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# GAS CHROMATOGRAPHIC AND MASS SPECTROMETRIC ANALYSIS OF BILE ACIDS AS TRIFLUOROACETYL-HEXAFLUOROISOPROPYL AND HEPTAFLUOROBUTYRYL DERIVATIVES

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## SUMMARY

The gas chromatographic retention times on QF-1 of 38 bile acids in the form of their trifluoroacetyl-hexafluoroisopropyl (TFA-HFIP), trifluoroacetyl-methyl, and heptafluorobutyryl derivatives are given. In general hexafluoroisopropyl ester trifluoroacetates proved superior with regard to simplicity of preparation, absence of artifacts, and resolution on QF-1. The main disadvantages of heptafluorobutyrates were the production of artifacts with some ketonic bile acids and the impossibility of separating any of the dihydroxy bile acids with substituents in the 3,6- and 3,7-positions. Mass spectra of TFA-HFIP derivatives were recorded with both direct and gas chromatographic inlet systems. The spectra of these derivatives are easily comparable with those of methyl ester trifluoroacetates and they enable the identification of positional isomers.

## INTRODUCTION

For gas chromatographic (GC) analysis bile acids are usually converted into methyl esters, methyl ester trimethylsilyl (TMS) or trifluoroacetyl (TFA) derivatives [1—6]. Among other derivatives only methyl ester acetates have gained any importance, because of their superior stability [7, 8]. Recently, however, the GC analysis of bile acids as hexafluoroisopropyl (HFIP) ester trifluoroacetyl derivatives [9] and most recently as heptafluorobutyrates (HFB) [10] has been proposed, mainly on the grounds of superior derivatization techniques and increased sensitivity by electron-capture detection. We checked the suitability of these new procedures for routine assays, and also the resolution on QF-1, which is the most promising liquid phase [5] for separation of these compounds. Furthermore we used TFA-HFIP derivatives of bile acids for combined gas chromatography—mass spectrometry (GC—MS) and compared their fragmentation patterns with those of methyl ester trifluoroacetates [11].

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## EXPERIMENTAL

### *Materials*

5β-Cholanoic acid, lithocholic acid (3α-hydroxy-5β-cholanoic acid), hyodeoxycholic acid (3α,6α-dihydroxy-5β-cholanoic acid), and 3α,7α-dihydroxy-12-keto-5β-cholanoic acid were obtained from Roth (Karlsruhe, G.F.R.), and deoxycholic acid (3α,12α-dihydroxy-5β-cholanoic acid), cholic acid (3α,7α,12α-trihydroxy-5β-cholanoic acid), dehydrocholic acid (3,7,12-triketo-5β-cholanoic acid), and cholesterol from Merck (Darmstadt, G.F.R.). Ursodeoxycholic acid (3α,7β-dihydroxy-5β-cholanoic acid) was purchased from Shuchardt (Munich, G.F.R.), while chenodeoxycholic acid (3α,7α-dihydroxy-5β-cholanoic acid), 7α,12α-, and 3-keto-7α,12α-dihydroxy-5β-cholanoic acids were supplied by Calbiochem (Giessen, G.F.R.). Hyocholic acid (3α,6α,7α-trihydroxy-5β-cholanoic acid) was from Serva (Heidelberg, G.F.R.), 3β-hydroxy-5α-, 3β-hydroxy-3-keto-, 3α,6β-dihydroxy-, 3α-hydroxy-6-keto-, 3α-hydroxy-7-keto-, 3α-hydroxy-12-keto-, 3,6-diketo-, 3,7-diketo-, 3,12-diketo-, 7,12-diketo-, 3α,12α-dihydroxy-7-keto-5β-cholanoic acids and 3β-hydroxy-$\Delta^5$-cholenoic acid, and 3α-hydroxy-12-keto-$\Delta^{9(11)}$-5β-cholenoic acid were obtained from Steraloids (Pawling, NY, U.S.A.) and 3α-hydroxy-6-keto-5α- and 3,6-diketo-5α-cholanoic acids from Makor (Jerusalem, Israel). 3α-Hydroxy-7,12-diketo-5β-cholanoic acid and apocholic acid (3α,12α-dihydroxy-$\Delta^{8(14)}$-5β-cholenoic acid) were available in the laboratory from previous studies. 3β,7α-Dihydroxy- and 3,7-diketo-12α-hydroxy-5β-cholanoyl methyl esters were generous gifts from Dr. J. Sjövall (Stockholm, Sweden), 3β,12α-dihydroxy-, 3-keto-7β-hydroxy-, and 3-keto-12α-hydroxy-5β-cholanoyl methyl esters were from Dr. P. Szczepanik (Argonne, IL, U.S.A.), and 3-keto-7α-hydroxy-5β- and 3α,7α,12α-trihydroxy-5α-cholanoyl methyl esters from Dr. W.H. Elliott (St. Louis, MO, U.S.A.). Trifluoroacetic anhydride and hexafluoroisopropanol were supplied from Merck—Shuchardt (Munich, G.F.R.), QF-1 phase was from Serva.

### *Gas chromatography and gas chromatography—mass spectrometry*

A Varian Model 1700 gas chromatograph equipped with dual flame-ionisation detectors and silanized glass columns (1.8 m × 3 mm I.D.) packed with 3% QF-1 on Chromosorb W AW DMCS (100—200 mesh) were used for all determinations. The operating conditions were: injection temperature 240°C, column temperature 230°C, detector temperature 300°C, nitrogen flow-rate 30 ml/min. For GC—MS analysis an instrument combination, consisting of a Varian Model 1700 gas chromatograph with a two-stage helium separator by Biemann-Watson, a Varian MAT mass spectrometer, Model CH 7A, and a Varian Spectro System 100 data system were used. Helium was substituted for nitrogen. The operating conditions were: separator temperature 220°C, accelerating voltage 3 kV, electron energy 70 eV, trap current 60 μA, and the ion-source temperature 180°C. Mass spectra of bile acid TFA-HFIP derivatives were obtained mostly with both direct and gas chromatographic inlet systems.

*Derivatization procedures*

Bile acid TFA-HFIP derivatives were prepared by dissolving each bile acid in 300 μl of a mixture of 200 μl of trifluoroacetic anhydride and 100 μl of hexafluoroisopropanol [9]. The mixture was incubated at 37°C for 30 min, evaporated under reduced pressure at room temperature and the dry residue dissolved in about 200 μl of acetonitrile. Aliquots of this solution were subjected to GC or GC—MS. Substances available as methyl esters only were first saponified with methanolic KOH, acidified with HCl, and extracted with diethyl ether (five times). Bile acid TFA-methyl derivatives were prepared in two steps. First methyl esters were prepared by the reaction of the acid, dissolved in diethyl ether—methanol (9:1), with diazomethane in an ethereal solution added in excess. Trifluoroacetates were obtained by dissolving the methyl ester in trifluoroacetic anhydride and subsequent treatment at 37°C for 30 min. Excess reagent was evaporated under reduced pressure and the residue dissolved in acetonitrile. Bile acid HFB derivatives were prepared according to the method of Musial and Williams [10].

## RESULTS

*Gas chromatography*

Table I summarizes the relative retention times (RRT) of 38 bile acids, tested as trifluoroacetates of hexafluoroisopropyl or methyl esters and as heptafluorobutyrates. The retention behaviour was characterized by RRT, to allow direct comparison of the values obtained with previous data. All but four bile acids were 5β-cholanoic acids. Separation of the 5α and 5β epimers was impossible for the 3,6-diketo-cholanoic acid because of poor resolution. The epimeric 3α-hydroxy-6-keto-cholanoic acids, however, could be separated as TFA-HFIP or HFB derivatives. 3α-Hydroxy-6-keto-5α-cholanoic acid was another rare exception from the general rule [5] that selective phases such as QF-1 retain the 5α-cholanoates longer than the corresponding 5β-cholanoates. It should be stressed that all dihydroxy bile acids with substituents in the 3,6- and 3,7-positions, isomers and epimers alike, were indistinguishable when analyzed as heptafluorobutyrates. However, separation of bile acids with epimeric hydroxyl functions as HFB derivatives was not impossible, as can be seen from 3α,12α- and 3β,12α-dihydroxy-5β-cholanoic acids.

The long retention times of ketonic acids are characteristic for the QF-1 phase. Most of them may be separated as esters or heptafluorobutyric anhydrides. It is of particular value that all ketonic bile acids tested were resolved as TFA-HFIP derivatives. As HFB or TFA-methyl derivatives, however, the two monoketo oxidation products of deoxycholic acid could not be separated, whereas the oxidation products of cholic acid with one keto group at carbon 3 or 12 were inseparable as methyl ester trifluoroacetates.

A few hydroxy bile acids [3α-hydroxy- and 3β-hydroxy-, 3α,6α- and 3α,7β-dihydroxy-cholanoic acids, and 3β,12α-dihydroxy-cholanoic acid and 3α,12α-dihydroxy-$\Delta^{8(14)}$-5β-cholenoic acid (apocholic acid)] were not resolved by any of the three methods, but most other bile acids were resolved well. Hyocholic acid could not be analyzed as the TFA or HFB derivative

TABLE I

RELATIVE RETENTION TIMES OF BILE ACID HEXAFLUOROISOPROPYL AND METHYL ESTER TRIFLUOROACETATES AND OF HEPTAFLUOROBUTYRATES ON 3% QF-1 AT 230°C

All relative retention times are referred to the corresponding deoxycholate derivatives. Mean absolute elution times were: TFA-HFIP derivative 642 sec; TFA-methyl derivative 1007 sec; HFB derivative 811 sec. The deoxycholate derivative was injected simultaneously with each of the esters. All data tabulated are mean values from 5—6 estimations. Mean standard deviation was 0.003 up to RRT = 1.00, 0.015 up to RRT = 2.5, 0.025 up to RRT = 5.0 and 0.04 for higher RRT values.

| Component* | Relative retention time | | |
|---|---|---|---|
| | TFA-HFIP | TFA-methyl | HFB |
| Cholesterol | 0.49 | 0.32 | 0.47 |
| Cholanoic | 0.20 | 0.25 | 0.19 |
| 3α | 0.60 | 0.69** | 0.54 |
| 3β | 0.59 | 0.68** | 0.56 |
| 3β-$\Delta^5$ | 0.66 | 0.73 | 0.66 |
| 3β,$\Delta^5$-22,23-bisnor | 0.35 | 0.36 | *** |
| 5α,3β | 0.74 | 0.81 | 0.73 |
| 3α,6α | 1.48 | 1.37** | 1.22 |
| 3α,6β | 1.38 | 1.24 | 1.21 |
| 3β,7α | 1.05 | 1.02** | 1.25 |
| 3α,7α | 1.29 | 1.22** | 1.21 |
| 3α,7β | 1.49 | 1.40 | 1.20 |
| 3β,12α | 0.86 | 0.88 | 0.76 |
| 3α,12α | 1.00 | 1.00 | 1.00 |
| 7α,12α | 0.62 | 0.63** | 0.60 |
| 3α,12α,$\Delta^{8(14)}$ | 0.86 | 0.85 | 0.85 |
| 3-keto | 1.37 | 1.49 | 1.19 |
| 3α,6-keto | 2.69 | 2.69 | 2.33§ |
| 3α,7-keto | 2.30 | 2.24** | 1.93§ |
| 3α,12-keto | 1.93 | 2.03** | 1.68 |
| 3-keto,7α | 2.62 | 2.37**,§ | 2.10 |
| 3-keto,7β | 2.74 | 2.84 | 2.32 |
| 3-keto,12α | 2.07 | 2.00** | 1.66 |
| 3α,12-keto,$\Delta^{9(11)}$ | 2.12 | 2.55 | 1.82 |
| 5α-3α,6-keto | 2.46 | 2.63§ | 2.01§ |
| 3,6-diketo | 5.89 | 5.96 | 4.99 |
| 3,7-diketo | 4.01 | 4.34 | 3.40 |
| 3,12-diketo | 3.63 | 4.29 | 3.10 |
| 7,12-diketo | 1.72 | 2.18 | 1.46 |
| 5α-3,6-diketo | 5.91 | 6.06 | 5.08 |
| 3α,6α,7α | 2.00§ (2.69) | 1.84§§ | 2.31§§ |
| 3α,7α,12α | 2.22 | 1.90** | 2.58 |
| 5α-3α,7α,12α | 2.44 | 2.02** | 2.80 |

TABLE I (continued)

| Component* | Relative retention time | | |
|---|---|---|---|
| | TFA-HFIP | TFA-methyl | HFB |
| 3α,7α,12-keto | 3.77 | 3.56** | 3.31 |
| 3α,12α,7-keto | 3.28 | 2.93** | 3.02§ |
| 3-keto,7α,12α | 4.30 | 3.59** | 3.71§ |
| 3α,7,12-diketo | 5.50 | 6.35 | 4.70 |
| 3,7-diketo,12α | 5.16 | 5.28 | 3.96§ |
| 3,7,12-triketo | 8.24 | 9.70 | 6.89 |

*The cholanoic acids are identified by the location (3, 6, 7 or 12) and configuration (α or β) of the hydroxyl groups as well as the location of the keto groups and double bonds (Δ). They are all 5β-acids if another ring junction of the A/B rings is not indicated by 5α.
**Data from ref. 6.
***No peak visible, probably because of unsuccessful derivatization procedure.
§The compounds give two peaks.
§§The compounds often give two peaks.

because of thermal decomposition, which is critical for most 3,6,7-trihydroxy acids on account of the vicinal 6- and 7-hydroxyl groups [6]. Thermal decomposition was observed occasionally with HFB derivatives of other hydroxy bile acids too. With the ketonic acids indicated in Table I, however, we always saw a second peak. This artifact accounted for up to about 30% of the original compound, but we do not know if it is a product of derivatization or of decomposition in the gas chromatograph. With TFA-HFIP derivatives we have not observed any artifacts so far.

*Mass spectrometry*

All mass spectra of bile acid TFA-HFIP derivatives were interpreted with the help of known fragmentation patterns of TFA or trimethylsilyl (TMS) derivatives of bile acid methyl esters [11]. Arithmetic considerations showed that the known fragmentation pathways can be distinctly seen with HFIP esters as well. It should be stressed that neither detailed mechanistic studies

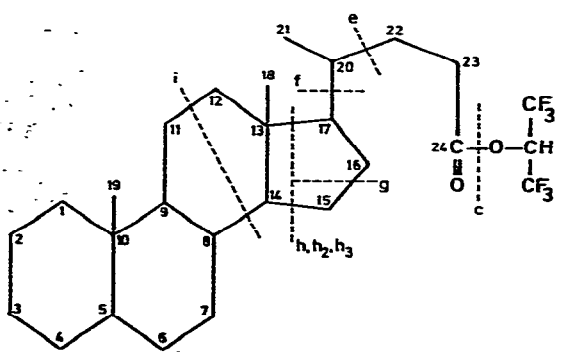


Fig. 1. Schematic illustration of diagnostically significant fragmentations of the carbon skeleton of hexafluoroisopropyl cholanoates according to Sjövall et al. [11].

with labeled acids nor high-resolution mass determination of particular ions were made. For an easier comparison of both published and measured data we used the same signs for fragment ions as Sjövall et al. [11]. Fig. 1 and Table II show, in a simplified way, these fragmentation patterns. Hexafluoroisopropyl cholanoates differ only by 136 mass units at ions containing the ester group.

TABLE II

SCHEMATIC REPRESENTATION OF THE TENTATIVE ORIGIN OF IONS FORMED IN THE FRAGMENTATION OF SUBSTITUTED METHYL AND HEXAFLUOROISOPROPYL CHOLANOATES

| Fragmentation | Methyl cholanoates* | | Hexafluoroisopropyl cholanoates | |
|---|---|---|---|---|
| | Lost fragment | Mass | Lost fragment | Mass |
| a | $H_2O$ | 18 | $H_2O$ | 18 |
| $a_1$ | $CF_3COOH$ | 114 | $CF_3COOH$ | 114 |
| $a_4$ | | | $CF_3COO$ | 113 |
| b | $CH_3$ | 15 | $CH_3$ | 15 |
| c | $CH_3O$ | 31 | $(CF_3)_2CHO$ | 167 |
| e | 22—24 | 87 | 22—24 | 223 |
| f | 20—24 | 115 | 20—24 | 251 |
| g | 16—17; 20—24 | 142 | 16—17; 20—24 | 278 |
| i | AB—11 | 166** | AB—11 | 166** |
| h | 15—17; 20—24 | 157 | 15—17; 20—24 | 293 |
| $h_2$ | 15—17; 20—24 | 155 | 15—17; 20—24 | 291 |
| $h_3$ | ABC | 220*** | ABC | 220*** |

*According to ref. 11.
**With no substituents in rings A, B.
***With no substituents in rings A, B, C.

*Dihydroxy bile acids.* The trifluoroacetates of hexafluoroisopropyl cholanoates generally cleaved in the same way as the methyl cholanoates (Table III). Trifluoroacetic acid (fragments $a_1$, $2a_1$) and the side-chain (e, f) were gradually lost, and ring D was cleaved (g, h), but these ions differed, as expected, in their relative intensities. The most striking change was an enhanced intensity of the $a_1$ ion, formed by the loss of one trifluoroacetic acid molecule.

In both 3,7-substituted HFIP cholanoates this ion became the base peak, whereas its intensity was significantly lower in the methyl ester (Table III, [11]). The subsequent loss of the side-chain with carbons of the ring D ($a_1g$) resulted in the second prominent fragment. In the spectrum of the 3α,12α-substituted dihydroxy bile acid the base peak $a_1f$ did not change, but the intensity of the fragment $a_1$ increased considerably, compared with the methyl ester (Table III, [11]).

The loss of the trifluoroacetyl group seemed to proceed in two ways, as $CF_3COOH$ ($a_1$) and $CF_3COO$ ($a_4$). The latter mode of elimination gave rise to the base peaks of the TFA-HFIP derivatives of chenodeoxycholic, ursodeoxycholic, and deoxycholic acids as reported by Imai et al. [9]. In our spectra

TABLE III

MASS SPECTRA OF THE TRIFLUOROACETATES OF METHYL AND HEXAFLUORO-ISOPROPYL DIHYDROXY-5β-CHOLANOATES

| Ion | Methyl esters* | | | HFIP esters* | | | |
|---|---|---|---|---|---|---|---|
| | *m/e* | Intensity (%) | | *m/e* | Intensity (%) | | |
| | | 3α,7α (CDC) | 3α,12α (DC) | | 3α,7α (CDC) | 3α,7β (UDC) | 3α,12α (DC) |
| $M^+$ | 598 | — | — | 734 | 5.5 | — | — |
| $a_1$ | 484 | 27.9 | 7.3 | 620 | 100.0 | 100.0 | 64.6 |
| $a_1b$ | 469 | 8.3 | — | 605 | 9.9 | 9.4 | 5.7 |
| $a_1a_4$ | 371 | 28.3 | 13.6 | 507 | 19.3 | 19.4 | 14.2 |
| $2a_1$ | 370 | 80.1 | 36.3 | 506 | 12.4 | 16.9 | 6.4 |
| $2a_1b$ | 355 | 42.3 | 6.0 | 491 | 4.2 | 5.2 | — |
| $a_1c$ | 453 | — | 2.8 | 453 | 11.0 | 5.6 | 7.8 |
| $a_4e$ | 398 | — | — | 398 | 7.9 | 5.5 | 4.4 |
| $a_1e$ | 397 | — | — | 397 | 4.9 | 4.5 | 4.8 |
| $a_1f$ | 369 | 54.7 | 100.0 | 369 | 21.9 | 22.7 | 100.0 |
| i | 208 | 10.6 | 3.7 | 344 | 17.1 | 14.7 | 7.2 |
| $a_1g$ | 342 | 7.4 | 20.4 | 342 | 43.8 | 26.3 | 14.1 |
| $a_4h$ | 328 | 7.7 | 2.6 | 328 | 36.9 | 67.4 | 14.4 |
| $a_1h$ | 327 | 12.9 | — | 327 | 18.5 | 17.3 | 6.7 |
| $h_3$ | 154 | 38.1 | — | 290 | 19.3 | 17.0 | 41.0 |
| $2a_1f$ | 255 | 53.7 | 42.9 | 255 | 13.5 | 18.3 | 7.3 |
| $a_1a_4g$ | 229 | 13.2 | 3.8 | 229 | 10.7 | 9.1 | 5.4 |
| $2a_1g$ | 228 | 12.5 | 3.7 | 228 | 12.8 | 10.8 | — |

*CDC = chenodeoxycholic acid; UDC = ursodeoxycholic acid; DC = deoxycholic acid.

of the hexafluoroisopropyl esters of these acids the fragments $2a_1$, $a_1e$, and $a_1h$ were accompanied by intense ions, one mass unit heavier. These peaks were significantly higher than the corresponding isotopic contributions of $^{13}C$ atoms and they may arise by the loss of the trifluoroacetyl group without a ring hydrogen.

Spectra of positional isomers differed distinctly. A major reaction in bile acids having a 12-hydroxyl group was the loss of one molecule of trifluoroacetic acid and the side-chain, whereas 3,6- and 3,7-dihydroxy bile acids preferentially lost two trifluoroacetic acid molecules [11]. The replacement of the methyl ester group by the hexafluoroisopropyl ester group did not change this rule. The base peak of deoxycholic acid ($a_1f$) was about fifteen times more intense than the $2a_1$ ion. With chenodeoxycholic and ursodeoxycholic acid the ratio of the fragments $a_1f$:$2a_1$ was smaller than 2.

*Trihydroxy bile acids.* Table IV gives spectra typical of cholanoic acids with three hydroxyl groups. The elimination of trifluoroacetyl groups ($a_1$, $2a_1$, $3a_1$), of the side-chain ($a_1f$, $2a_1f$, $3a_1f$), and the cleavage of the ring D ($2a_1g$) were seen. The molecular ion was absent. The spectrum, obtained with the GC inlet, shows, as expected, a shift of dominant ions to lower mass units on account of thermal excitation. The ion $2a_1f$ became the base peak, whereas in the direct inlet spectrum this ion was less intense than its precursor $2a_1$. The intensity ratio of $2a_1f$ and $3a_1$ ions was in both spectra in agreement

TABLE IV

MASS SPECTRUM OF THE TRIFLUOROACETATE OF HEXAFLUOROISOPROPYL 3α,7α,12α-TRIHYDROXY-5β-CHOLANOATE (CHOLIC ACID)

| *m/e* | Ion | Intensity (%) | |
|---|---|---|---|
| | | Direct inlet | GC inlet |
| 846 | $M^+$ | — | — |
| 732 | $a_1$ | 6.4 | — |
| 619 | $a_1a_4$ | 31.6 | 17.4 |
| 618 | $2a_1$ | 88.4 | 24.4 |
| 603 | $2a_1b$ | 5.6 | 4.9 |
| 505 | $2a_1a_4$ | 12.1 | 9.4 |
| 504 | $3a_1$ | 8.0 | 7.2 |
| 489 | $3a_1b$ | 4.9 | 6.3 |
| 481 | $a_1f$ | 14.6 | 5.2 |
| 367 | $2a_1f$ | 53.6 | 100.0 |
| 340 | $2a_1g$ | 4.8 | 5.0 |
| 290 | $h_3$ | 100.0 | 45.7 |
| 253 | $3a_1f$ | 11.6 | 35.5 |

with the finding by Sjövall et al. [11]. In the presence of a trifluoroacetoxy group the former ion was more intense than the latter. The base peak $h_3$ in the direct inlet spectrum was observed neither by Sjövall et al. [11] nor by us in other spectra recorded with the GC—MS combination. An impurity enhancing its intensity to 100% cannot be excluded. As in the case of dihydroxy bile acids, the elimination of the trifluoroacetoxy group could also occur without a ring hydrogen (ions $a_1a_4$, $2a_1a_4$).

*Ketonic bile acids.* The usual fragmentation pattern of the 3α-hydroxy-7-keto-cholanoic acid could be easily recognized (Table V). The molecular ion at *m/e* 636 was pronounced in both direct and GC inlet records. Major fragmentation pathways were loss of water (a), of a trifluoroacetyl group ($a_1$, $a_4$), and their combination ($aa_1$), of the hexafluoroisopropyl ester group (ac) and the side-chain (f, af, $aa_1f$). The fission of rings C (i) and D ($aa_1h$) was also pronounced.

Sjövall et al. [11] emphasized the directing effect of a 7-keto group for fragmentation reactions. In the spectrum of the silyl ether of 3α-hydroxy-7-keto-5β-methyl-cholanoate a diagnostically important peak at *m/e* 292 was noted (fission of the 5,6 and 9,10 carbon bonds). The corresponding peak of HFIP ester at *m/e* 428 was a dominant signal. The cleavage of the 8,14 and 12,13 bonds of the methyl ester trifluoroacetate of 3α-hydroxy-7-keto-cholanoate gave rise to the peak at *m/e* 303. This ion was seen also in the spectrum of the HFIP ester. Last, ketonic bile acids with 7-keto groups lost water more readily than other ketocholanoates. The intensity of the "a" ion in the HFIP ester was also in accordance with this finding.

The simultaneous presence of two hydroxyl groups in 3α,12α-dihydroxy-7-keto-5β-cholanoic acid changed the directing effect of the 7-keto group (Table VI). The spectrum was characterized by the usual fragments due to loss of trifluoroacetyl groups, water, and side-chain. Loss of water occurred, however, less readily than in 3α-hydroxy-7-keto-5β-cholanoic acid and the

ion at *m/e* 303 was missing. The intense fragment at *m/e* 426 might have been formed by fission of the 9,10 and 5,6 carbon bonds and additional loss of the 12-trifluoroacetyl group (analogous to the typical peak at *m/e* 428 in Table V).

TABLE V

MASS SPECTRUM OF THE TRIFLUOROACETATE OF HEXAFLUOROISOPROPYL 3α-HYDROXY-7-KETO-5β-CHOLANOATE

| *m/e* | Ion | Intensity (%) | |
|---|---|---|---|
| | | Direct inlet | GC inlet |
| 636 | $M^+$ | 100.0 | 19.4 |
| 618 | a | 40.6 | 4.9 |
| 603 | ab | 4.9 | 13.9 |
| 523 | $a_4$ | 11.9 | 13.9 |
| 522 | $a_1$ | 4.6 | 35.9 |
| 504 | $aa_2$ | 6.4 | 15.8 |
| 489 | $aa_1b$ | 17.2 | 15.6 |
| 451 | ac | 32.4 | 13.1 |
| 428 | | 87.9 | 43.2 |
| 385 | f | 16.4 | 23.1 |
| 367 | af | 34.4 | 18.3 |
| 303 | | 27.1 | 12.2 |
| 290 | i | 18.6 | 8.4 |
| 253 | $aa_1f$ | 13.3 | 22.7 |
| 211 | $aa_1h$ | 17.6 | 17.7 |

TABLE VI

MASS SPECTRA OF THE TRIFLUOROACETATES OF HEXAFLUOROISOPROPYL 3α,12α(3α,7α-DIHYDROXY-7(12)-KETO-CHOLANOATES

| *m/e* | Ion | Intensity (%) | | |
|---|---|---|---|---|
| | | 3α,12α-7-keto (direct inlet) | 3α,12α-7-keto (GC inlet) | 3α,7α-12-keto (GC inlet) |
| 748 | $M^+$ | 18.3 | — | 20.5 |
| 730 | a | 6.3 | — | — |
| 635 | $a_4$ | 56.4 | 21.0 | 5.6 |
| 634 | $a_1$ | 98.9 | 41.3 | 11.3 |
| 616 | $aa_1$ | 46.6 | 16.1 | — |
| 521 | $2a_4$ | 17.3 | 14.3 | — |
| 520 | $2a_1$ | 7.1 | 19.3 | 5.5 |
| 487 | $2a_1ab$ | 6.8 | 7.2 | — |
| 457 | $h_2$ | — | — | 26.2 |
| 426 | | 71.3 | 41.4 | — |
| 343 | $a_1h_2$ | 56.3 | 28.6 | 38.9 |
| 383 | $a_1f$ | 100.0 | 92.4 | — |
| 365 | $aa_1f$ | 24.1 | 17.0 | — |
| 269 | $2a_1f$ | 13.7 | 64.1 | 6.5 |
| 251 | $2a_1af$ | 14.8 | 33.8 | — |
| 229 | $2a_1h_2$ | 9.3 | 8.4 | 100.0 |

The spectrum of 3α,7α-dihydroxy-12-keto-5β-cholanoic acid (Table VI) was recorded with the GC inlet only and originated from a biological sample. The number and the kind of substituents could be easily recognized (molecular ions and fragments $a_1$, $2a_1$). The position of the keto group was clearly indicated, analogous to the TMS derivative [11], by the intense fragment $h_2$ and the base peak $2a_1h_2$.

## DISCUSSION

### *Gas chromatography*

Derivatives of bile acids suitable for GC analysis should be easily and quantitatively prepared without by-products, and should be stable during storage and chromatographic analysis. TFA-HFIP derivatives meet these criteria very well. Preparation is fast and simple and no by-products arise during derivatization. This is true even in complex mixtures of extraneous compounds. During GC, all compounds tested, except hyocholic acid, gave a single peak. TFA-HFIP derivatives were found to be stable for at least five days (ref. 9 and our own experience) when stored in the refrigerator, provided moisture was excluded.

Methyl esters are mostly prepared with diazomethane, which is, however, unstable and therefore not a suitable reagent for routine assays. Moreover, diazomethane may convert hydroxyl groups of the steroid nucleus to methoxy groups [12, 13]. Besides TFA derivatives methyl esters are also converted into TMS derivatives for GC analysis, but side reactions have been observed with silylating agents too [14, 15]. TFA-methyl derivatives are stable for at least two days, when they are stored in the refrigerator and moisture is excluded. They may, however, undergo thermal decomposition in the chromatograph. TFA derivatives of 3,6,7-trihydroxy bile acids are reported to be particularly sensitive compounds [6]. This should be true for TFA-HFIP derivatives too, although our experimental experience is limited to hyocholic acid (3α,6α,7α-trihydroxy-5β-cholanoic acid). These types of derivatives, therefore, are probably not suited for the analysis of bile acids in mice, rats, and pigs.

Preparation of HFB derivatives is more laborious and time-consuming. By-products of the derivatization procedure were not observed with hydroxy bile acids, but thermal decomposition during GC was occasionally seen. With ketonic bile acids we often saw two peaks during GC, but we do not know if these artifacts originate from derivatization or from thermal decomposition. This represents a serious disadvantage in the use of HFB derivatives, which otherwise enable the analysis of conjugated bile acids without prior deconjugation [10]. Musial and Williams [10] report HFB derivatives to be stable for one year.

In spite of their higher molecular weights, bile acid hexafluoroisopropyl esters and heptafluorobutyrates (except the cholic acid HFB derivative) are eluted earlier than the corresponding methyl esters. This is probably due to interference of the fluoro groups in the compound with the fluoro groups of QF-1. Besides the nature of the carboxyl derivative, the retention behaviour depends on the number, nature and position of substituents. From the RRTs

of hydroxy bile acids it is concluded that introduction of an hydroxyl group brings about an effect opposite to the fluorinated ester or anhydride groups. The RRTs of monohydroxy bile acid TFA-HFIP and HFB derivatives are all less than those of TFA-methyl derivatives. With dihydroxy bile acids there is little variation, whereas the TFA-HFIP and HFB derivatives of trihydroxy acids show longer RRTs than the corresponding methyl ester trifluoroacetates. Similar effects can be seen with hydroxyketo acids. So TFA-HFIP derivatives of most bile acids are more effectively resolved than the TFA-methyl derivatives considering the number of overlapping peaks. The resolution of heptafluorobutyrates on QF-1 is in general less efficient, but sufficient for the separation of many bile acids.

On the fluorosilicone QF-1, selective for carbonyl groups [5], keto bile acids are effectively separated and resolution is again best for TFA-HFIP derivatives. In this respect QF-1 is superior to non-polar phases such as OV-1, SE-30, and to the less polar OV-17. Separation on OV-210, another fluorosilicone, is similar to that on QF-1 [16]. Data for OV-225, a cyanopropylphenylsilicone, are available for methyl ester acetates only [7]. The results are generally comparable with our results, but the separation of ketonic bile acids is less efficient.

Capillary GC [17—19] and high-performance liquid chromatography (HPLC) of bile acids [20—22] can not yet substitute for the well-established packed column GC methodology for routine assays. Capillary GC is hampered mainly by insufficient resolution on many commercial stationary phases, limited life of columns and adsorptive effects of bile acid derivatives [18], whereas HPLC of bile acids is still in an early developmental stage.

*Mass spectrometry*

Substitution of the methyl ester group by the more polar hexafluoroisopropyl ester group did not result in any disadvantages for mass spectrometric identification. General fragmentation pathways, indicating number and kind of substituents, were retained. Neither did the presence of an HFIP group influence fragmentation rules characterizing positional isomers. In dihydroxy bile acids the intensity ratio of $a_1f$:$2a_1$ fragments indicates the presence of the 12-hydroxyl group; similarly the ratio of $2a_1f$:$3a_1$ fragments in trihydroxy bile acids shows the presence of the 12-hydroxyl group. The directing effect of the 7-keto group is also distinct (typical ions *m/e* 303, 428, and 618 in 3$\alpha$-hydroxy-7-keto-cholanoic acid and probably the ion *m/e* 426 in 3$\alpha$, 12$\alpha$-dihydroxy-7-keto-cholanoic acid). The fission of ring D, typical of 12-keto acids, is also seen (ions $h_2$, $2a_1h_2$ in 3$\alpha$,7$\alpha$-dihydroxy-12-keto-cholanoic acid).

HFIP esters show a more intense $a_1$ fragment in hydroxy bile acids than the corresponding methyl esters. In ketohydroxy bile acids this tendency was not observed. For both types of substituents a more intense cleavage of the $CF_3COO$ group instead of the $CF_3COOH$ group is typical.

HFIP esters are suitable for combined GC—MS analysis with packed columns. In spite of their higher polarity they were not retained in the GC—MS interface and only a minor tailing of their GC peaks was observed. Due to their higher molecular weights more fragments in the higher mass range are found. A continuous ion registration in this mass range shows some drawbacks,

for example, a decline of sensitivity due to the decreased resolution, but the enhanced specificity of these ions is more convenient for selective ion monitoring.

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