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 trimethylsilyl ether 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 polylethylene glycol 20000 as liquid phase according to Grob et al. (Chromatographia 10: 181, 1977).

Supplementary key words glass capillary column · cholesterol · trimethylsilyl ether 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.

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. Polyethylene glycol 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 columns. The internal surface of the glass capillary column (20 mm × 0.3 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 polyethylene glycol 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) 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 trimethylsilyl ether 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.
TABLE 1. Retention times for bile acid methyl ester TMS derivatives

<table>
<thead>
<tr>
<th>Bile Acid</th>
<th>Absolute Retention (min)</th>
<th>Relative Retention Time*</th>
<th>Relative Retention Time*</th>
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</thead>
<tbody>
<tr>
<td>Cholic acid</td>
<td>6.03 ± 0.14</td>
<td>0.547 ± 0.001</td>
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<tr>
<td>Deoxycholic acid</td>
<td>8.79 ± 0.21</td>
<td>0.797 ± 0.001</td>
<td></td>
</tr>
<tr>
<td>Chenodeoxycholic acid</td>
<td>9.34 ± 0.22</td>
<td>0.847 ± 0.001</td>
<td></td>
</tr>
<tr>
<td>Hyodeoxycholic acid</td>
<td>11.03 ± 0.27</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lithocholic acid</td>
<td>11.39 ± 0.28</td>
<td>1.051 ± 0.002</td>
<td></td>
</tr>
<tr>
<td>Ursodeoxycholic acid</td>
<td>13.28 ± 0.32</td>
<td>1.204 ± 0.002</td>
<td></td>
</tr>
<tr>
<td>3β-Hydroxy-5-cholenoic acid</td>
<td>15.08 ± 0.36</td>
<td>1.368 ± 0.004</td>
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</tr>
</tbody>
</table>

* Values are means ± SD of 10 observations. Methyl ester TMS derivatives of bile acid standards were injected into the same polyethylene glycol 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).

Related to hyodeoxycholic acid (internal standard).

Results and discussion

For the separation of bile acid methyl ester trimethylsilyl-ether (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 (polyethylene glycol, 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 polyethylene glycol 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 derivatization 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
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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References


