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# GAS CHROMATOGRAPHY OF BILE ACIDS AS THEIR HEXAFLUOROISOPROPYL ESTER–TRIFLUOROACETYL DERIVATIVES

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## SUMMARY

Bile acids, such as cholic, chenodeoxycholic, deoxycholic, lithocholic and ursodeoxycholic acids, were allowed to react with hexafluoroisopropanol and trifluoroacetic anhydride at 37° for 30 min. The resulting derivatives were gas chromatographed on QF-1, with flame ionization detection, and were identified by gas chromatography–mass spectrometry. Separation was good. By using this method, these acids were detected in samples of human duodenal fluid; the ratios of each were 24.4, 41.5, 24.9, 2.3 and 6.9%, respectively.

## INTRODUCTION

For determination by gas chromatography (GC), the bile acids, cholic acid (C), chenodeoxycholic acid (CDC), deoxycholic acid (DC), lithocholic acid (LC) and ursodeoxycholic acid (UDC) are usually converted into methyl ester[1,2], methylester–trifluoroacetyl[3] or methyl ester–trimethylsilyl[4] derivatives. In all these methods, formation of the methyl ester is carried out with diazomethane. However, diazomethane is unstable and should be freshly generated before use; thus, it is not a suitable reagent for routine assays.

Recently, hexafluoroisopropanol together with trifluoroacetic anhydride was used successfully for the derivatization of acidic metabolites of catecholamines[5]. In this paper, we describe the preparation of hexafluoroisopropyl ester–trifluoroacetyl derivatives of bile acids with those reagents for subsequent determination of the acids by GC on QF-1. The application of the method to the estimation of the acids in human bile is suggested.

## EXPERIMENTAL

### *Materials*

The cholic, chenodeoxycholic, deoxycholic and lithocholic acids were a generous gift from Dr. O. W. Portman (Oregon Regional Primate Research Center, Beaverton, Ore., U.S.A.). Ursodeoxycholic acid was kindly supplied by Tokyo Tanabe Seiyaku (Tokyo, Japan), and trifluoroacetic anhydride was obtained from Nakarai Chemical (Kyoto, Japan). All other reagents were of reagent grade.

### *Apparatus and conditions*

A Shimadzu GC-3BF gas chromatograph equipped with a flame ionization detector was used, with a glass column (1.7 m × 3 mm I.D.) packed with 1% of QF-1 on Chromosorb W (80–100 mesh). The operating conditions were as follows: injection temperature, 240°; column temperature, 220°; nitrogen flow-rate, 45 ml/min. For gas chromatography–mass spectrometry (GC–MS), a Shimadzu LKB 9000 combined gas chromatograph–mass spectrometer was used; for GC, a glass tube (2 m × 3 mm I.D.) packed with 1% of QF-1 on Chromosorb W (80–100 mesh) was fitted. The flow-rate of helium was 28 ml/min, and the column temperature was 220°. For MS, the separator temperature was 270° and that of the ion source was 290°. The trap current was 60 μA. The electron energy was 20 eV, and accelerating potential was 3.5 kV.

### *Preparation of samples for GC or GC–MS*

To an adequate amount of a standard mixture of bile acids was added a specified amount of *d,l*-α-tocopheryl octanoate in ethyl acetate as internal standard. After evaporation under reduced pressure, the bile acids were dissolved in 100 μl of hexafluoroisopropanol and 200 μl of trifluoroacetic anhydride, and the mixture was set aside at 25° or 37° on a water bath. After an appropriate period, the mixture was evaporated under reduced pressure at room temperature, the residue was dissolved in 100 μl of acetonitrile, and 5 μl of this solution were subjected to GC or GC–MS. The acetonitrile solution was stored in a refrigerator in order to test its stability.

### *Preparation of samples from bile fluid*

A portion (1 ml) of bile-rich duodenal fluid obtained by duodenal tube from a fasting normal human was diluted to 20 ml with methanol–chloroform (1:2) (see ref. 6). After 1 h at 37°, the solution was filtered, 2 ml of filtrate were evaporated at 70°–80°, and the residue was dissolved in 10 ml of 15% sodium hydroxide solution in a nickel crucible. This solution was heated at 120° for 4 h in an autoclave, then cooled and acidified to pH ≃ 3 (methyl orange indicator) by addition of 6 *N* hydrochloric acid; it was then extracted with diethyl ether (2 × 20 ml). The organic layer was dried over anhydrous sodium sulphate and evaporated under reduced pressure after the addition of 0.1 ml of *d,l*-α-tocopheryl octanoate solution (2 mg/ml in ethyl acetate). The residue was treated as for the standard samples and submitted to GC.

## RESULTS

The reaction products of each of the bile acids tested, and that of *d,l*-α-toco-

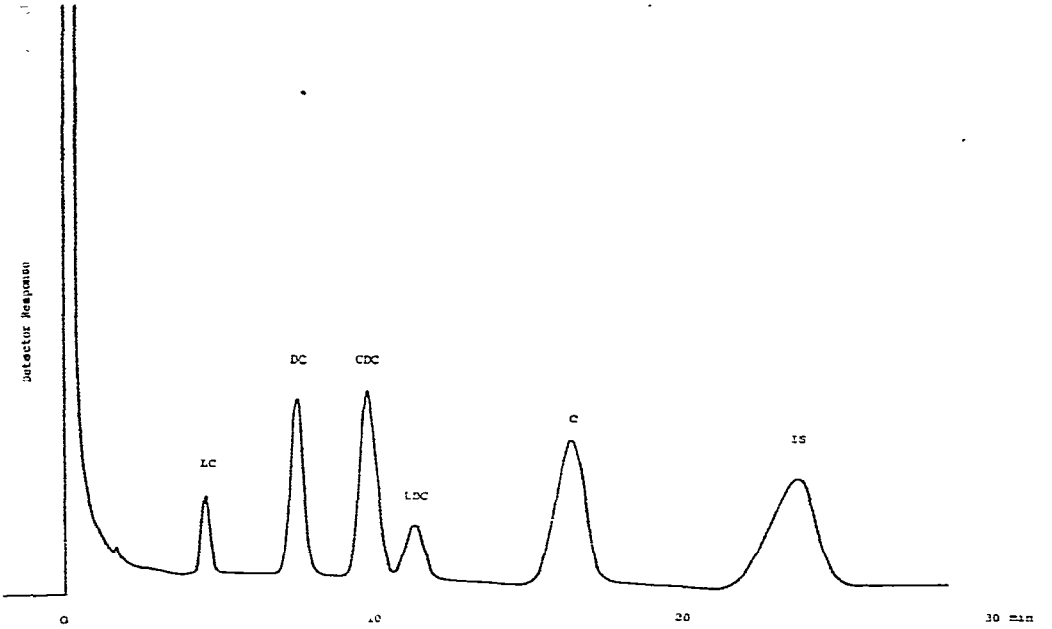


Fig. 1. Gas chromatogram of the reaction products of bile acids with hexafluoroisopropanol and trifluoroacetic anhydride; for abbreviations and GC conditions, see text. The amounts (μg) of the compounds present were: LC, 35.2; DC, 137; CDC, 218; UDC, 66.4; C, 266; internal standard (IS), 600.

TABLE I

SELECTED IONS FROM MASS SPECTRA OF HEXAFLUOROISOPROPYL ESTER–TRIFLUOROACETATE DERIVATIVES OF BILE ACIDS

M = molecular ion.

| *Value of m/e* | *Derivative of bile acid* | | | | |
|---|---|---|---|---|---|
| | *C* | *CDC* | *DC* | *UDC* | *LC* |
| 290 | 100 | | | | |
| 329 | | 46.1 | | | |
| 330 | | 26.9 | | | 100 |
| 331 | | 34.6 | | | 48.8 |
| 343 | | 53.8 | | | |
| 344 | | | | | 37.0 |
| 345 | | 26.9 | | | |
| 368 | 76.6 | | | | |
| 370 | | 30.8 | 100 | 25.3 | |
| 371 | | | 21.6 | | |
| 619 | 61.7 | | | | |
| 620 | 22.4 | | | | |
| 621 | | 100 ($M-OCOCF_3$) | 45.9 ($M-OCOCF_3$) | 100 ($M-OCOCF_3$) | |
| 622 | | 34.6 | 16.2 | 26.3 | 27.4 (M) |
| 623 | | | | 5.3 | 9.5 |
| 733 | 2.9 ($M-OCOCF_3$) | | | | |
| 734 | | 7.7 (M) | | | |

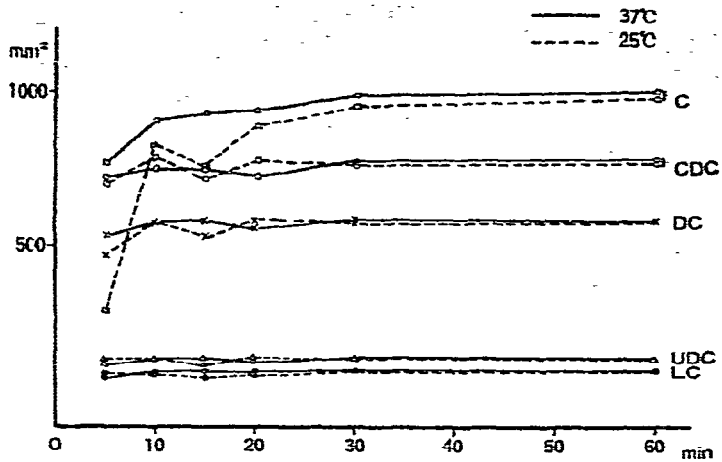


Fig. 2. Formation of hexafluoroisopropyl ester–trifluoroacetyl derivatives of bile acids; for abbreviations, see text.

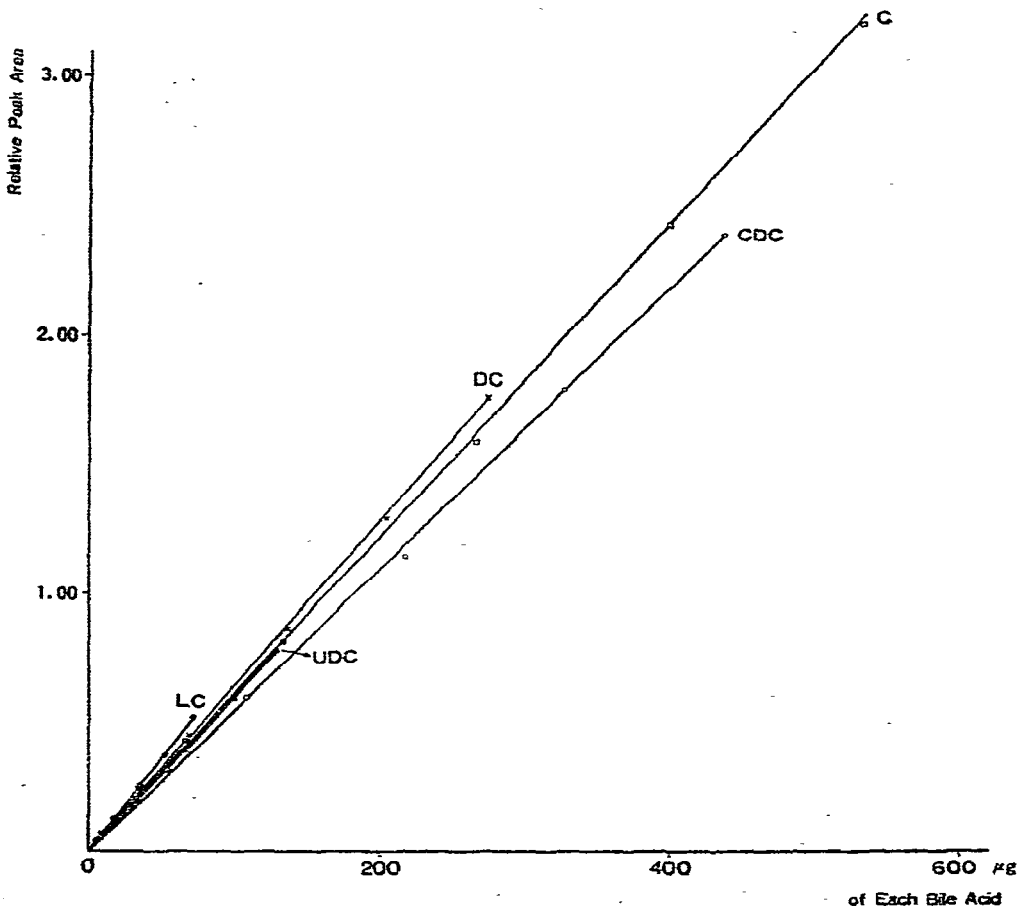


Fig. 3. Calibration graphs for bile acids with *d,l*-α-tocopheryl octanoate as internal standard.

pheryl octanoate, with hexafluoroisopropanol and trifluoroacetic anhydride gave a single peak on the 1% QF-1 column; the derivatives were well separated from each other, as shown in Fig. 1. The retention times relative to that of the internal standard (actual retention time, 24 min) were as follows: LC, 0.177; DC, 0.291; CDC, 0.380; UDC, 0.430; C, 0.650.

The spectrum obtained during GC–MS showed, for the CDC derivative, ions of *m/e* 734 (M) and 621 (M—$OCOCF_3$) (see ref. 7), which lend support for the structure of the hexafluoroisopropyl (HFIP) ester of the trifluoroacetyl (TFA) derivative of CDC. Similar results were obtained with the LC derivative, but for C, DC and UDC, the molecular ions were not apparent and the ions M—$OCOCF_3$ and/or M—$HOCOCF_3$ were detected (see Table I).

The bile acids were easily derivatized at the lower temperature, except for C, which should be allowed to react for at least 30 min at 37° (see Fig. 2). A higher temperature (*e.g.*, 60°) did not give reproducible results, probably because of loss of the reagents. Thus, the reaction was carried out at 37° for 30 min.

The derivatives were stable in acetonitrile solution for at least 5 days when stored in a refrigerator.

The relationships between the amounts of bile acids and the peak-area ratios of their HFIP ester–TFA derivatives to the TFA derivative of the internal standard were rectilinear in the ranges of 33.2–532 μg for C, 27.2–436 μg for CDC, 17.0–274 μg for DC, 4.4–70.4 μg for LC and 8.30–133 μg for UDC, as shown in Fig. 3.

A chromatogram of an extract from human bile is shown in Fig. 4; it can be seen that the sample contained C, CDC, DC, LC and UDC; their contents were

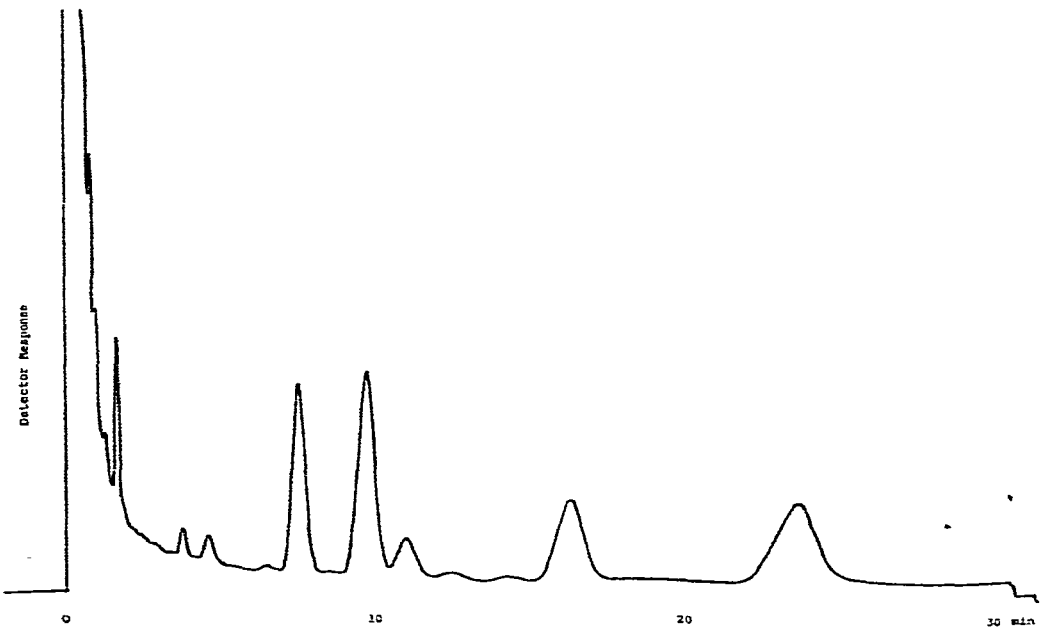


Fig. 4. Gas chromatogram of hexafluoroisopropyl ester–trifluoroacetyl derivatives of bile acids obtained from human bile.

calculated to be 1.06, 1.80, 1.08, 0.10 and 0.30 mg/ml, respectively, disregarding losses (about 10%) during the extraction.

## DISCUSSION

As with acidic metabolites of catecholamine[5], C, CDC, DC, LC and UDC were easily derivatized with use of hexafluoroisopropanol and trifluoroacetic anhydride at the lower temperature. The results obtained by GC–MS show that the carboxyl groups of bile acids are selectively esterified, and the hydroxyl groups acylated, with this reagent mixture.

The values estimated for bile acids in human bile by this method, *i.e.*, 24.4, 41.5, 24.9, 2.3 and 6.9% for C, CDC, DC, LC and UDC, respectively, are consistent with those obtained by colorimetry after paper-chromatographic separation[8]. Because it does not involve use of diazomethane, the method should be useful for the routine assay of bile acids in biological fluids.

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