculated the molar percentages of biliary cholesterol, bile, salts, and phospholipids in an *in vitro* model bile system, and demonstrated a micellar or supersaturated zone on triangular coordinates. The curve for cholesterol saturation was modified by Hegardt and Dam [2], and further modified by Holzbach *et al.* [3]. In addition, a lithogenic index was calculated by Thomas and Hofmann [4] and Carey and Small [5].

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0378-4347/94/$07.00
SSDI 0378-4347(93)E0422-M

Knowledge of the relative concentrations of the various biliary lipids is necessary for an accurate determination of cholesterol solubility. When the concentrations of the different biliary lipids are determined separately, only a poor estimate of cholesterol solubility in bile is obtained. Furthermore, errors can be caused by the influence of viscosity or a high bilirubin concentration in the bile.

The accurate relative concentrations of the three classes of biliary lipids can be measured if we use the same condition and technique to determine each of them simultaneously. In previous studies by Yunker *et al.* [6] and by our group [7], simultaneous quantification of these three lipid classes was performed using a gas chromatograph equipped with a glass column packed with either QF-1 or OV-1, after bile acids were methylated but not acetylated. However, the resolution was poor and the sensitivity of detection of the bile acids and fatty acids was inadequate.

In the present study, the method of determining biliary lipids was improved by performing methyl acetylation of the bile acids and subsequent analysis by gas chromatography (GC) on a fused-silica capillary column. Also, the accuracy in calculating the bile cholesterol saturation index was enhanced by rapid computation. Further, the quantitative assessment of fatty acids in lecithin provided a unique insight into bile lithogenicity.

2. Experimental

2.1. Chemicals

Sodium salts of lithocholic acid (LCA), deoxycholic acid (DCA), chenodeoxycholic acid (CDCA), cholic acid (CA), and ursodeoxycholic acid (UDCA) were generous gifts from Tokyo Tanabe (Tokyo, Japan). They were more than 99% pure when examined by high-performance liquid chromatography. Cholesterol, 5α-cholestane, and fatty acids (myristic acid, palmitic acid, palmitoleic acid, stearic acid, oleic acid, linoleic acid, arachidic acid, eicosatrienoic acid, arachidonic acid, and docosahexaenoic acid) were purchased from Sigma (St. Louis, MO, USA). The solvents used were of analytical-reagent grade and were redistilled prior to use.

2.2. Bile sample collection

Gall bladder bile samples were obtained from 76 patients during surgery by aseptic needle aspiration. These patients were divided into four groups on the basis of the presence or absence and type of stones. The stones were analysed by infrared spectrometry, and a stone having a cholesterol content of more than 70% was defined as a "cholesterol stone". The group with cholesterol stones contained 41 patients, 10 of which had been treated before surgery with ursodeoxycholic acid (600 mg/day for at least 6 months). The group with non-cholesterol stones consisted of 25 patients. Informed consent was obtained before operation in all cases.

2.3. Analysis of biliary lipids by capillary column gas chromatography

An amount of 100 μg of 5α-cholestane in 1 ml of chloroform–methanol (2:1, v/v) was added as an internal standard to a 0.1-ml bile sample, followed by quick vortex-mixing and then evaporation of the organic solvent under a nitrogen stream. After 10 ml of 1 *M* NaOH had been added to the dried sample, followed by quick vortex-mixing, hydrolysis of conjugated bile acids and lecithin was performed by autoclaving at 1.5 kg/cm^2 and 120°C for 3 h. Subsequently, 1.5 ml of 1 *M* HCl were added for acidification, after the sample had been transferred to a test-tube with a ground-glass stopper.

The lipids were extracted three times with 5 ml of diethyl ether, and collected in a test-tube with a ground-glass stopper. The residual diethyl ether was evaporated under a nitrogen stream, and the dried sample was resolubilized by the addition of 0.5 ml of methanol and 5 ml of diazomethane in diethyl ether to methylate the extracted lipids. After incubation for 30 min at room temperature, the solution developed a

yellow colour. The organic solvent was then evaporated under a nitrogen stream, after which 2 ml of the acetylation solution (acetic anhydride–acetic acid–70% perchloric acid, 10:14:1, v/v) was added [8]. The mixture was cooled in ice-water for 1.5 h, and lipids were extracted with diethyl ether after the addition of 4 ml of 20% NaCl.

The extract was analysed with a Shimadzu GC-9A gas chromatograph (Shimadzu, Tokyo, Japan) equipped with a flame ionization detector and a solventless glass solid injector. The column was a fused-silica capillary column (25 m × 0.24 mm I.D.) coated with OV-1 (phase layer thickness, 0.25 μm; Chromatopackings Center, Tokyo, Japan). The initial column temperature was set at 170°C, and this was increased at 3°C/min to 290°C, where it was maintained for 15 min. The carrier gas was nitrogen at a flow-rate of 3 ml/min. A 5-μl aliquot of the sample, resolubilized with *n*-hexane, was injected into the column with a microsyringe. Peak areas were electronically integrated, and molar concentrations of fatty acids, cholesterol, and bile acids were calculated by the internal standard technique using a Shimadzu C-RIB Chromatopack.

2.4. Computation of calculation of bile lithogenic index

Since glycerophospholipids contain two fatty acid molecules, the molar concentration of phospholipids is calculated by dividing the total molar concentration of the total fatty acids by two. Calculations for the molar percentages of phospholipids, cholesterol, and total bile acids and for the bile lithogenecity were performed using the personal computer technique. Briefly, Microsoft Excel (Version 4.0 or 3.0, Microsoft) was employed to create the calculating program. The program is written in macro-command of Excel and is able to run on both Microsoft Windows and Macintosh computers. The program was designed to estimate the conventional concentrations of cholesterol, bile acids, and phospholipids by inputting the GC data. We used the fifth-degree polynomial equations generated by Carey [9] to calculate the cholesterol saturation index, and the third-degree polynomial equations generated by Admirand and Small [1] to calculate the lithogenic index. Also, the calculated cholesterol saturation was appropriately corrected for the presence of ursodeoxycholic acid according to the previous proposal [10].

2.5. Calibration graphs for cholesterol, bile acids, and fatty acids

A mixture of cholesterol, bile acids, and fatty acids was prepared as follows. Cholesterol, LCA, DCA, CDCA, CA, UDCA, and authentic fatty acids (myristic acid, palmitic acid, palmitoleic acid, stearic acid, oleic acid, linoleic acid, arachidic acid, eicosatrienoic acid, arachidonic acid and docosahexaenoic acid) were dissolved in chloroform–methanol (2:1, v/v) (100 μg/ml), and the solution was further diluted 10- and 100-fold with the same organic solvent. For the internal standard, 5α-cholestane in chloroform (10 μg/ml) was pipetted into each Kleisen flask and evaporated to dryness. The residue was derivatized as described above and subjected to GC analysis.

2.6. Recovery of cholesterol in bile

A known amount of cholesterol was added to bile to determine the recovery. Cholesterol was determined by GC following hydrolysis and derivatization, as described above, using 5α-cholestane as an internal standard.

2.7. Comparison of results for biliary lipids determined by the GC method and by other methods using spectrophotometry

Values of cholesterol, total bile acid and phospholipid determined by the present GC method were compared with those obtained by the enzymic method for cholesterol [11] and for total bile acid [12] and by the determination of inorganic phosphorus for phospholipid [13].

3. Results

3.1. Gas chromatography of biliary lipids

The GC elution patterns obtained with the present method using a glass capillary column are shown in Fig. 1 (standard and human gall bladder bile sample). The lipids were eluted in the following order: methyl ester derivatives of fatty acids that were constituents of phospholipids; 5α-cholestane (the internal standard); free cholesterol; and acetylated methyl ester derivatives of bile acids. Retention times, relative retention times, and the reproducibility of peak areas are summarized in Table 1. Also, the intra- and inter-assay accuracy and precision were satisfactory.

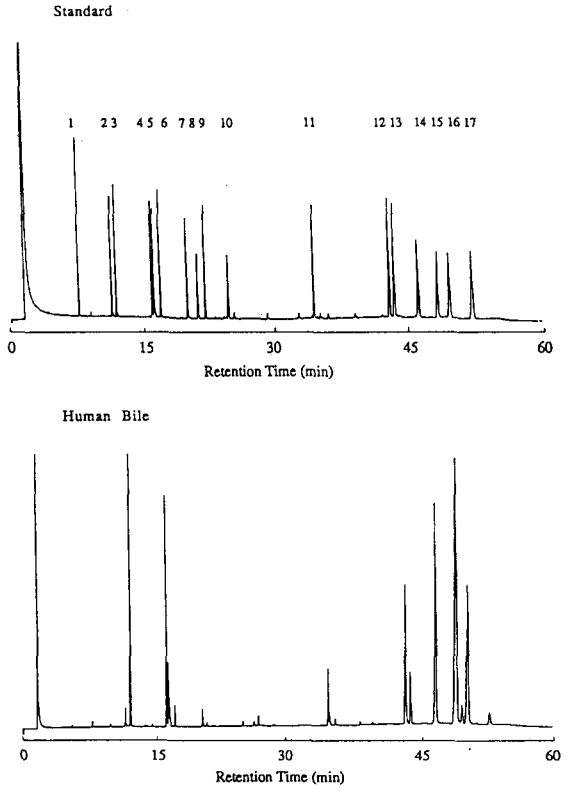

Fig. 1. Gas chromatographic elution patterns of fatty acids, the internal standard (I.S.), cholesterol, and bile acids for the standard mixture (upper) and for a bile sample (lower). Peaks: 1 = myristic acid; 2 = palmitoleic acid; 3 = palmitic acid; 4 = linoleic acid; 5 = oleic acid; 6 = stearic acid; 7 = arachidonic acid; 8 = arachidic acid; 9 = eicosatrienoic acid; 10 = docosahexaenoic acid; 11 = 5α-cholestane (internal standard); 12 = cholesterol; 13 = lithocholic acid; 14 = deoxycholic acid; 15 = chenodeoxycholic acid; 16 = cholic acid; 17 = ursodeoxycholic acid.

3.2. Recovery of cholesterol added to bile

Table 2 shows the recovery of cholesterol added and the calculated value of cholesterol in bile by the GC method and those given by the enzymic method after solvent extraction. The accuracy and precision of the GC method were satisfactory.

3.3. Comparison of results for biliary lipids determined by the GC method and by spectrophotometric methods

Values for cholesterol, total bile acids and phospholipids obtained by the GC method agreed well with those given by the enzymic methods and the determination of inorganic phosphorus. Correlation coefficients were 0.93 for cholesterol (GC method–enzymic method), 0.96 for total bile acid (GC method–enzymic method), and 0.94 for phospholipid (GC method–inorganic phosphorus determination).

3.4. Analysis of human gall bladder bile

Table 3 shows data for human gall bladder bile samples obtained by the GC method. The highest cholesterol saturation index was found in bile samples from cholesterol gallstone patients. In addition, the proportion of polyunsaturated fatty acids in biliary phospholipid was significantly higher in the same group when compared with others (Table 4). Also, the proportion of UDCA was highest in cholesterol gallstone patients treated with UDCA prior to operation, and that of CDCA was highest in those treated with CDCA (data not shown).

4. Discussion

Accurate determination of the relative concentrations of the three classes of biliary lipids is

Table 1
Retention data of biliary lipids and the internal standard by capillary column GC

Lipid	Retention time (min)	Relative retention time	C.V. of peak area ($n = 10$) (%)
Myristic acid	7.37	0.21	6.6
Palmitoleic acid	11.03	0.32	5.8
Palmitic acid	11.57	0.34	6.0
Linoleic acid	15.59	0.45	5.3
Oleic acid	15.82	0.46	5.3
Stearic acid	16.57	0.48	5.2
Arachidonic acid	19.72	0.57	4.5
Arachidic acid	20.92	0.61	4.2
Eicosatrienoic acid	21.79	0.63	3.8
Docosahexaenoic acid	24.53	0.71	2.4
5α-Cholestane (internal standard)	34.40	1.00	
Cholesterol	42.93	1.25	2.2
LCA	43.48	1.26	2.5
DCA	46.15	1.34	2.5
CDCA	48.35	1.41	2.6
CA	49.70	1.44	2.7
UDCA	52.30	1.53	2.7

important for calculating the maximal cholesterol solubility. Currently, biliary lipids are estimated individually either by GC, enzymic methods, or high-performance liquid chromatography. However, small errors in pipetting while determining one lipid molar percentage will upset the lithogenic index, which is calculated from an equation of higher power. The influence on colorimetry of a high bilirubin content in concentrated bile may also cause errors in the determination of biliary lipids. In this regard, a simultaneous determination of biliary lipids is certainly useful and advantageous. Yunker *et al.* [6] reported a method for simultaneously measuring cholesterol, bile acids, and phospholipids in bile (analysed as their component fatty acids after hydrolysis) by temperature-programmed GC. They used 3% QF-1 for the equipped column, methylated and esterified biliary lipids derivatives, and a three-step increase in temperature.

Table 2
Recovery of cholesterol added to bile

Sample	Amount added to bile (mg/ml of bile)			
	0	5	10	15
1	5.9	10.1	16.5	21.3
2	6.5	10.9	15.9	22.1
3	5.6	10.5	15.8	21.0
4	4.9	10.2	14.5	19.3
5	7.1	11.9	17.0	21.6
Recovery (%)	–	94.4	99.4	100.4

The present method provides several improvements by using a fused-silica capillary column coated with OV-1, 5α-cholestane as the internal standard, a one-step method for increasing column temperature from 170°C to 290°C, and computation of the calculation of the bile lithogenic index. A previous communication from our laboratory [7] reported that the cholesterol, bile acids, and fatty acids composing the phospholipids in bile could be measured simultaneously by GC. However, our previous method still had some problems with measuring bile acids. Methylation of biliary lipid derivatives increased the sensitivity of detection of fatty acids, but not that of bile acids, particularly CA.

Table 3
Lithogenecity in human gall bladder bile

	Cholesterol saturation index			
	Cholesterol stone			Non-cholesterol stone
	UDCA (−)	UDCA (+)	CDCA (+)	
Ref. 1				
Mean	1.46[a]	0.97	0.78	1.01
S.E.	0.28	0.21	0.22	0.69
Ref. 9				
Mean	2.23[a]	1.36	1.14	1.57
S.E.	0.39	0.29	0.12	0.88

[a] $p < 0.05$ compared with other group (Mann–Whitney U test).

Although methyl acetylation or methyl propionation of biliary lipid derivatives markedly increased the sensitivity of detection of bile acids, the resolution of the biliary lipids (especially between cholesterol and LCA) was decreased by using a one-step temperature increase. Therefore, the method was varied by the use of a silicone OV-1 capillary column. Both the sensitivity of detection of bile acids and the resolution of cholesterol from LCA were improved with the present method. However, the sample preparation procedure in the present study is still time-consuming and, therefore, further technical devices are needed to simplify such a process.

Another advantage of the present method was that the fatty acid composition was simultaneously provided. The molecular packing of cholesterol lecithin monolayers is related to the degree of acyl chain unsaturation of lecithin, which also governs the cholesterol distribution between biliary lipid particulate species [14,15]. Thus, an assessment of the fatty acid composition in lecithin provides more useful information on the bile metastability. In fact, this method revealed

Table 4
Fatty acid composition in biliary lecithin

Fatty acid	Composition (mol. %)			
	Cholesterol stone			Non-cholesterol stone
	UDCA (−)	UDCA (+)	CDCA (+)	
Saturated				
Mean	57.5	62.6	58.1	62.3
S.E.	3.4	2.9	3.5	3.2
Monounsaturated				
Mean	15.8	16.3	15.9	19.0
S.E.	1.9	1.6	1.8	2.0
Polyunsaturated				
Mean	26.7[a]	21.0	25.9[a]	18.7
S.E.	1.8	1.9	2.1	1.1

[a] $p < 0.05$ compared with non-cholesterol stone samples.

that the proportion of polyunsaturated fatty acid, especially linoleate and archidonate, in biliary lecithin was higher in cholesterol gallstone patients than in patients with non-cholesterol gallstones or without stones. This disagrees with previous results [16], and such a discrepancy might be due to differences in analytical methods or in the race or dietary condition of the subjects. A study of a much larger group may provide an explanation for this. In any event, data obtained from the present method are primarily in agreement with recent scientific investigations.

Thus, data on the fatty acid composition in lecithin provided by the present GC method can help to estimate the bile metastability. In addition, the rapid computation improved the accuracy in calculating the bile cholesterol saturation.

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