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notes on methodology

Analysis of bile acids in serum and bile by capillary gas–liquid chromatography

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Summary Various liquid phases for glass capillary columns have been evaluated for gas–liquid chromatographic analysis of methyl ester trimethylsilylether derivatives of bile acids from serum and bile. Bile acid analysis is rapid and exhibits high separation efficiency with a 20 × 0.3 mm glass capillary column whose internal surface is covered with a crystal layer of barium carbonate and coated with polyethyleneglycol 20000 as liquid phase according to Grob et al. (*Chromatographia* **10:** 181, 1977).

Supplementary key words glass capillary column · cholesterol · trimethylsilylether derivatives

Gas–liquid chromatography of bile acid derivatives on packed columns is a well established and widely used method (1). However, it is difficult to separate all the individual bile acids occurring in human serum by a single chromatographic step. The analysis of bile acids in biological fluids is further complicated by the presence of interfering compounds such as cholesterol which cannot be completely eliminated during the extraction procedure. To obtain sufficient analytical security in regard to compound identification and quantification, analysis of bile acids as two different derivatives has been suggested (2). Capillary gas–liquid chromatography offers high separation efficiency and may make such an approach unnecessary. Laatikainen and Hesso (3) reported on bile acid analysis by an OV-101 glass capillary column. However, lithocholic acid and cholesterol could not be separated on this column (3). We have, therefore, tested glass capillary columns with different polar stationary phases for analysis of bile acids in serum and bile.

Abbreviations and trivial names: lithocholic, 3α-hydroxy-5β-cholanoic; hyodeoxycholic, 3α,6α-dihydroxy-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.

Materials and methods

Reagents. All reagents were of analytical grade. Amberlite XAD-2, mesh 100–200 μm, was obtained from Serva Laboratories, Heidelberg, Germany. Bile acid standards were purchased from Supelco Inc., Bellefonte, PA. Polyethyleneglycol 20000 was obtained from Fluka AG, Buchs, Switzerland. HI-EFF-8BP and SILAR-10C were purchased from Applied Science Laboratories Inc., State College, PA. OV-101 and OV-61 glass capillary columns were manufactured by H. and G. Jaeggi, Laboratory for Gas Chromatography, Trogen, Switzerland.

Apparatus. The gas chromatograph used was a Fractovap 2301-AC equipped with a flame ionization detector, a model 1976 Grob-type injector (Carlo Erba S.p.A., Milan, Italy) and a glass capillary column. Peak areas were integrated with a Supergrator-2 programmable computing integrator (Columbia Scientific Industries, Austin, TX). Peaks were recorded on a Pye Unicam AR 25 linear recorder at a chart speed of 1 cm/min.

Preparation of glass capillary column. The internal surface of the glass capillary column (20 m × 0.30 mm ID) was covered with a crystal layer of barium carbonate at room temperature according to Grob et al. (4). The barium carbonate layer was then coated with a 0.2% solution of polyethyleneglycol 20000 in methylene chloride by the method of static coating (5).

Chromatographic conditions. The injection port temperature was 300°C, the column temperature was 230°C. Hydrogen was used as carrier gas at a flow rate of 4.4 ml/min. The flow rate at the split outlet was 22 ml/min (split ratio 1:5).

Preparation of samples. For analysis of bile acids in serum, 6.36 nmol of hyodeoxycholic acid (3α,6α-dihydroxy-5β-cholanoic acid) was added to 2 ml of serum as internal standard. Bile acids were extracted with XAD-2 according to the method of Makino and Sjövall (6) as modified by Schwarz, von Bergmann, and Paumgartner (7). Solvolysis of bile acid sulfates was achieved by incubation of the sample in dry 1 M hydrochloric acid in methanol–acetone 1:9 for 3 hr at room temperature. After alkaline hydrolysis and extraction with ethylacetate (8), the free bile acids were methylated according to Schlenk and Gellerman (9) and converted to their trimethylsilylether derivatives (10). The residue was dissolved in 20–50 μl of hexane and an aliquot of 2 μl was injected. For analysis of bile acids in bile, 127 nmol of hyodeoxycholic acid was added to 20 μl of bile as internal standard. The sample was then subjected to alkaline hydrolysis and processed as described above.

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TABLE 1. Retention times for bile acid methyl ester TMS derivatives[a]

Bile Acid	Absolute Retention Time (min)	Relative Retention Time[b]
Cholic acid	6.03 ± 0.14	0.547 ± 0.001
Deoxycholic acid	8.79 ± 0.21	0.797 ± 0.001
Chenodeoxycholic acid	9.34 ± 0.22	0.847 ± 0.001
Hyodeoxycholic acid	11.03 ± 0.27	
Lithocholic acid	11.59 ± 0.28	1.051 ± 0.002
Ursodeoxycholic acid	13.28 ± 0.32	1.204 ± 0.002
3β-Hydroxy-5-cholenoic acid	15.08 ± 0.36	1.368 ± 0.004

[a] Values are means ± SD of 10 observations. Methyl ester TMS derivatives of bile acid standards were injected into the same polyethyleneglycol 20000 glass capillary column at different days over a period of 3 months (column temperature 230°C; hydrogen carrier gas flow 4.4 ml/min).
[b] Related to hyodeoxycholic acid (internal standard).

Results and discussion

For the separation of bile acid methyl ester trimethylsilylether (TMS) derivatives the following stationary phases on glass capillary columns were tested: OV-101 (methylsilicone), OV-61 (phenyl(33%)methylsilicone), HI-EFF-8BP (cyclohexanedimethanol succinate), SILAR-10C (100% cyanoethylsilicone), and PG 20m (polyethyleneglycol, mol wt 20,000). Of those only PG 20m coated on to a layer of barium carbonate proved to be satisfactory for separation of bile acid TMS derivatives. The other columns exhibited short life expectancy and/or insufficient resolution. The PG 20m capillary column offered better separation efficiency, threefold shorter analysis times (15 min), and higher durability than HI-EFF-8BP in conventionally packed columns. Column performance did not show any deterioration over a 5-month period. The constancy of relative retention times of bile acids over a 3-month period is documented in **Table 1.** A decrease of separation efficiency, mainly between deoxycholic and chenodeoxycholic acid and between hyodeoxycholic and lithocholic acid, was observed after 5 months of use. Column performance was then restored by washing the column with methylene chloride and recoating with polyethyleneglycol 20000. In our hands the success rate in recoating was 100%.

Linearity of response was demonstrated over the range of 100–800 pmol of bile acid derivatives injected for cholic, deoxycholic, chenodeoxycholic acid, and 20–160 pmol of bile acid derivatives injected for lithocholic, ursodeoxycholic, and 3β-hydroxy-5-cholenoic acid. Standard curves of the two major bile acids of man, cholic and chenodeoxycholic acid, are shown in **Fig. 1.**

A chromatogram of a standard bile acid mixture is shown in **Fig. 2.** The methyl ester TMS derivatives of cholic, deoxycholic, chenodeoxycholic, hyodeoxycholic (internal standard), lithocholic, ursodeoxycholic, 3β-hydroxy-5-cholenoic acid, and cholesterol could be completely separated on the 20-m PG 20m glass capillary column. Therefore, it was not necessary to employ two different stationary phases or two derivation procedures (2) for analysis of all six bile acids in the same sample. The application of our method to normal human serum and bile is demonstrated in **Fig. 3** and **Fig. 4,** respectively.

In contrast to packed HI-EFF-8BP columns, cholesterol and cholic acid could be clearly separated on the PG 20m glass capillary column. Therefore, a relatively simple procedure for sample preparation without a special purification step for elimination of cholesterol (e.g. thin-layer chromatography (8) or alumina columns (3)) could be employed.

The coating of glass capillary columns with PG 20m does not require expensive equipment and can be

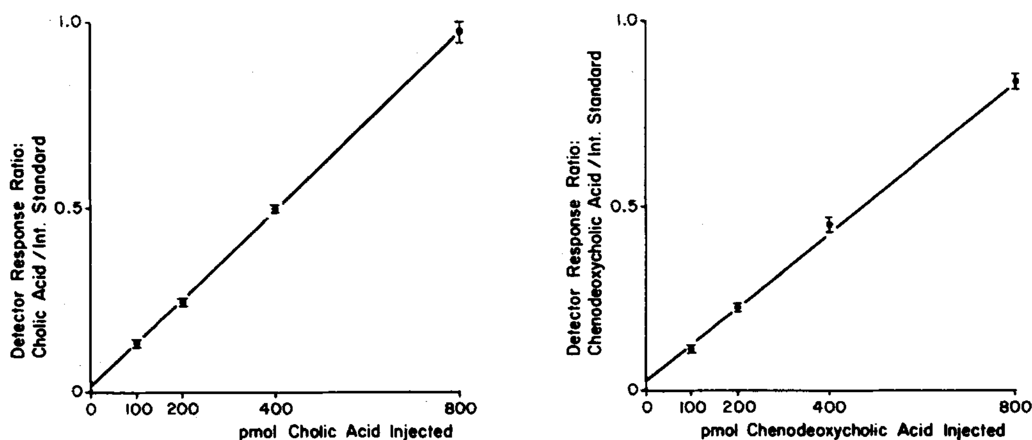

Fig. 1. Standard curves of TMS ethers of methyl esters of cholic and chenodeoxycholic acid. Hyodeoxycholic acid was used as internal standard. Each point represents the mean ± SEM of six injections of different samples. A 20-m PG 20m glass capillary column was used at 230°C.

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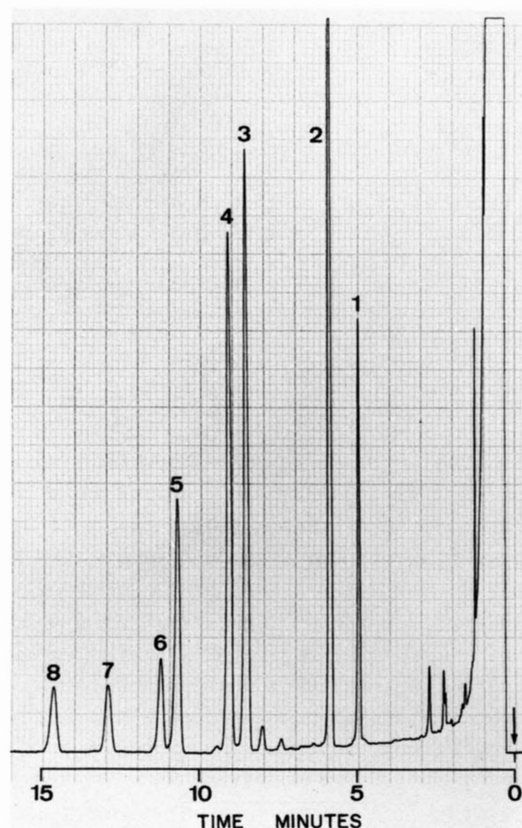
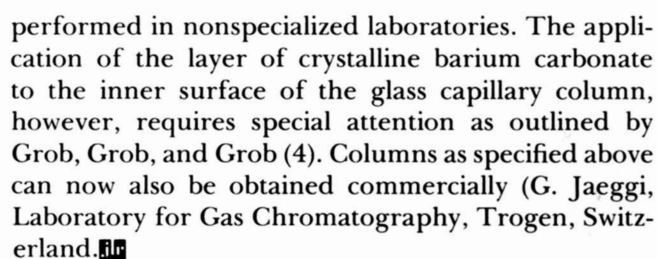


Fig. 2. Capillary gas chromatogram of TMS ethers of methyl esters of a standard bile acid mixture containing cholesterol (*1*), cholic (*2*), deoxycholic (*3*), chenodeoxycholic (*4*), hyodeoxycholic (internal standard, 250 pmol per injection) (*5*), lithocholic (*6*), ursodeoxycholic (*7*), and 3β-hydroxy-5-cholenoic acid (*8*). GLC conditions are the same as in Fig. 1.


performed in nonspecialized laboratories. The application of the layer of crystalline barium carbonate to the inner surface of the glass capillary column, however, requires special attention as outlined by Grob, Grob, and Grob (4). Columns as specified above can now also be obtained commercially (G. Jaeggi, Laboratory for Gas Chromatography, Trogen, Switzerland.

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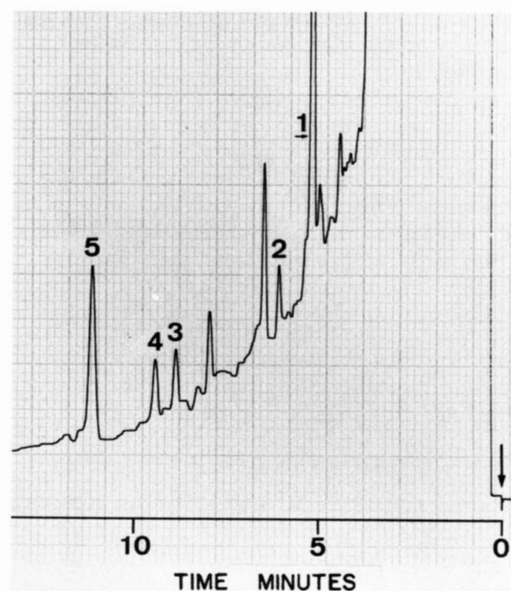

Fig. 3. Capillary gas chromatogram of TMS ethers of methyl esters of bile acids extracted with XAD-2 from 2 ml of normal human serum containing 6.36 nmol hyodeoxycholic acid as internal standard. GLC conditions and peak numbers are the same as in Fig. 2.

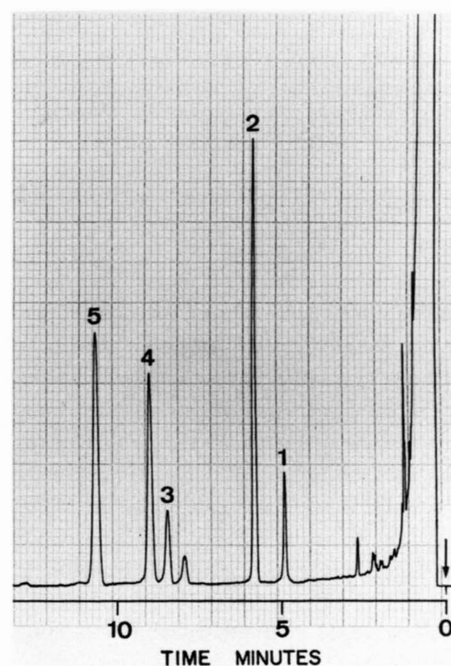

Fig. 4. Capillary gas chromatogram of TMS ethers of methyl esters of bile acids extracted from 20 μl of human bile containing 127 nmol of hyodeoxycholic acid as internal standard. GLC conditions and peak numbers are the same as in Fig. 2.

REFERENCES

1. Eneroth, P., and J. Sjövall. 1971. Extraction, purification and chromatographic analysis of bile acids in biological materials. *In* The Bile Acids. Chemistry, Physiology, and Metabolism, Vol. 1: Chemistry. P. P. Nair and D. Kritchevsky, editors. Plenum Press, New York, N.Y. 121–168.
2. Galeazzi, R., E. Kok, and N. Javitt. 1976. Gas–liquid

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chromatography of bile acids: a new liquid phase for both acetate and trimethylsilyl derivatives. *J. Lipid Res.* **17:** 288–290.
3. Laatikainen, T., and A. Hesso. 1975. Determination of serum bile acids by glass capillary gas–liquid chromatography. *Clin. Chim. Acta.* **64:** 63–68.
4. Grob, K., G. Grob, and K. Grob, Jr. 1977. The barium carbonate procedure for the preparation of glass capillary columns; further informations and developments. *Chromatographia.* **10:** 181–187.
5. Grob, K., and G. Grob. 1976. A new, generally applicable procedure for the preparation of glass capillary columns. *J. Chromatogr.* **125:** 471–485.
6. Makino, I., and J. Sjövall. 1972. A versatile method for analysis of bile acids in plasma. *Anal. Lett.* **5:** 341–349.
7. Schwarz, H. P., K. von Bergmann, and G. Paumgartner. 1974. A simple method for the estimation of bile acids in serum. *Clin. Chim. Acta.* **50:** 197–206.
8. Ali, S. S., and N. B. Javitt. 1970. Quantitative estimation of bile salts in serum. *Can. J. Biochem.* **48:** 1054–1057.
9. Schlenk, H., and J. L. Gellerman. 1960. Esterification of fatty acids with diazomethane on a small scale. *Anal. Chem.* **32:** 1412–1414.
10. Makita, M., and W. W. Wells. 1963. Quantitative analysis of fecal bile acids by gas–liquid chromatography. *Anal. Biochem.* **5:** 523–530.

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