Size exclusion chromatography for extraction of serum bile acids[1]

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Summary A major problem in the measurement of serum bile acids is their quantitative extraction from the high molecular protein matrix. In our hands, the standard techniques of adsorption and reversed-phase chromatography have yielded incomplete recovery for different bile acids (33–93%) and poor reproducibility. In contrast, with the novel extraction procedure of size exclusion chromatography, recovery was nearly quantitative (75–104%) and reproducibility was satisfactory. The described method allowed for a reliable determination of serum bile acids in healthy subjects and patients with liver cirrhosis. We conclude that size exclusion chromatography for serum bile acid extraction is more reliable than alternative techniques, because the separation by size is independent of solubility, charge, and polarity. —**Nuber, R., H. Maucher, and E. F. Stange.** Size exclusion chromatography for extraction of serum bile acids. *J. Lipid Res.* 1990. **31:** 1517–1522.

Supplementary key words high resolution gas chromatography liver cirrhosis

Adsorption chromatography (1–3) or, during recent years, reversed-phase chromatography (4–7) represent the standard techniques for the extraction of bile acids from biological materials. In our hands, serum extraction with these methods has yielded unsatisfactory results with poor recovery and reproducibility. Therefore, we developed a novel technique, size exclusion chromatography, and compared it with extraction using Amberlite XAD-2 or C_{18}-cartridges. With Sephadex G-75 gel chromatography, quantitative extraction of all serum bile acids was achieved because separation from the protein matrix is determined by size rather than solubility, charge, and polarity.

After extensive validation, this method was applied to the gas chromatographic determination of serum bile acids in healthy subjects and patients with alcoholic liver cirrhosis. In the latter group the disturbed bile acid metabolism is reflected in a change of the profile of serum bile acids (8–10).

METHODS

Materials and instrumentation

Methanol, hexane, acetone, ethylacetate, toluene, and N-(trimethyl-silyl-)imidazole were obtained from Merck, Darmstadt, FRG. Bile acid standards of >98% purity, 2,2-dimethoxypropane and cholylglycine hydrolase (EC 3.5.1.24) were from Sigma Chemical Co. Ltd., Deisenhofen, FRG. Column chromatography was performed on Amberlite XAD-2, Amberlyst A-15 (Serva Feinbiochemica, Heidelberg, FRG), Lipidex 1000 (Packard, Groningen, the Netherlands), Sephadex G-75 (Pharmacia, Fine Chemicals, Uppsala, Sweden), and Silica Gel 60 (Merck, Darmstadt, FRG). Bond-Elut C_{18}-cartridges (reversed-phase octadecylsilane bonded silica, 500 mg) were obtained from Analytichem International (Harbor City, CA). The radiolabeled bile acids [24-^{14}C]chenodeoxycholic acid (sp act 50 mCi/mmol) and [24-^{14}C]taurocholic acid (sp act 46.7 mCi/mmol) were purchased from Amersham Buchler, Brauschweig, FRG. [24-^{14}C]Glycodeoxycholic acid (sp act 50 mCi/mmol) and [11,12-^{3}H]ursodeoxycholic acid (sp act 37.0 mCi/mmol) were from NEN Research, Dreieich, FRG.

Capillary GLC analysis was carried out with a Fractovap 2150 gas chromatograph (Carlo Erba, Milano, Italia) or a Hewlett-Packard 5890 gas chromatograph (Hewlett-Packard, Palo Alto, CA) equipped with a flame ionization detector using a cross-linked SE-54 column (95% dimethyl–5% diphenylsiloxane), 25 m, i.d. 0.25 mm, fused silica, film thickness 0.25 μm (Machery-Nagel, Düren, FRG).

Subjects

The three healthy subjects were of both sexes, and their ages ranged from 29 to 37 years; the age of the five female liver cirrhosis patients was between 48 and 67 years. The blood samples were all taken in the morning in the postabsorptive state. In all patients liver cirrhosis was proven by liver biopsy. Total serum bilirubin ranged from 22 to 205 μmol/l. As judged from serum albumin, cholinesterase activity, and prothrombin time, the patients suffered from mild to severe disturbance of hepatic function.

Analytical procedure

A mixture of free bile acids (lithocholic acid 22.7 μg, deoxycholic acid 19.8 μg, chenodeoxycholic acid 24.5 μg, cholic acid 24.8 μg, hyodeoxycholic acid 1.6 μg, and ursodeoxycholic acid 27.4 μg) or of conjugated bile acids (glycodeoxycholic acid 11.7 μg, glycocholic acid 13.7 μg, taurochenodeoxycholic acid 10.0 μg, taurocholic acid 12.9 μg, and tauroursodeoxycholic acid 1.9 μg) was added to 2 ml of serum from healthy subjects to yield final concentrations in the range observed in patients with liver cirrhosis. The sample was diluted with 8 ml of 0.1 M NaOH and heated in a water bath at 64°C for 30 min. The

Abbreviations: GLC, gas–liquid chromatography; HRGC, high resolution gas chromatography.

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heated serum was applied to a column of Sephadex G-75 in water (bed size 10.5 × 2.5 cm). The column was then washed with 70 ml distilled water and eluted with 40 ml of methanol–water 3:1. 7-Keto-deoxycholic acid was then added as internal standard. The extraction of bile acids with C_{18}-cartridges and the XAD-resin was performed exactly as described (1, 4). In the three extraction methods the further clean-up was done in identical fashion according to the following steps. After extraction the eluates were evaporated to a final volume of approximately 500 μl, diluted with 1.5 ml of methanol, and applied to a strongly acidic cation exchange column (Amberlyst A-15, bed size 5 × 1 cm) which was eluted with 30 ml methanol–water 3:1 (3). Conjugated bile acids were hydrolyzed enzymatically with cholylglycine hydrolase (EC 3.5.1.24) (11); the deconjugated bile acids were extracted by liquid-gel chromatography on Lipidex 1000 (bed size 5 × 1 cm) (5). Prior to the determination by GLC, the free bile acids were derivatized. The carboxyl group was methylated (12), and the hydroxyl groups were converted to trimethylsilyl ethers (13). To eliminate interfering reaction products after derivatization, a final clean-up on silica gel was performed. The derivatized bile acids were applied to a column (i.d. 1 cm), filled with 1 g of silica gel 60 (35–70 mesh ASTM, water content 3%) in toluene. The elution was done with 20 ml toluene–ethyl acetate 9:1. The eluates were analyzed under the following gas chromatographic conditions. In the Carlo Erba gas chromatograph, the oven temperature was raised from 200 to 295°C at 2°C/min. The injector was set at 250°C and the detector at 295°C. Carrier gas was hydrogen at 0.7 m/sec. In the Hewlett-Packard gas chromatograph the oven temperature was set at 100°C, the temperature program was 10°C/min to 260°C, 1°C/min to 280°C with the injector at 250°C and the detector at 280°C. Carrier gas was helium at 0.27 m/sec. Peak areas were calculated by use of an integrator (Shimadzu, Chromatopac C-R 3 A, Kyoto, Japan) and were linear with respect to injected bile acid mass up to 0.15 nmol. A representative chromatogram of a standard bile acid mixture is shown in **Fig. 1.**

For measuring the recovery rates of the three different extraction procedures with radioactive bile acids, the initially described mixtures of free and conjugated reference bile acids and the respective radiolabeled bile acids [^{14}C]chenodeoxycholic, [^{3}H]ursodeoxycholic, [^{14}C]glycodeoxycholic, or [^{14}C]taurocholic acid were added to serum from health subjects. Radioactive determinations were made by liquid scintillation counting before and after extraction of bile acids according to the three described extraction methods. Subsequently, size exclusion chroma-

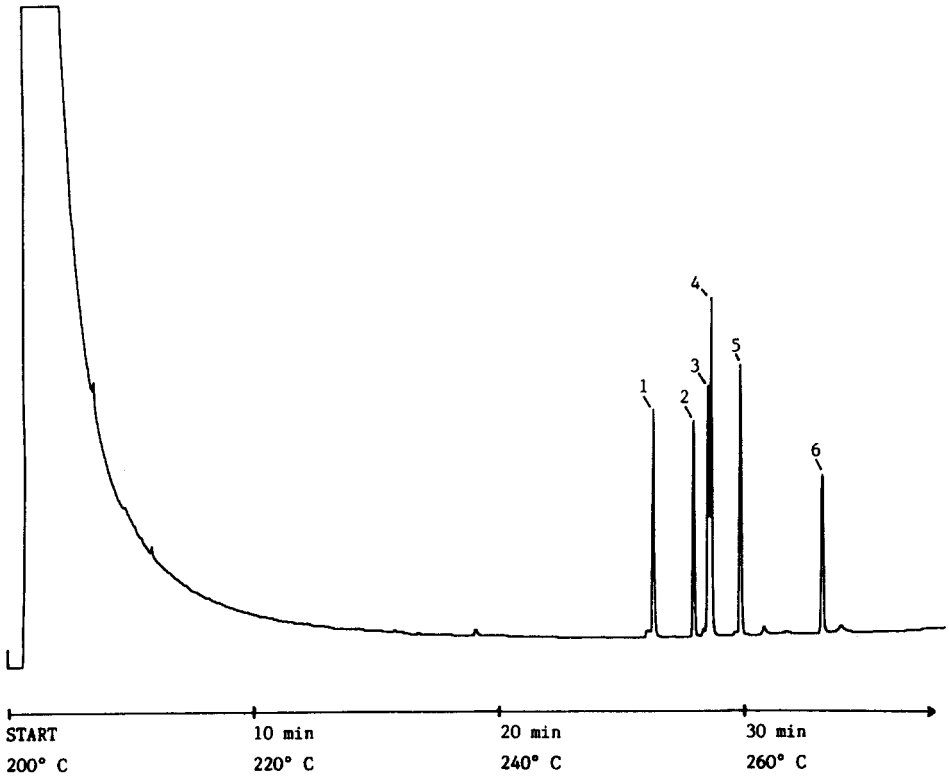

Fig. 1. HRGC/FID-chromatogram of methyl ester trimethylsilyl ether derivatives of standard bile acid mixture containing lithocholic acid (1), deoxycholic acid (2), chenodeoxycholic acid (3), cholic acid (4), ursodeoxycholic acid (5), 7-keto-deoxycholic acid (internal standard) (6). HRGC was performed with the Carlo Erba gas chromatograph Fractovap 2150.

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TABLE 1. Recovery of standard bile acids added to serum after extraction with XAD-2, Bond-Elut C_{18}, or Sephadex G-75

Added Bile Acid	Recovery % (mean ± SEM)		
	XAD-2	Bond-Elut C_{18}	Sephadex G-75
Lithocholic acid	33 ± 9.6 (17-66)[a]	37 ± 7.6 (21-67)	75 ± 3.3 (65-87)
Deoxycholic acid	51 ± 3.9 (38-57)	48 ± 7.8 (33-88)	90 ± 2.9 (81-98)
Chenodeoxycholic acid	44 ± 8.2 (23-65)	46 ± 7.9 (31-78)	90 ± 3.0 (97-100)
Cholic acid	80 ± 1.8 (77-82)	88 ± 5.9 (74-111)	98 ± 3.2 (86-108)
Hyodeoxycholic acid	61 ± 6.1 (24-81)	78 ± 8.9 (42-110)	98 ± 3.3 (85-110)
Ursodeoxycholic acid	62 ± 5.2 (51-78)	59 ± 6.7 (43-84)	95 ± 2.2 (89-102)
Glycodeoxycholic acid	77 ± 10.6 (39-95)	65 ± 5.9 (44-80)	100 ± 3.2 (92-108)
Glycocholic acid	82 ± 2.8 (74-92)	58 ± 5.0 (41-76)	82 ± 0.7 (80-84)
Taurochenodeoxycholic acid	90 ± 10.4 (44-105)	71 ± 10.8 (44-107)	104 ± 2.9 (96-110)
Taurocholic acid	93 ± 5.7 (79-111)	71 ± 3.7 (61-85)	96 ± 4.4 (80-108)
Tauroursodeoxycholic acid	84 ± 11.7 (44-100)	71 ± 10.4 (40-105)	92 ± 4.9 (77-102)

Capillary GLC analysis was carried out with the Hewlett-Packard 5890 gas chromatograph.
[a]Range (%) of five separate experiments.

tography and the further clean-up, described above, were used to measure the individual bile acids in sera from healthy subjects and patients with alcoholic liver cirrhosis. For that purpose 2 ml of serum was taken as initial sample volume, and the internal standard 7-ketodeoxycholic acid was added before extraction and further analytical procedures.

RESULTS

In the three different extraction methods the recovery of bile acid mass was calculated by subtracting the amounts of bile acids determined in serum of health subjects from the amounts in their serum following supplementation with the respective bile acid mixtures. Using adsorption chromatography with XAD-resin the recovery for single bile acids ranged from 33% to 93% (**Table 1**). In particular, the recovery of the less polar free bile acids was unsatisfactory. Conjugated bile acids showed higher recovery, but the reproducibility was poor in all instances. Comparable data were obtained for the extraction of radioactive bile acids (**Table 2**). The recovery of single bile acids, extracted by reversed-phase chromatography using Bond-Elut C_{18}-cartridges, ranged from 37% to 88% (Table 1). Free bile acids showed highly variable recovery; only the results for taurine-conjugated bile acids were acceptable. Again, reproducibility was unsatisfactory. The

TABLE 2. Recovery of radiolabeled reference bile acids added to serum after extraction with XAD-2, Bond-Elut C_{18}, and Sephadex G-75

Added Bile Acid	Recovery %		
	XAD-2	Bond-Elut C_{18}	Sephadex G-75
[^{14}C]Chenodeoxycholic acid	69 (66-72)[a]	85 (78-93)	85 (78-97)
[^{3}H]Ursodeoxycholic acid	56 (46-69)	44 (20-61)	97 (95-102)
[^{14}C]Glycocholic acid	84 (79-86)	58 (51-72)	93 (87-98)
[^{14}C]Taurocholic acid	89 (87-92)	72 (60-80)	89 (85-93)

[a]Range (%) of three separate experiments.

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TABLE 3. Serum bile acids in healthy subjects and patients with alcoholic liver cirrhosis

Subjects	Concentrations of Bile Acids			
	Deoxycholic Acid	Chenodeoxycholic Acid	Cholic Acid	Total
		μmol/l		
Healthy (n = 3)	0.7	1.3	1.0	3.0
Range	0.1–1.2	1.1–1.5	0.7–1.3	1.9–3.9
Patient I	7.7	96.0	210.0	313.7
Patient II	7.9	106.4	128.6	242.9
Patient III	3.0	14.5	14.5	32.0
Patient IV	3.9	20.0	26.1	50.0
Patient V	2.8	3.0	7.1	12.9

Capillary GLC analysis was carried out with the Carlo Erba gas chromatograph Fractovap 2150.

radioactive experiments yielded similar results (Table 2). There appeared to be some relationship between recovery of single bile acids and their hydrophobicity.

In contrast, using Sephadex G-75, the single bile acids added to serum could be extracted by size exclusion chromatography with a recovery ranging from 75% to 104% and high reproducibility (Table 1). No significant difference between free and conjugated bile acids was observed. There was also a nearly complete extraction of radioactive bile acids (Table 2).

When the latter technique was applied to normal serum, the concentration of total bile acids was about 3 μmol/l (**Table 3**). Chenodeoxycholic acid predominated, the ratio cholic/chenodeoxycholic acid was 1:1.3, and deoxycholic acid amounted to about 50% of chenodeoxycholic acid. In liver cirrhosis patients, the total serum bile acids were elevated, due mainly to a rise in the primary bile acids, cholic and chenodeoxycholic acids. In nearly all cases the concentration of cholic acid in serum exceeded that of chenodeoxycholic and deoxycholic acids (Table 3). The ratio of cholic/deoxycholic acid ranged from 1:0.03 to 1:0.4. The highest concentrations of total serum bile acids (242.9 and 313.7 μmol/l) were measured in those patients with poor liver function as documented by hyperbilirubinemia, and with the lowest serum albumin concentration, cholinesterase activity, and prothrombin time (**Fig. 2**). Patients with a moderate increase of serum bilirubin had intermediate serum bile acids between 12.9 and 50.0 μmol/l.

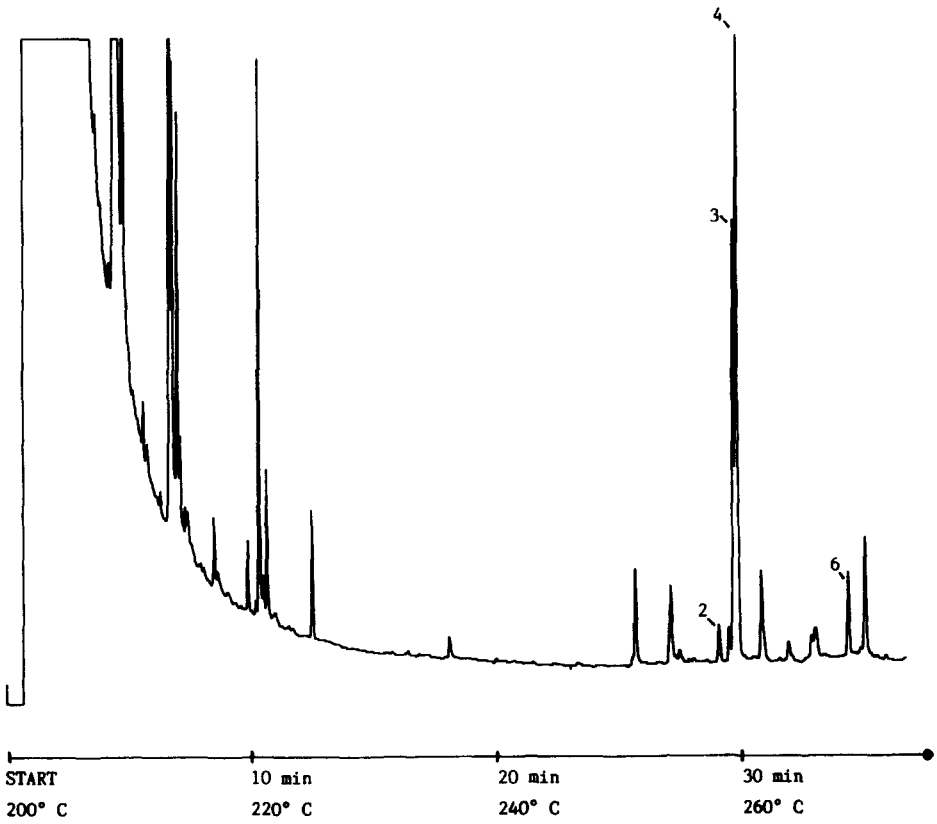

Fig. 2. HRGC/FID-chromatogram of methyl ester trimethylsilyl ether derivatives of serum bile acids of a patient with alcoholic liver cirrhosis. Peak numbers are as in Fig. 1. HRGC was also performed with the Carlo Erba gas chromatograph.

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DISCUSSION

When revalidating established techniques for serum extraction of bile acids using XAD-2 adsorption (1) or C_{18} reversed-phase chromatography (4), we obtained poor recovery and reproducibility. These methodological deficiencies were observed with two separate methods based on radiolabeled bile acids or various mixtures of standard bile acids detected by HRGC. Since techniques identical to those used in previous work (1, 4) were used, the reasons for the differences are not readily obvious. It is conceivable that the variable recovery of bile acids using Bond-Elut cartridges may be due to variations in quality from batch to batch.

To optimize the methodology for analyzing serum bile acids by HRGC, size exclusion chromatography was used for extraction of serum bile acids. The advantages of size exclusion chromatography for extraction of serum bile acids may be summarized as follows. First, because of the large differences in molecular weight of all bile acids compared to the high molecular serum carrier proteins, it is possible to achieve a quantitative serum extraction. Second, the similar size of the different bile acids assures comparable extraction because, for example, dipole–dipole or van der Waals type interactions with the stationary phase are negligible. Thus, in contrast to reversed-phase extraction, polarity plays a minor role. Third, because of the good recovery and reproducibility, it is possible to add an internal standard to blood samples before bile acid extraction. Only by using size exclusion chromatography can it be assumed that the internal standard behaves like the other bile acids of the sample and suffers from the same loss during the analytical procedure.

The high recovery in size exclusion chromatography applies to all bile acids, regardless of conjugation, polarity, and solubility. This novel technique allows for a qualitative and quantitative determination of bile acids in human serum, even in trace amounts. After the quantitative separation of serum bile acids from the sample matrix by size exclusion chromatography and the further clean-up, only a few non-bile acid contaminants remain in the sample. Therefore, the described method should also be applicable to the determination of unusual bile acids in different liver diseases or complex biological materials.

In this work serum bile acids were analyzed by HRGC in sera from healthy subjects and from patients with alcoholic liver cirrhosis using size exclusion chromatography for serum extraction. As expected (8–10), in liver cirrhosis elevated concentrations of total serum bile acids could be measured, caused mainly by the primary bile acids, cholic and chenodeoxycholic acids. Cholic acid consistently showed the highest concentrations in serum. When cirrhotic patients were compared with healthy subjects, the secondary bile acid, deoxycholic acid, was increased, but much less than the primary bile acids. Since total serum bile acids in liver cirrhosis reflect hepatic function (14–16) and are probably good prognostic indices (17, 18), it seems important to better define the relevance of individual bile acids in this disease. It may be concluded that this newly developed extraction technique may be helpful in further studies on serum bile acids and their metabolism.

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