notes on methodology

Analysis of serum bile acids by capillary gas–liquid chromatography–mass spectrometry

Georg Karlaganis, René P. Schwarzenbach, and Gustav Paumgartner

Department of Clinical Pharmacology, University of Berne,[1] Berne, and Institute for Water Resources and Water Pollution Control, Swiss Federal Institute of Technology,[2] Dübendorf, Switzerland

SUMMARY A method for quantitative analysis of serum bile acids by capillary gas–liquid chromatography–mass spectrometry is described. The main features of this method are a Grob-type barium carbonate/polyethyleneglycol 20,000 glass capillary column, an all-glass capillary interface, use of the lipophilic anion exchanger DEAP-Sephadex-LH-20 for purification of the serum extract, and chenodeoxycholic-11,12-d_2 acid as internal standard. Linearity of the response (ratio of intensities of the fragment ions diagnostic for the bile acid to be measured and for the internal standard) was demonstrated for four different bile acids. The method is sufficiently sensitive for measurement of bile acids in serum of healthy humans.—**Karlaganis, G., R. P. Schwarzenbach, and G. Paumgartner.** Analysis of serum bile acids by capillary gas–liquid chromatography–mass spectrometry. *J. Lipid Res.* 1980. **21:** 377–381.

Supplementary key words glass capillary column · DEAP-Sephadex-LH-20 · trimethylsilylether derivatives · stable isotopes

The advantages of using a mass spectrometer as a selective detector for the gas–liquid chromatographic analysis of bile acids in serum have been well documented (1–3). Except for a short report on glass capillary GLC–MS of bile acid standards (4), so far only packed columns have been used for GLC–MS of bile acids. Because of the higher separation power of glass capillary columns as compared to packed columns, we have adapted our previously published high resolution capillary GLC method for bile acid analysis in serum (5, 6) to combined GLC–MS.

MATERIALS AND METHODS

Reagents

All reagents were of analytical grade. Bile acid standards were purchased from Supelco Inc., Bellefonte, PA, with the exception of 3β-hydroxy-5-cholenoic acid, which was obtained from Steraloids Inc., Wilton, NH. Chenodeoxycholic-11,12-d_2 acid was purchased from Merck, Sharp and Dohme, München, F.R.G. Polyethyleneglycol 20,000 was obtained from Fluka AG, Buchs, Switzerland. Cholylglycine hydrolase from *Clostridium perfringens* (*welchii*), 50% glycerol solution, 250 units/ml, was purchased from Sigma, Saint Louis, MO. Amberlite XAD-2, mesh 100–200 μm, was obtained from Serva Laboratories, Heidelberg, F.R.G. DEAP-Sephadex-LH-20 (DEAP-Lipidex) was purchased from Packard Instrument International SA, Zürich, Switzerland.

Sample preparation

Chenodeoxycholic-11,12-d_2 acid (internal standard, 20 nmol, 7.88 μg) in 50 μl methanol was pipetted into a glass tube and evaporated to dryness. One ml of serum and 9 ml of 0.1 M sodium hydroxide in 0.9% sodium chloride were added. The mixture was kept in an ultrasonic bath for 15 min and was then passed at 4°C and a flow-rate of about 0.2–0.3 ml/min over a column (35/5 mm) of approximately 0.5 g Amberlite XAD-2 in water, which had been washed with methanol, acetone, and water. The column was subsequently washed with 1 ml 0.1 M sodium hydroxide in 0.9% sodium chloride and 5 ml of water, and then eluted with 5 ml of methanol. The eluate was evaporated to dryness at 50°C under a stream of nitrogen. For solvolysis, 1 ml of 1 M dry methanolic hydrogen chloride and 9 ml of acetone (dried over molecular sieve 3 Å) were added to the dry sample and kept at room temperature for 3 hr. The mixture was then neutralized by 1 ml of 1 M dry methanolic sodium hydroxide and evaporated to dryness at 50°C under a stream of nitrogen. The residue was dissolved in 4 ml of glass-distilled water, and the pH was adjusted to 5.8–6.0 with a few drops of 0.2 M acetic acid, followed by 0.4 ml of 0.1 M sodium acetate buffer (pH 5.6). Twenty μl (=5 units) cholylglycine hydrolase solution and 0.1 ml each of freshly prepared solutions of disodium salt of ethylenediaminetetraacetic acid (0.2 M) and 2-mercaptoethanol (0.2 M) were added. The mixture was incubated at 37°C overnight for at least 12 hr,

Abbreviations and trivial names: Lithocholic, 3α-hydroxy-5β-cholanoic; chenodeoxycholic, 3α,7α-dihydroxy-5β-cholanoic; ursodeoxycholic, 3α,7β-dihydroxy-5β-cholanoic; deoxycholic, 3α,12α-dihydroxy-5β-cholanoic; and cholic, 3α,7α,12α-trihydroxy-5β-cholanoic acid. TMS, trimethylsilyl; GLC, gas–liquid chromatography; MS, mass spectrometry.

[1] G. Karlaganis and G. Paumgartner.

[2] R. P. Schwarzenbach.

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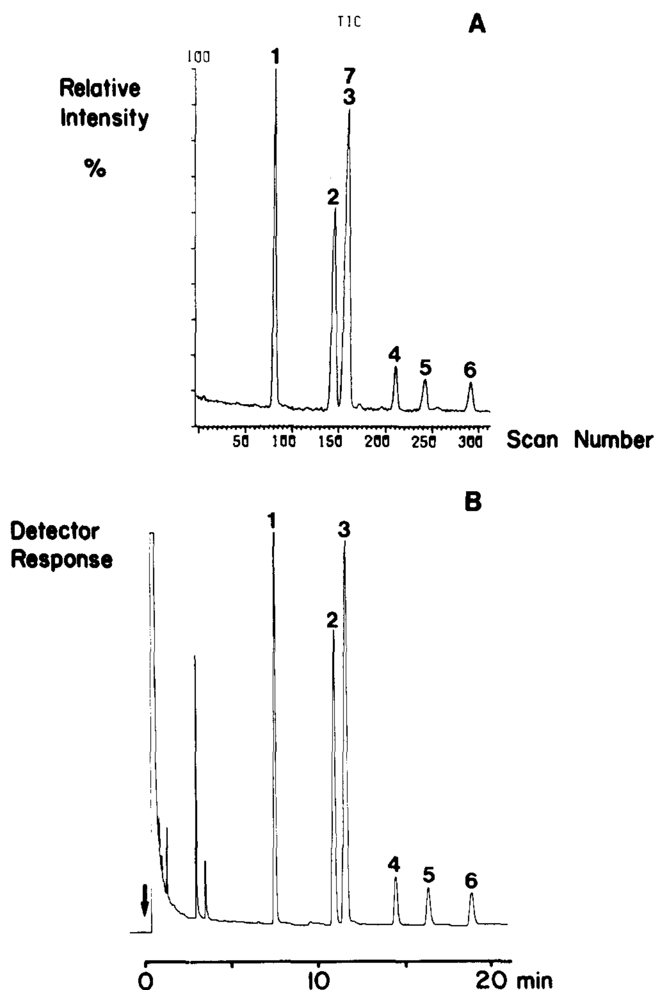

Fig. 1. A) Total-ion current chromatogram (capillary GLC–MS) of TMS ethers of methyl esters of a standard bile acid mixture containing cholic 1), deoxycholic 2), chenodeoxycholic 3), lithocholic 4), ursodeoxycholic 5), 3β-hydroxy-5-cholenoic 6), and chenodeoxycholic-11,12-d_2 acid (internal standard, 800 pmol per injection) 7). The internal standard 7) has the same retention time as chenodeoxycholic acid 3). B) Capillary gas chromatogram of TMS ethers of methyl esters of a standard bile acid mixture. Peak numbers are the same as in Fig. 1A. A 20 m polyethyleneglycol 20,000 glass capillary column was used at 230°C with hydrogen as carrier gas (1.0 atm).

cooled at room temperature, and acidified to pH 1.0 with 6 M hydrochloric acid. The aqueous solution was extracted three times with 10 ml of diethylether. The ether extract was evaporated to dryness with nitrogen at 35°C after addition of 0.5 ml of methanol. The residue with the free bile acids was dissolved in 72% ethanol (3 × 3 ml) and transferred to a DEAP-LH-20 column (200 × 4.4 mm bed volume), which was connected to a nitrogen pressure line. The column was washed with 15 ml 72% ethanol and the free bile acids were eluted with 8 ml 0.1 M acetic acid in 72% ethanol and taken to dryness. The residue was dissolved in 0.1 ml methanol and 0.9 ml diethylether. A stream of gaseous diazomethane (prepared from *N*-methyl-*N*-nitroso-p-toluenesulfonamide) was bubbled through the solution at room temperature for at least 2 min. The sample was then placed into an ice-bath for 15 min and taken to dryness under a stream of nitrogen at 35°C. The silylating reagent was prepared by mixing three volumes of pyridine (refluxed over barium oxide and distilled) with two volumes of hexamethyldisilazane (distilled) and one volume of trimethylchlorosilane (dried over molecular sieve 4 Å). The silylating reagent (100 μl) was added to the dry sample and left at room temperature for 15 min. The sample was then evaporated under a stream of nitrogen at room temperature. The residue was dissolved in 50 μl hexane, and an aliquot of 2 μl was injected into the GLC–MS system.

Capillary gas–liquid chromatography–mass spectrometry

A Finnigan mass spectrometer model 1015D was used together with a Finnigan 6000 interactive data system and a gas-chromatograph Fractovap GI (Carlo Erba, Milano, Italy), equipped with a double splitting injector and a glass capillary column (20 m × 0.3 mm i.d.). The Grob-type glass capillary column was coated with barium carbonate/polyethyleneglycol 20,000 (0.2%) as previously described (5). An all glass interface according to Blum and Richter (7) was used to connect the polyethyleneglycol 20,000 glass capillary column to the ion source of the mass spectrometer. The column was connected to the interface by a Teflon

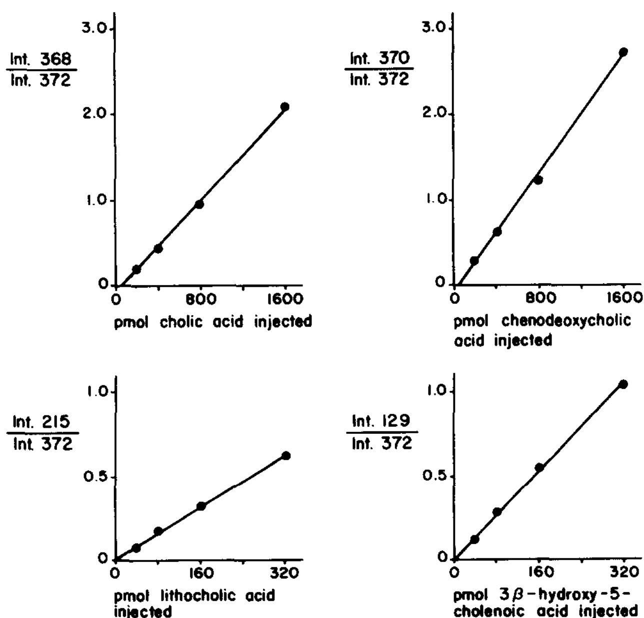

Fig. 2. Standard curves of TMS ethers of methyl esters of four different bile acids using capillary GLC–MS. Chenodeoxycholic-11,12-d_2 acid was used as internal standard. Each point represents the mean of three injections of different samples.

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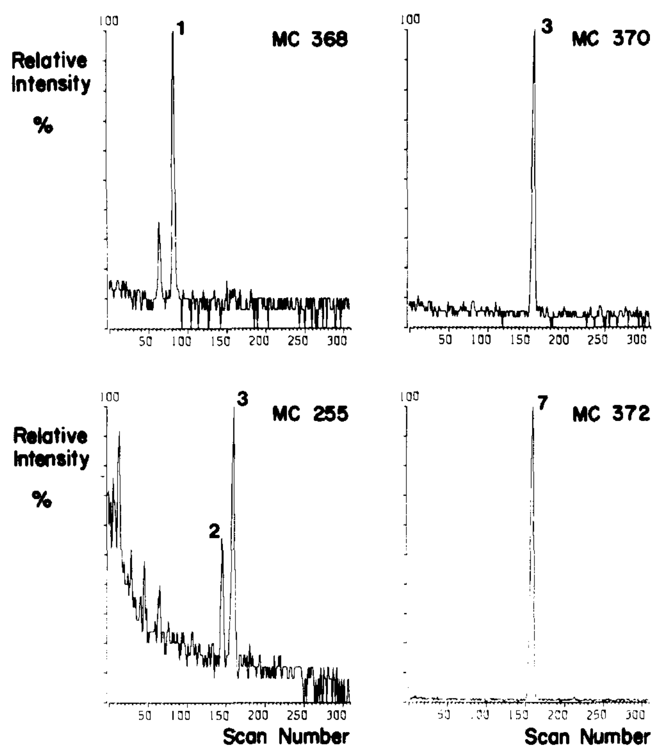

Fig. 3. Mass chromatograms of TMS ethers of methyl esters of bile acids extracted from 1 ml of normal human serum containing 20 nmol of chenodeoxycholic-11,12-d_2 acid as internal standard. Peak numbers are the same as in Fig. 1A.

shrinking tube. The glass capillary of the interface had an inner diameter of 0.1 mm, was edged with hydrochloric acid and deactivated with polyethylene-glycol 1000. The operating parameters were: electron energy 70 eV, emission current 310 μA, mass range 100–470 m/e, integration times: 2 ms (100–299 m/e), 3 ms (300–470 m/e), repetitive scanning mode. Helium was used as carrier gas (2.0 atm). The temperatures of the injector, the GLC oven, and the GLC–MS all glass capillary interface were 265, 230, and 250°C, respectively.

The mass spectral data of each analysis were stored in the data system of the GLC–MS apparatus. Afterwards, the mass chromatograms of the diagnostic ions were depicted on a display. Peak areas in the mass chromatograms corresponding to the bile acid derivatives were integrated by the data system and listed. The areas of the diagnostic ions of the bile acids to be measured were divided by the area of the internal standard chenodeoxycholic-11,12-d_2 acid of the mass chromatogram 372. The area of the ion 372 diagnostic for the internal standard was not corrected for the M + 2 contribution of the unlabeled chenodeoxycholic acid derivative. This M + 2 contribution to the ion 372 due to the natural abundance of ^{2}H and ^{13}C is 4.35% of the ion 370.

TABLE 1. Retention times and diagnostic fragment ions of bile acid derivatives in GLC–MS

Bile Acid Methyl Ester TMS Ether	Retention Time	Diagnostic Fragment Ion[a]
	min	*m/e*
Cholic acid	11	368
Deoxycholic acid	17	255
Chenodeoxycholic acid	18	370
Chenodeoxycholic-11,12-d_2 acid (internal standard)	18	372
Lithocholic acid	22	215
3β-Hydroxy-5-cholenoic acid	28	129

[a] For structure of fragment ions see Sjövall, Eneroth and Ryhage (11).

RESULTS AND DISCUSSION

Using an all-glass interface according to Blum and Richter (7), it was possible to combine our glass capillary GLC method for bile acid analysis with MS without significant loss of separation efficiency (**Fig. 1**). This interface was far superior to the formerly used platinum interface (8), which adsorbed and destroyed part of the bile acid derivatives. Even worse results were obtained using the commercial glass-lined stainless steel capillary interface in a Finnigan 4000 GLC–MS system. The high performance of the all-glass interface was further documented by the linearity of responses shown in the standard curves (**Fig. 2**).

TABLE 2. Reproducibility of bile acid measurements by GLC–MS in serum of a normal subject and of a patient with cholestasis

		Bile Acid Concentration			Coefficient of Variation		
	n[a]	Cholic Acid	Deoxycholic Acid	Chenodeoxy-cholic Acid	Cholic Acid	Deoxycholic Acid	Chenodeoxy-cholic Acid
			μM			%	
Normal subject	5	2.8	1.2	3.9	4.4	1.7	5.6
Patient with cholestasis	5	155.4		92.1	5.6		8.6

[a] Number of injections.

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TABLE 3. Bile acid measurements by GLC–MS in serum of patients with liver disease

Diagnosis	Cholic Acid	Deoxycholic Acid	Chenodeoxy-cholic Acid	Lithocholic Acid	3β-Hydroxy-5-cholenoic Acid
			concentration, μM		
Chronic active hepatitis	13.3	2.5	59.7	7.6	0.8
Primary biliary cirrhosis	93.2	3.2	73.3	1.6	4.7
Primary biliary cirrhosis	5.8	1.2	6.9		3.0
Cholestasis of pregnancy	146.4	2.0	85.7	3.6	26.8
Thrombosis of the portal vein	21.8	6.3	51.0	3.1	

It must be pointed out that no significant bleeding of the liquid phase (polyethyleneglycol 20,000), frequently observed with polar phases coated onto silica surfaces, did occur. Silica catalyzes the breakdown of polyglycols. However, if the silica surface is covered with a crystal layer of barium carbonate, the liquid film of polyethyleneglycol 20,000 is stable up to 250°C, as reported by Grob, Grob, and Grob (9). With higher temperatures than 250°C, the following masses of bleed ions must be expected (10): 219, 203, 177, 161, 147, 133, 117, 103, and fragments smaller than 100. These bleed ions do not have the same masses as the ions diagnostic for bile acid TMS derivatives.

Retention times and diagnostic fragment ions used in this study are listed in **Table 1** for the TMS ethers of methyl esters of six human bile acids. Because helium was used for capillary GLC–MS, the retention times were about twice as long as during capillary GLC, for which hydrogen was used as carrier gas (5).

In contrast to GLC, combined GLC–MS offers the possibility to use bile acids labeled with stable isotopes as internal standard. Accordingly, the commercially available deuterated bile acid, chenodeoxycholic-11,12-d_2 acid, was used as internal standard. It has the same retention time in the gas chromatograph and contains the same diagnostic fragment ion in the mass spectrum of chenodeoxycholic acid. The ratio of the intensities of the diagnostic fragment ion for the bile acid to be measured and the ion 372 (internal standard) was taken as response. This response was linear over the range of 200 to 1600 pmol of bile acid derivatives injected for cholic and chenodeoxycholic acid, and 40 to 320 pmol of bile acid derivatives injected for lithocholic and 3β-hydroxy-5-cholenoic acid (Fig. 2). Theoretically, the use of deuterium-labeled conjugated bile acids as internal standards could improve the precision of the analysis. So far, these bile acids are not commercially available. However, recovery experiments using conjugated bile acids for capillary GLC analysis with a similar sample preparation procedure showed recoveries of 86% for glycocholic, 89% for glycochenodeoxycholic, 87% for taurochenodeoxycholic, and 88% for glycolithocholic acid using unconjugated hyodeoxycholic acid as internal standard (6).

Purification of the serum sample sufficient for capillary GLC, was inadequate for capillary GLC–MS. Therefore, an additional step had to be added to the purification procedure in order to improve the signal to noise ratio. The serum sample containing the deuterated internal standard was extracted with Amberlite XAD-2, solvolyzed, and hydrolyzed as described for capillary GLC alone (6). This procedure was followed by an additional purification using the lipophilic anion exchanger diethylaminohydroxypropyl-Sephadex-LH-20 according to Almé and coworkers (1). Employing this sample purification procedure, satisfactory mass chromatograms of extracts of normal human serum were obtained (**Fig. 3**). There were no other peaks in the mass chromatograms that disturbed the analysis because of insufficient separation. This was due to the high separation efficiency of the glass capillary column and the high selectivity of the mass spectrometer.

Reproducibility of GLC–MS measurements was similar for bile acids extracted from healthy serum and from serum of a patient with cholestasis (coefficients of variation between 1.7 and 8.6%; **Table 2**). Examples of actual serum data are given in **Table 3.** The major bile acids measured were cholic and chenodeoxycholic acid, as expected by the diagnosis of the patients.

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