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SIMULTANEOUS MICROANALYSIS OF BILE ACIDS AND CHOLESTEROL IN BILE BY GLASS CAPILLARY COLUMN GAS CHROMATOGRAPHY

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SUMMARY

Simultaneous quantitative microanalysis of bile acids and cholesterol was carried out by enzymatic hydrolysis, the formation of the ethyl ester dimethylethylsilyl ether derivatives and subsequent analysis by glass capillary gas chromatography. A complete separation and satisfactory recovery of cholesterol and the five major bile acids commonly occurring in human and hamster bile were obtained. The method is applicable to individual small animal models such as hamster from which only a small amount of bile is available.

INTRODUCTION

Routine analysis of bile usually includes the determination of the three major constituents, i.e., cholesterol, bile acids and phospholipid. Cholesterol has been determined either spectrophotometrically [1–4], enzymatically [5–7] or by gas chromatography (GC) [8–10] after solvent extraction in order to separate interfering substances such as bile pigment. Bile acids presented more difficult problems. Human bile contains five major bile acids, i.e., cholic, chenodeoxycholic, deoxycholic, ursodeoxycholic and lithocholic acid. They are conjugated mainly with glycine or taurine. Bile acids can be determined either spectrophotometrically [11–13], by thin-layer chromatography (TLC), by high-performance liquid chromatography [14] or by GC. GC has been considered as a standard method of analysis for bile acids, but it requires hydrolysis and derivatization steps to render naturally occurring bile acids volatile. Each of these steps requires a critical evaluation as they are found to be either incomplete or to lead to further degradation and the formation of side-products. The analysis of phospholipids is usually performed by the determination of organic phosphorus after solvent extraction [15], which is sensitive enough to be applied to very small volumes of bile.

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In this work, each step in the routine chemical analysis used in this laboratory was critically examined in order to determine cholesterol and major bile acids (cholic, chenodeoxycholic, deoxycholic, ursodeoxycholic and lithocholic acid) present in hamster and human bile simultaneously. The method is useful for determining individual biliary lipids in hamster gall bladder bile.

EXPERIMENTAL

Chemicals

5β-Cholanic acid-3α-ol (lithocholic acid, LCA), 5β-cholanic acid-3α,12α-diol (deoxycholic acid, DCA), 5β-cholanic acid-3α,7α-diol (chenodeoxycholic acid, CDCA) and 5β-cholanic acid-3α,7α,12α-triol (cholic acid, CA) and sodium salts of their glycine- and taurine-conjugated bile acids were purchased from Calbiochem (San Diego, CA, U.S.A.) or Steraloid (Wilton, NH, U.S.A.). 5β-Cholanic acid-3α,7β-diol (ursodeoxycholic acid, UDCA) and its glycine- and taurine-conjugated bile acids were kindly supplied by Tokyo Tanabe (Tokyo, Japan). 23-Nor-5β-cholanic acid-3α,12α-diol (23-nordeoxycholic acid, NDCA), 5β-cholanic acid-3,7,12-trione, 5β-cholanic acid-3α-ol-7-one, 5β-cholanic acid-3α,12α-diol-7-one, 5β-cholanic acid-3α,7α-diol-12-one, 5β-cholanic acid-3-one and 5β-cholanic acid were purchased from Steraloid. Their purities were greater than 98% as determined by TLC and GC. The purity of NDCA was more than 95% and the extraneous peak observed on the gas chromatogram was its methyl ester. Cholylglycine hydrolase, a partially purified lyophilized powder, was purchased from Sigma (St. Louis, MO, U.S.A.). Sephadex LH-20 (25–100 μm) was purchased from Pharmacia (Uppsala, Sweden). Ethanolic hydrogen chloride was prepared by bubbling dry hydrogen chloride through cold ethanol. Dimethylethylsilylimidazole (DMESI) was purchased from Tokyo Kasei Kogyo (Tokyo, Japan). The solvents used were of analytical-reagent grade and were redistilled prior to use.

Human gall bladder bile was obtained by puncture of the gall bladder with a syringe during laparotomy for hepatobiliary disease. Hamster gall bladder bile was obtained by puncture of the gall bladder with a pre-weighed glass capillary tube.

Gas chromatography

A Shimazu GC-6AM gas chromatograph equipped with a flame ionization detector and a solventless glass solid injector was employed. The column was a WCOT glass capillary (25 m×0.35 mm I.D.) coated with SE-30 (LKB, Stockholm, Sweden). The temperature of the column oven was maintained at 275°C and those of the injection port and the detection were 290°C. The carrier gas was helium at a flow-rate of 1.5 ml/min. Peak areas were calculated by use of a digital integrator (Shimazu Chromatopac E1A).

Hydrolysis

Glycine-conjugated bile acids, especially glycolithocholic acid (GLCA), gave low recoveries and needed longer incubation time owing to poor solubility in the aqueous phase, as described previously [16,17]. To investigate the optimal con-

ditions for the enzymatic hydrolysis we used a mixture of GLCA and NDCA. The mixture was placed in 12-ml centrifuge tube and the solvent was evaporated completely under a stream of nitrogen. The residue was dissolved in various amounts of ethanol and then 0.4 mmol of acetate buffer (pH 5.6), 40 μmol of Na_2EDTA, 40 μmol of β-mercaptoethanol and 0.5 U of cholylglycine hydrolase were added to give a final volume of 2.7 ml. The final percentages of ethanol were 0, 2, 4, 6, 8, 10, 12, 14, 16 and 18%.

For each ethanol percentage, enzymatic hydrolysis was performed in triplicate at 37°C for 18 h under continuous stirring and it was found that the ethanol used to dissolve GLCA did not improve the recovery. Lower recoveries were observed when more than 10% ethanol was used. Therefore, ethanol was not used in the subsequent study.

Enzymatic and alkaline hydrolysis were compared. A mixture of five kinds of bile acids conjugated with glycine or taurine were dissolved in ethanol in 10-ml volumetric flasks. NDCA was added to each flask as an internal standard. The mixture was made up to volume with ethanol and an aliquot was used for a comparative study between enzymatic and alkaline hydrolysis in PTFE and glass tubes.

Enzymatic hydrolysis was performed as follows. The mixture was transferred into a 12-ml centrifuge tube with a glass stopper and the solvent was evaporated under a stream of nitrogen. The mixture contained about 100 μg of total bile acid. The residue was dissolved in 0.4 mmol of acetate buffer (pH 5.6), 40 μmol of Na_2EDTA, 40 μmol of β-mercaptoethanol and 0.5 U of cholylglycine hydrolase in a final volume of 2.7 ml and the mixture was incubated at 37°C under continuous stirring for 18 h.

Alkaline hydrolysis was performed as follows. The mixture was placed in a PTFE or glass tube and the solvent was evaporated under a stream of nitrogen. The mixture contained about 100 μg of total bile acids. The residue was dissolved in 2.0 ml of 5% (1.25 *M*) aqueous sodium hydroxide. The PTFE or glass tube was sealed and kept at 120°C for 7 h [18].

The stability of authentic free bile acids and conjugated bile acids was also examined and the stability of keto bile acid during alkaline hydrolysis was investigated by TLC and GC.

Derivatization

After hydrolysis, the solution was acidified to pH 1 with concentrated hydrochloric acid and the bile acids were extracted three times with 5 ml of ethyl acetate. The combined ethyl acetate mixture was transfered into a Kleisen flask and evaporated to dryness. To each hydrolysed sample 1 ml of 5% (w/v) ethanolic hydrogen chloride solution was added and the mixture allowed to stand for 60 min at room temperature. The solvent was evaporated under a stream of nitrogen and the residue was treated with 50 μl of DMESI and allowed to stand for 30 min.

Excess of silylating reagent was removed using a Sephadex LH-20 column 50 mm × 7 mm I.D. with *n*-hexane–chloroform–methanol (10:10:1, v/v/v) as eluent [19]. The DMES ether derivatives of cholesterol and bile acid ethyl esters were recovered in the first 2.5 ml of eluate. This eluate was evaporated to dryness and

the residue was dissolved in 5% (v/v) pyridine–*n*-hexane and subjected to glass capillary GC.

Calibration graphs for bile acids and cholesterol

A mixture of authentic bile acids and cholesterol was prepared as follows. Cholic, deoxycholic, ursodeoxycholic, chenodeoxycholic, lithocholic acid and cholesterol (100 μg/ml) were dissolved in ethanol and the solution was further diluted 2-, 5-, 10- and 100-fold with ethanol. For the internal standard, nordeoxycholic acid (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.

Recovery of cholesterol

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 NDCA as an internal standard. In a separate study, cholesterol in bile was determined spectrophotometrically [1,2] and by the described GC method and the values was compared.

Application to human and hamster gall bladder bile

A 5-μl sample of hamster gall bladder bile was pipetted into 5-ml volumetric flask filled about two thirds to capacity with ethanol. The mixture was brought to boiling on a hot water-bath and cooled to room temperature. The mixture was made up to volume with ethanol and transferred into a 10-ml centrifuge tube with a stopper and centrifuged at 400 *g* for 10 min to precipitate the protein. The ethanol extract of bile was used for analysis of biliary lipids.

Briefly, 0.02 and 0.04 ml of the ethanol extract of bile were used for the total bile acid determination by the 3α-hydroxysteroid dehydrogenase method according to Mashige et al. [20]. A 1-ml volume was used for phospholipid determination according to the modification by Bartlett [15]. A 2-ml volume of the ethanol extract of bile were used for the determination of cholesterol and each bile acid. A 2-ml sample was pipetted into a 12-ml centrifuge tube with a glass stopper and 5 μg of NDCA were added as an internal standard and mixed well. The solvent was evaporated and the enzymatic hydrolysis was carried out. After hydrolysis, extraction and derivatization was performed as described above and the sample was analysed by glass capillary GC.

Comparison of results for total bile acid determined by the GC method and by the 3α-HSD method

We compared the bile acid value calculated by the GC method with those given by the enzymatic method using 3α-HSD according to Mashige et al. [20].

RESULTS

Hydrolysis

Enzymatic and alkaline hydrolysis in PTFE and glass tubes were compared. Table I shows the recovery of five kinds of glycine-conjugated bile acids. Enzy-

TABLE I

COMPARISON OF ENZYMATIC AND ALKALINE HYDROLYSIS IN PTFE AND GLASS TUBES

Recoveries of five glycine-conjugated bile acids (mean ± S.D., $n=6$).

Hydrolysis	Tube	Recovery (%)				
		GLCA	GDCA	GCDCA	GUDCA	GCA
Alkaline	PTFE	83.6 ± 0.3	90.5 ± 0.5	84.1 ± 0.4	100 ± 0.3	93.7 ± 0.6
Alkaline	Pyrex glass	72.4 ± 0.5	87.9 ± 0.7	81.0 ± 0.5	100 ± 0.4	85.3 ± 0.9
Enzymatic	Centrifuge glass	88.2 ± 0.1	96.5 ± 0.1	89.0 ± 0.3	100 ± 0.2	96.0 ± 0.1

TABLE II

COMPARISON OF ENZYMATIC AND ALKALINE HYDROLYSIS IN PTFE AND GLASS TUBES

Recoveries of five taurine-conjugated bile acids (mean ± S.D., $n=6$).

Hydrolysis	Tube	Recovery (%)				
		TLCA	TDCA	TCDCA	TUDCA	TCA
Alkaline	PTFE	100 ± 0.9	99.6 ± 0.5	87.4 ± 0.8	85.9 ± 0.5	96.1 ± 0.7
Alkaline	Pyrex glass	95.5 ± 1.8	99.3 ± 1.7	84.0 ± 1.5	82.7 ± 0.6	90.3 ± 1.4
Enzymatic	Centrifuge glass	100 ± 0.4	100 ± 0.3	92.1 ± 0.3	92.5 ± 0.6	98.5 ± 0.3

matic hydrolysis was better with regard to both recovery and reproducibility. In alkaline hydrolysis, the PTFE tube was preferable to the glass tube. Table II shows the recovery of five kinds of bile acids conjugated with taurine. Generally good recoveries and satisfactory reproducibility were obtained in enzymatic hydrolysis. 3-Keto bile acid and triketo bile acid were markedly decomposed under alkaline hydrolysis, as indicated by TLC and GC. These results show that enzymatic hydrolysis is better than alkaline hydrolysis, but if alkaline hydrolysis is performed, a PTFE tube must be used for quantitative analysis.

Derivatization

The DMES ether derivatives of cholesterol and bile acid ethyl esters could be completely separated on the SE-30 glass capillary column and appeared in order of the number of hydroxy groups present. Table III shows methylene unit values of the DMES derivatives of bile acid ethyl esters and cholesterol. Complete separation, satisfactory reaction at room temperature and good reproducibility were obtained.

The derivatives were stable for more than six months. This is better than the stability of other derivatives such as trimethylsilyl (TMS) and hexafluoroisopropyl ester-trifluoroacetyl (HFIP-TFA) derivatives.

TABLE III

METHYLENE UNIT VALUES OF THE DMES ETHER DERIVATIVES OF BILE ACID ETHYL ESTERS AND CHOLESTEROL

Compound	Methylene unit value
Cholesterol	32.86
LCA	33.27
NDCA	33.48
DCA	34.71
CDCA	35.02
UDCA	35.39
CA	36.23

TABLE IV

RECOVERY OF CHOLESTEROL ADDED TO BILE

Sample (X_0+na)* ($n=0, 1, 2, 3$)		Amound added (mg/ml of bile)	Amount found (mg/ml of bile)	Recovery (mean ± S.D.) (%)	Estimated value ± 95% confidence limit (mg/ml of bile)
A	(X_0)	0	6.05, 6.15, 6.35		5.82 ± 0.61
B		0	6.35, 6.50, 6.40		
C	(X_0+a)	5.04	10.70, 10.80, 10.70	85.5 ± 5.9	
D		5.04	10.50, 10.40, 10.50		
E	(X_0+2a)	10.08	16.25, 16.70, 16.25	97.3 ± 5.0	
F		10.08	15.95, 15.80, 15.70		
G	(X_0+3a)	15.12	22.55, 22.35, 22.10	100.0 ± 4.8	
H		15.12	20.65, 20.75, 21.15		

*X_0 = amount of cholesterol present in bile; na = amount of cholesterol added to bile.

Recovery of cholesterol in bile

Table IV shows the recovery of cholesterol added, the calculated value of endogenous cholesterol and the 95% confidence limits, the last two parameters being obtained using the orthogonal polynomial equation.

Satisfactory accuracy and precision were obtained by this method. The cholesterol concentration in bile obtained by GC and the spectrophotometric method agreed well ($r=0.91$).

Application to hamster gall bladder bile

Fig. 1 shows the chromatogram of bile acids and cholesterol of hamster gall bladder bile. Cholesterol and each bile acid were quantitatively analyzed. An unknown minor peak eluted before DCA.

Comparison of results for total bile acid determined by the GC method and by the 3α-HSD method

Table V shows the analytical data for human gall bladder bile and individual hamster gall bladder bile. Slightly lower values were obtained by the GC method,

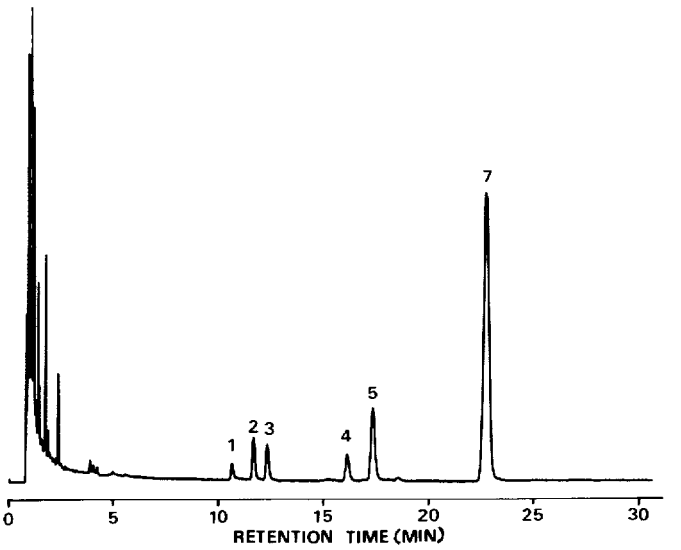

Fig. 1. Chromatogram of the DMES ether derivatives of cholesterol and bile acid ethyl esters from hamster gall bladder bile. Peaks: 1 = cholesterol; 2 = lithocholic acid; 3 = nordeoxycholic acid (internal standard); 4 = deoxycholic acid; 5 = chenodeoxycholic acid; 7 = cholic acid. The methylene unit values of these compounds are given in Table III.

TABLE V

COMPARISON OF THE DETERMINATION OF BILE ACIDS BY THE GC AND 3α-HSD METHODS

N.D. = not detectable.

Sample*	Concentration (μg/μl)					Total bile acids (μg/μl)		Correlation coefficient (GC method/ 3α-HSD method)
	LCA	DCA	CDCA	UDCA	CA	GC method	3α-HSD method	
HGB-1	N.D.	1.32	37.90	4.02	42.63	85.87	86.00	0.998
HGB-2	1.28	14.72	22.81	2.97	19.16	60.94	64.60	0.943
HGB-3	1.63	8.00	39.27	4.69	21.80	75.39	80.80	0.933
HGB-4	N.D.	N.D.	22.75	1.68	18.90	43.33	45.20	0.959
HGB-5	1.18	21.61	26.74	3.43	19.98	72.94	79.60	0.916
HaGB-1	0.317	4.112	3.049	N.D.	20.040	27.52	30.60	0.899
HaGB-2	1.163	0.893	3.067	N.D.	12.636	17.76	19.72	0.901
HaGB-3	0.561	4.532	4.009	N.D.	15.095	24.20	25.16	0.962
HaGB-4	0.281	2.356	3.395	N.D.	20.591	26.62	27.80	0.958
HaGB-5	0.341	2.742	1.877	N.D.	8.624	13.58	14.80	0.918
Mean ± S.D.								0.939 ± 0.031

*HGB = human gall bladder bile; HaGB = hamster gall bladder bile.

probably owing to hydrolysis and the presence of other bile acids [21,22]. The total bile acids determined by the GC method were in close agreement with those obtained by the enzymatic method.

DISCUSSION

The hydrolysis of conjugated bile acids presented a major obstacle to the accurate determination of bile acids in biological fluids. Alkaline hydrolysis has been known to lead to low recoveries if glass containers are used, as shown in Tables I and II. The use of PTFE vessels is said to improve the recovery, probably by inducing less decomposition of bile acids. The later introduced enzymatic hydrolysis using cholylglycine hydrolase from *Clostridium perfringens* is mild, gives good recoveries (Tables I and II) and seems to produce no further degradation. This is most advantageous when analysing ketone-containing bile acids, especially 3-keto bile acid [23,24], which undergoes extensive degradation under alkaline hydrolysis. However, it may introduce unknown contaminants from the enzyme preparation used, which may interfere with the subsequent structural analaysis. Further, as the rate of hydrolysis differs for each conjugated bile acid and some ketonic bile acids are poor substrates, a long incubation period is necessary to ensure satisfactory hydrolysis of major conjugated bile acids present in bile.

After hydrolysis, either alkaline or enzymatic, free bile acids have to be esterified. The use of diazomethane has been shown to introduce artefacts, probably the diester, which can be avoided by using methanolic or ethanolic hydrogen chloride [25]. The use of the ethyl ester improves the separation when used in conjunction with dimethylethylsilyl ether derivatives. Several groups of bile acids derivatives, such as formyl, acetyl, trifluoroacetyl, hexafluoromethyl, trimethylsilyl and dimethylethylsilyl have been used [18,26–30].

The ethyl ester dimethylethylsilyl ether derivatives are baseline-separated on a glass capillary column with high efficiency. Hence the five kinds of major bile acids commonly occurring in human bile and cholesterol can be determined simultaneously and the solvent extraction step previously used for preliminary separation of cholesterol from bile acids can be eliminated, which greatly simplifies the analytical procedure. Further, the use GC of instead of either spectrophotometry or enzymatic reactions for the determination of cholesterol enhances the sensitivity and enables the present procedure to be scaled down to the analysis of the small volumes of bile available when using small animals such as the hamster. The presence of ketonic bile acids such as 7-ketodeoxycholic acid, 5β-cholanic acid-7α,12α-diol-3-one and 7-ketolithocholic acid should be considered in hamster bile [21,22]. Although the bile acid composition depends on the diet, the above ketonic bile acids represented a few percent in the bile of hamsters fed a standard diet, as reported [22]. However, when the analysis of ketonic bile acids and other conjugates such as sulphates is required, further manipulations (oximation, solvolysis) are necessary. As little as 5 μl of bile can be processed. Bartlett's [15] modification for the determination of organic phosphorus is sensitive enough to be compatible with such a small volume of bile.

The described procedure is not only sensitive but also reproducible and gives good recoveries. The current study should be useful in determining cholesterol and the predominant bile acids simultaneously.

REFERENCES

1 H. Burchard, Chem. Zentralbl., 61 (1890) 25.
2 C. Liebermann, Ber. Dtsch. Chem. Ges., 18 (1885) 1803.
3 W.M. Sperry and M. Webb, J. Biol. Chem., 187 (1950) 97.
4 L.L. Abell, B.B. Levy, B.B. Brodie and F.E. Kendall, J. Biol. Chem., 195 (1952) 357.
5 R.J. Morin, Clin. Chim. Acta, 71 (1976) 75.
6 H. Fromm, P. Amin, H. Klein and I. Kupke, J. Lipid. Res., 21 (1980) 259.
7 J.G. Heider and R.L. Boyett, J. Lipid. Res., 19 (1978) 514.
8 J.P. Blomhoff, Clin. Chim. Acta, 43 (1973) 257.
9 C.H. Bolton, T.S. Low-Beer, E.W. Pomare, A.C.B. Wicks, J. Yeates and K.W. Heaton, Clin. Chim. Acta, 83 (1978) 177.
10 R. Haeckel, O. Sonntag, W.R. Kulpmann and U. Feldmann, J. Clin. Chem. Clin. Biochem., 17 (1979) 553.
11 J.L. Irvin, C.G. Johnston and J. Kopala, J. Biol. Chem., 153 (1944) 439.
12 C.R. Szalkowski and W.J. Mader, Anal. Chem., 24 (1954) 1602.
13 B. Isaksson, Acta Chem. Scand., 8 (1954) 889.
14 F. Nakayama and M. Nakagaki, J. Chromatogr., 183 (1980) 287.
15 G.R., Bartlett, J. Biol. Chem., 234 (1959) 466.
16 O.J. Roseleur and C.M. Van Gent, Clin. Chim. Acta, 66 (1976) 269.
17 T. Yamamoto, Yonago Acta Med., 28 (1977) 114.
18 J. Yanagisawa, M. Itoh, M. Ishibashi, H. Miyazaki and F. Nakayama, Anal. Biochem., 104 (1980) 75.
19 A.G. Smith, W.A. Harland and C.J.W. Brooks, J. Chromatogr., 142 (1977) 533.
20 F. Mashige, K. Imai and T. Osuga, Clin. Chim. Acta, 70 (1976) 79.
21 F. Bergman, W. van der Linden and J. Sjovall, Acta Physiol. Scand., 74 (1968) 480.
22 S. Kuroki, E.H. Mosbach, B.I. Cohen, R.J. Stenger and C.K. McSherry, J. Lipid Res., 28 (1987) 856.
23 G. Lepage, A. Fontaine and C.C. Roy, J. Lipid Res., 19 (1978) 505.
24 P. Eneroth and J. Sjovall, The Bile Acids, Plenum Press, New York, 1971.
25 H. Miyazaki, M. Ishibashi, M. Inoue, M. Itoh and T. Kubodera, J. Chromatogr., 99 (1974) 553.
26 K. Imai, Z. Tamura, F. Mashige and T. Osuga, J. Chromatogr., 120 (1976) 181.
27 R. Edenharder and J. Slemr, J. Chromatogr., 222 (1981) 1.
28 G. Karlaganis and G. Paumgartner, J. Lipid Res., 19 (1978) 771.
29 H. Miyazaki, M. Ishibashi and K. Yamashita, Biomed. Mass Spectrom., 6 (1979) 57.
30 A. Fukunaga, Y. Hatta, M. Ishibashi and H. Miyazaki, J. Chromatogr., 190 (1980) 339.