Quantitative assay of conjugated and free bile acids as heptafluorobutyrate derivatives by gas–liquid chromatography

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Abstract Quantitative analyses of individual bile acids in biological samples are limited by the lengthy multistep preparations necessary. Using heptafluorobutyric acid anhydride in pyridine as a derivatizing agent, we reduced several steps to one. Bile acids and their glycine and taurine conjugates form stable heptafluorobutyrate derivatives, eliminating the need for deconjugation and preparation of methyl esters. The derivatives have been characterized by mass spectrometry, and optimum reaction yields have been determined. Operating conditions for analyzing the bile acid heptafluorobutyrates by gas–liquid chromatography on various column packings were investigated, and 0.5% QF-1 or 3% OV-225 was found suitable. The bile acid derivatives were identical whether starting with the free bile acid or the glycine or taurine conjugates. The procedure was applied to a quantitative analysis of artificial mixtures of bile acids and bile conjugates, and also of human bile. The results compared favorably to those obtained with a 3α- and 7α-hydroxysteroid dehydrogenase fluorimetric method.

Supplementary key words deconjugation · mass spectrometry · electron-capture detection · 3α- and 7α-hydroxysteroid dehydrogenase · human bile

The recent improvements in trace steroid determinations and new derivation techniques for steroids (1–10) led us to an improved and rapid analysis of bile acids and bile conjugates using heptafluorobutyrate derivatives. Lithocholic, deoxycholic, chenodeoxycholic, and cholic acids all form single, well-characterized, and stable derivatives which exhibit complete esterification at all possible centers including the C_{24}-carboxylic acid group. Moreover, both the taurine and glycine conjugates of each bile acid react with the same derivatizing agent giving products identical to those of the free acids.

The analyses of bile acids by previous methods cited in the literature involve a number of procedures prior to GLC analysis. Among these are deconjugation by alkaline hydrolysis under pressure (11–15) or enzymic hydrolysis using clostridial peptide-bond hydrolase (12, 15–20), preliminary separations of bile acids by thin-layer chromatography or column chromatography (13, 14, 17, 20–23), and the formation of methyl esters, trimethylsilyl or trifluoroacetyl derivatives (11–25).

With heptafluorobutyric acid anhydride, it is now possible to derivatize and deconjugate a crude sample of bile conjugates in bile in one easy step prior to GLC analysis, without depending on an initial thin-layer chromatographic separation of the individual bile acids before analysis.

MATERIAL AND METHODS

Chemicals

All solvents, glass-distilled and dried, were obtained from Caledon Laboratories Ltd., Georgetown, Ontario, Canada, and were used without further purification. Heptafluorobutyric acid anhydride was obtained from Pierce Chemical Company, Rockford, IL. All bile acids and bile conjugates (as sodium salts) were from Supelco, Inc., Bellefonte, PA, with the exception of sodium taurolithocholate which was from Calbiochem, Los Angeles, CA. Only those standards found to be 98–99% pure by TLC were used for quantitative work. Supelcosil was from Supelco, Inc.

Abbreviations: Trivial names of hydroxy-substituted-5β-cholanoic acids are denoted by LC, lithocholic acid, 3α-OH; DC, deoxycholic acid, 3α-OH, 12α-OH; CDC, chenodeoxycholic acid, 3α-OH, 7α-OH; C, cholic acid, 3α-OH, 7α-OH, 12α-OH. The sodium salts of glycine or taurine conjugates of bile acids are referred to by, for example, LC-gly-Na or LC-tau-Na. HFB, heptafluorobutyrate; GLC, gas–liquid chromatography or gas–liquid chromatographic; HSDH, hydroxysteroid dehydrogenase.

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Gas–liquid chromatography

A Hewlett-Packard Model 5830A, with a 18850A integrator and a ^{63}Ni-electron capture detector, was used for all determinations. Glass columns, 1.8 m long and 2 mm i.d., were silanized with dimethyldichlorosilane and packed with factory-prepared, coated column supports. All packings were obtained from Chromatographic Specialties, Brockville, Ontario, Canada except for OV-225 which was from Supelco, Inc. Carrier gas was high purity argon–methane (95:5) from Union Carbide.

Quantitation of peaks on the chromatograms was carried out using external standards.

Derivatization procedure

For analytical determinations, microgram samples of bile acid or conjugate in chloroform–methanol 2:1 (v/v) were placed in glass vials with Teflon cap liners and the solvent was evaporated. To this benzene (250 μl), pyridine (10 μl), and heptafluorobutyric acid anhydride (20 μl) were added. The vial and contents were heated in a Temp-block heater at 105°C for 2 hr. After cooling, the contents were diluted to 1.0 ml with additional benzene. Then water (1.0 ml) and 5% aqueous ammonia (1.0 ml) were added successively to extract unused reagents. Microliter samples of the organic layer were withdrawn for GLC injection. Minor contaminants eluted close to the solvent peak without interference with the bile acid derivative peaks.

Bile studies

Twelve samples of human bile-rich duodenal aspirate, obtained by nasoduodenal intubation after intravenous cholecystokinin (26), were analyzed by GLC of the bile acid heptafluorobutyrate derivatives. The samples were also subjected to thin-layer chromatography to isolate the individual glycine and taurine bile acid conjugates (27, 28). These were then quantitated using a 3α- and 7α-hydroxysteroid dehydrogenase (HSDH) assay for comparison purposes (28, 29).

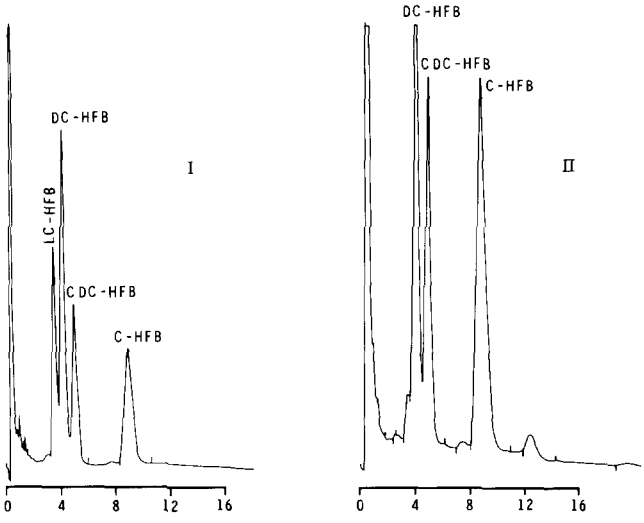

Fig. 1. GLC analyses of bile acid heptafluorobutyrates: I, artificial mixture of standards; II, sample of normal bile from a healthy female. 3% OV-225, 230°C.

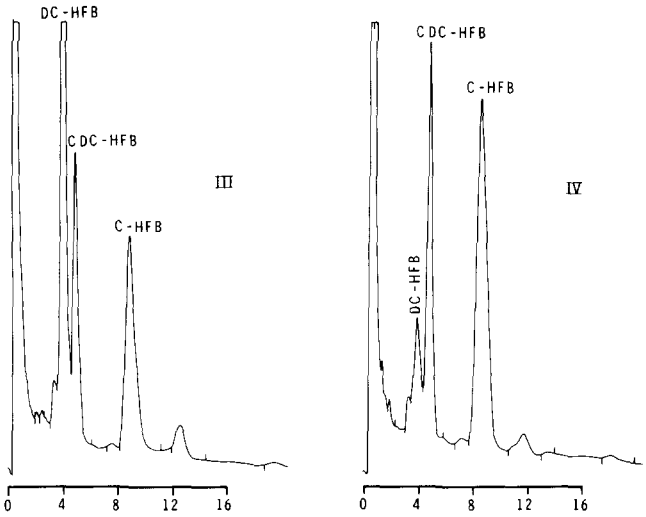

Fig. 2. GLC analyses of bile acid heptafluorobutyrates: III, sample of bile from a male patient with gallstones; IV, bile sample from a healthy female with a lower than average amount of deoxycholic acid. 3% OV-225, 230°C.

The correlation coefficients between the paired observations obtained by the two methods for C, CDC, and DC were determined.

Bile was extracted as previously described (28). One ml of crude bile was shaken with 20 ml of chloroform–methanol 2:1 and then extracted by further shaking with 4 ml of water. On standing 6–8 hr, the aqueous top phase was removed and the lower phase was re-extracted with 4 ml of additional water. The combined top phases were evaporated to dryness and redissolved in 1 ml of methanol–3% hydrogen peroxide 4:1 (v/v). Microliter samples of this crude bile extract were then derivatized in the same manner as the conjugated and unconjugated bile acid standards. After derivatization, the bile extract was present in 1.0 ml of benzene, of which 1.0 μl was injected for GLC analysis (equivalent to 1×10^{-9} liter of crude bile). Three examples of the bile analyses are shown in **Figs. 1** and **2**.

Products were prepared on a larger scale to carry out mass spectral studies and to make standard solutions. Ten-mg samples of bile acids were reacted with pyridine (0.5 ml) and heptafluorobutyric acid anhydride (1.0 ml) in 1.0 ml of benzene. Heating and extraction were similar to the previous procedure. However, it was necessary to separate the bile acid deriva-

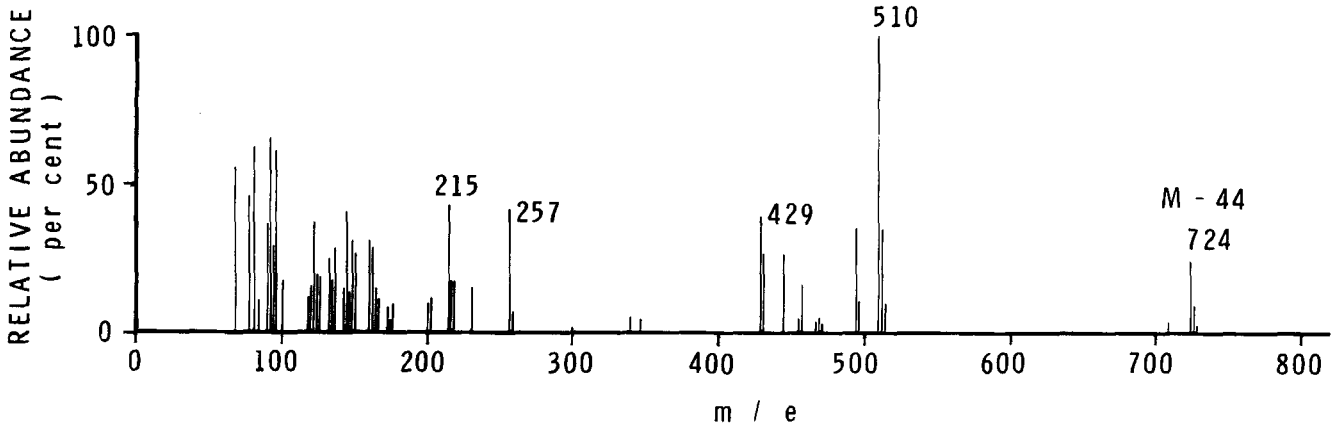

Fig. 3. Mass spectra of lithocholic acid-bis-heptafluorobutyrate. Inlet temp., 102°C and source block, 208°C.

tives from reaction by-products. Supelcosil-ATF 120, mesh 100/200, with hexane–benzene gradient elution was effective in isolating the products. No attempt was made to maximize yields for these larger scale reactions.

Bile acid heptafluorobutyrate derivatives, as standard solutions in benzene, were stored for one year at 4°C without any trace of decomposition as judged by GLC analysis.

The only examples of product decomposition occurred during purification on acidic absorbants of alumina, silica gel, or highly activated Supelcosil. Low activity Supelcosil and basic or neutral alumina allowed the products to elute with hexane or hexane–benzene 9:1 with little or no decomposition.

Mass spectrometry

High-resolution mass spectra were obtained on a CEC-21-110 instrument at 70 eV ionization voltage. Operating parameters were recorded with the appropriate graphs as shown in Figs. 3–6. Accurate mass measurements were determined with "peak-matching" using electrical detection. The standard was tris-(pentadecafluoroheptyl)-*S*-triazine (PCR-8) with a molecular weight of 1185 as standard reference for peak-matching.

RESULTS AND DISCUSSION

Figs. 3–6 show the high-resolution mass spectra of the bile acid heptafluorobutyrate derivatives. The molecular ion peaks for each of the derivatives were not seen; however, an M − 44 peak was the highest *m/e* value present in each spectrum, indicating a probable loss of CO_2 as the first step in fragmentation. The spectrum for LC-HFB had the highest *m/e* value at 724 (M − 44), corresponding to $C_{31}H_{38}O_3F_{14}$ by mass measurement. Accurate mass measurements on each bile acid derivative are listed in **Table 1** along with the calculated mass measurements for various fragments. The largest peak occurred at *m/e* 510 (M − 258), corresponding to a probable loss of CO_2, C_3F_7CO,HO. Other major fragments occurred at *m/e* 444, 257, and 215.

The spectrum for DC-HFB exhibited the highest *m/e* value at 936 (M − 44), indicating loss of CO_2. The most intense peak occurred at *m/e* 722 (M − 258), corresponding to $C_{31}H_{36}O_3F_{14}$ by accurate mass measurement; i.e., loss of CO_2,C_3F_7CO,HO. Other major peaks occurred at *m/e* 509, 469, 442, 253, and 213. The spectrum of CDC-HFB was somewhat similar to that of DC-HFB, as expected. The molecular ion peak occurred at *m/e* 936 (M − 44), and accurate

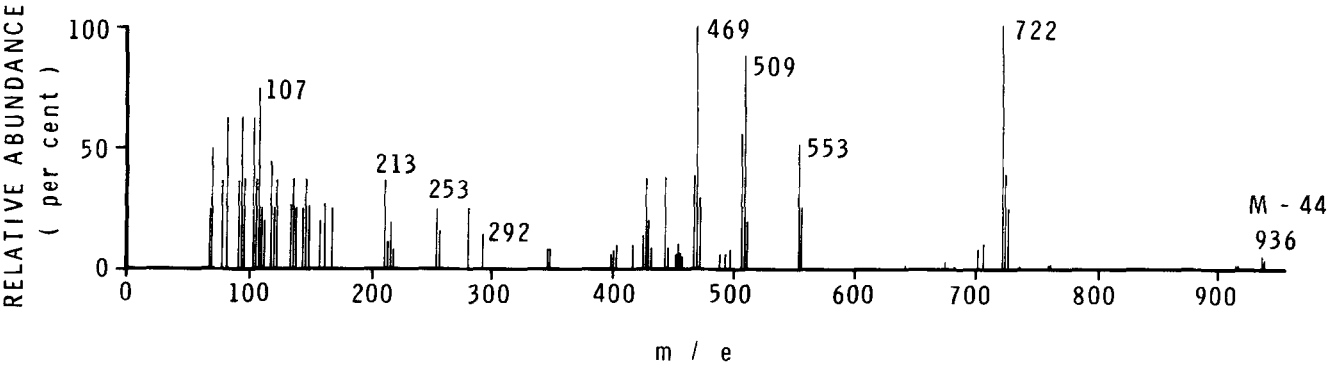

Fig. 4. Mass spectra of deoxycholic acid-tris-heptafluorobutyrate. Inlet temp., 116°C and source block, 282°C.

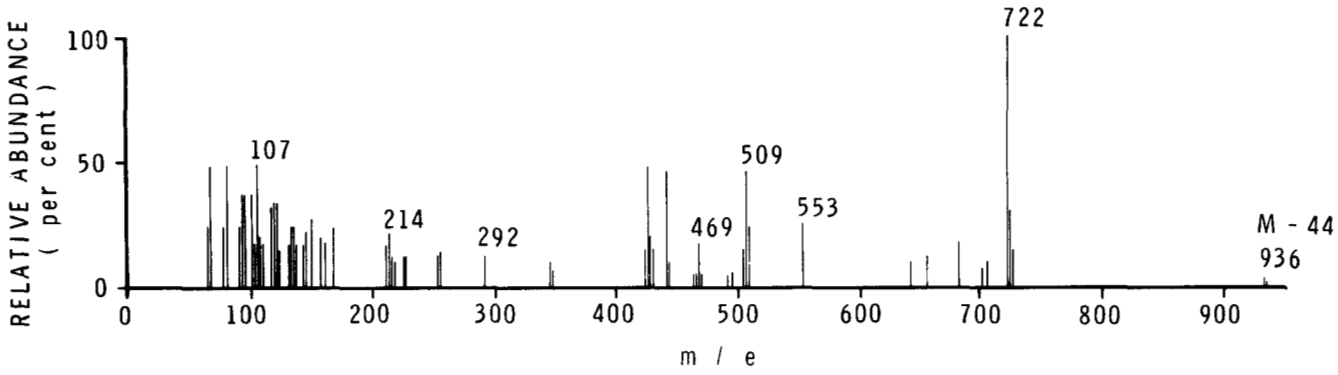

Fig. 5. Mass spectra of chenodeoxycholic acid-tris-heptafluorobutyrate. Inlet temp., 109°C and source block, 150°C.

mass measurement corresponded to $C_{35}H_{37}O_5F_{21}$; i.e., loss of CO_2. The largest peak occurred at *m/e* 722 (M − 258), indicating loss of CO_2, C_3F_7CO, and HO. Other major peaks were at *m/e* 509, 442, 255, and 214.

The spectrum for C-HFB had the highest *m/e* value at 1148 (M − 44), showing loss of CO_2. Accurate mass measurement was achieved at *m/e* 934, (M − 258), indicating loss of CO_2, C_3F_7CO, and HO, as occurred with the other bile derivatives. Other major peaks occurred at *m/e* 720, 508, 467, 292, and 253.

Projected structures of the products from the derivatization reactions are shown in **Fig. 7**, with the assumption that the stereochemistries of the starting material are maintained.

Two column packings were found suitable for analysis of the fluorinated derivatives, either 0.5% QF-1 on Gas Chrom Q, 100–200 mesh, AW-DMCS or 3% OV-225 on Gas Chrom Q, 100–120 mesh, AW-DMCS. Other packings were either not sufficiently specific to distinguish the individual bile acids or caused some decomposition of the derivatives as indicated in **Table 2**. **Table 3** shows a complete description of the operating conditions under which the relative retention times were measured, and gives the absolute retention times for LC-HFB on the various columns investigated.

It is likely that the multiple peaks obtained for a single derivatized bile acid on SE-30 and OV-17 are breakdown products on the column rather than multiple products from the derivatization that were not separated on QF-1 or OV-225. In support of this, no steroidal-HFB products could be isolated by thin-layer chromatography other than the major derivative when the reactions were performed on a larger scale.

The retention times and breakdown patterns of the product from either glycine or taurine conjugates (trihydroxy, dihydroxy, or monohydroxy) during GLC on five different column packings were identical to those of the respective free acids. This is strong evidence that deconjugation had occurred during the derivatization.

The percentage yields of bile acid or conjugate converted to HFB- derivative were investigated so that the heptafluorobutyryl derivatization would be useful for quantitative analysis of bile acids in biological samples. Optimum product yields were determined by GLC, using external standards for the quantitation. The results are shown in **Tables 4** and **5.** The percentage yields were determined by analyzing artificial mixtures of bile acids and bile conjugates, and the standard deviations from the means were calculated.

A concentration range, 0.1–10.0 μg, of bile acid or conjugate substrates was used, as well as different glycine–taurine ratios. The low standard deviations indicate that the linearity and reproducibility of response for the HFB-derivatizations are within the

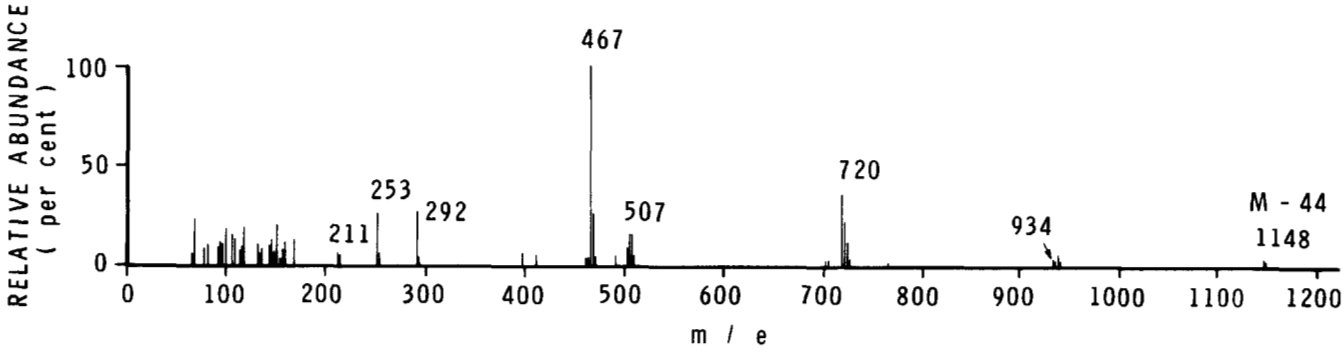

Fig. 6. Mass spectra of cholic acid-tetra-heptafluorobutyrate. Inlet temp., 128°C and source block, 218°C.

TABLE 1. Accurate mass measurements on each bile acid derivative

Sample	Observed Mass Measurement	Calculation for Fragment	Calculated Mass Measurement	Fragment(s) lost
LC-HFB	724.2607	$C_{31}H_{38}O_3F_{14}$	724.2596	CO_2
DC-HFB	722.2413	$C_{31}H_{36}O_3F_{14}$	722.2441	CO_2, C_3F_7CO, HO
CDC-HFB	936.2304	$C_{35}H_{37}O_5F_{21}$	936.2305	CO_2
C-HFB	934.2427	$C_{35}H_{35}O_5F_{21}$	934.2403	CO_2, C_3F_7CO, HO

expected precision limits for this concentration range. When the glycine and taurine conjugates of bile acids were derivatized singly, it appeared that the taurine amide linkage was hydrolyzed to a greater extent than the glycine linkage. However, as mixtures of glycine and taurine conjugates in different proportions (simulating bile G:T ratios of 0.5 to 5) the overall yield for a conjugated bile acid was consistently one value only. It may be that some kind of equilibration mechanism is operating when the glycine and taurine conjugates react together with the heptafluorobutyric acid anhydride. However when the G:T ratio was 10 or greater, yields fell to 15% (±5), which is the same as the yield for the glycine conjugate reacted singly.

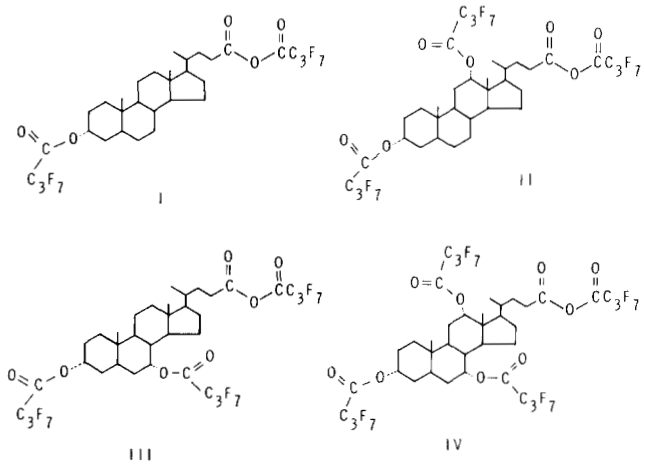

Fig. 7. Projected structures of the heptafluorobutyrate derivatives: I, LC-HFB; II, DC-HFB; III, CDC-HFB; IV, C-HFB.

The derivatization of bile acids in actual biological samples was investigated. A series of bile extracts was chosen that had been analyzed by the enzymatic–fluorimetric method used routinely in this laboratory (26, 28, 29). **Table 6** summarizes the analyses of bile extracts by GLC. Both the absolute amounts of HFB-products and the calibrated amounts, using the derivatization yields as correction factors, are shown. These results were then compared to the analyses of the same bile extracts by enzyme assay, as indicated in **Table 7**.

The correlation coefficients between the paired observations obtained by the two methods were 0.954, 0.960, and 0.988 for cholic, chenodeoxycholic, and

TABLE 2. Retention times of bile acid derivatives on various columns relative to lithocholic acid-HFB[a]

Derivative from	3% OV-225	0.5% QF-1	0.75% SE-30	4% SE-30/ 6% QF-1	1% OV-17
LC-acid	1.00	1.00	0.72, 1.00,[b] 1.48	1.00	1.00
LC-gly-Na	1.00	1.04	0.72, 1.00, 1.48	1.00	1.00
LC-tau-Na	1.01	1.00	0.71, 1.00, 1.48	0.99	1.00
DC-acid	1.18	1.57	0.79, 1.01, 1.25	1.31	0.94, 1.22, 1.44
DC-gly-Na	1.17	1.56	0.78, 1.00, 1.24	1.32	0.94, 1.25, 1.44
DC-tau-Na	1.18	1.59	0.79, 1.01, 1.24	1.31	0.94, 1.24, 1.44
CDC-acid	1.42	1.77	1.01, 1.46	1.00, 1.48	1.02, 1.25
CDC-gly-Na	1.41	1.78	1.01, 1.45	1.01, 1.50	1.02, 1.26
CDC-tau-Na	1.41	1.77	1.01, 1.46	1.01, 1.50	1.02, 1.26
C-acid	2.55	3.53	0.92[c]	2.38[c]	1.21, 2.85, 3.10
C-gly-Na	2.56	3.55	0.92	2.38	1.20, 2.84, 3.09
C-tau-Na	2.55	3.53	0.92	2.38	1.20, 2.85, 3.09

[a] Operating parameters for the GC data obtained are listed in Table 3, as well as absolute retention times of LC-HFB on the various columns.
[b] Underlined values constitute more than 80% of peak areas.
[c] Major peak of unresolved multiplet.

TABLE 3. Operating parameters for the relative retention times listed in Table 2[a]

Coating	Support	Column Temp. °C	Injection Temp. °C	Flow ml/min.	Absolute R.T. of LC-HFB min.
QF-1 0.5%	GC-Q,AW-DMCS 100–120 mesh	215	250	26	1.52
SE-30 0.75%	GC-Q,AW-DMCS 100–120 mesh	240	265	25	3.73
SE-30/QF-1 4%/6%	Chromosorb W-HP 80–100 mesh	260	275	24	6.46
OV-17 1%	Chromosorb W-HP 80–100 mesh	235	260	25	2.36
OV-225 3%	GC-Q,AW-DMCS 100–120 mesh	230	245	27	3.40

[a] The detector was maintained at 300°C. Column packings were conditioned for 2–3 days before use.

deoxycholic acid, respectively. This minimal difference in results obtained by two very diverse analytical methods supports our GLC procedure using heptafluorobutyric acid anhydride as a derivatizing and deconjugating agent.

Representative GLC traces of the bile analyses are shown in Fig. 1 and 2. Trace I is an artificial mixture of the four bile acid heptafluorobutyrate derivatives. Trace II is a sample of normal bile from a healthy female (sample 1 in Table 6). Trace III is bile from a male patient with gallstones (sample 2 in Table 6). Trace IV is a bile sample from a healthy female with a lower than average amount of deoxycholic acid (sample 9 in Table 6).

COMMENTS

The use of heptafluorobutyric acid anhydride appears to offer considerable advantages for GLC analysis of bile acids and conjugates. Ease of preparation and stability of the products as well as increased volatility are all desirable assets. On a qualitative or quantitative basis, the present derivatization–deconjugation method is simpler and faster as a final step before GLC or for other chromatographic analyses, such as thin-layer. The preparation of methyl esters of bile acids by diazomethane is no longer necessary.

The former deconjugation method by hydrolysis under pressure is limited by some destruction of bile acids, and it has been proposed that the enzymatic hydrolysis allows a more complete deconjugation under less drastic conditions (12, 15, 17). A modification of the alkaline hydrolysis procedure utilizing milder chemical conditions has been developed for sulfolithocholyltaurine and sulfolithocholylglycine, but not yet for other bile conjugates (30).

For quantitative analysis, however, deconjugation of bile conjugates by heptafluorobutyric acid anhydride has a relative disadvantage since glycine conjugates do not hydrolyze to the same extent as taurine conjugates. This limitation is compensated by the fact that, in mixtures of glycine and taurine conjugates, a particular bile conjugate is reproducibly derivatized to a constant degree. The actual amounts of bile acid derivatives detected by GLC can then be corrected to reflect the total amounts present. This procedure is

TABLE 4. Analysis of artificial mixtures of bile acids

Starting Material[a]	Product[b]	Yield[c]
LC-acid	LC-HFB	82 ± 5
DC-acid	DC-HFB	75 ± 4
CDC-acid	CDC-HFB	87 ± 4
C-acid	C-HFB	94 ± 7

[a] Reactions were carried out over a 0.1–10.0 μg range of substrates, and a mixture of the four bile acids was used in each determination.

[b] Analyses were performed on 3% OV-225/Gas Chrom Q at 220°C oven temperature; flow rate 26 ml/min; quantitation by external standards.

[c] Each yield (mean ± SD) is the mean of eight determinations.

TABLE 5. Analysis of artificial mixtures of bile conjugates

Starting Material[a]	Product[b]	% Yield[c]
DC-gly-Na DC-tau-Na	DC-HFB	34 ± 3
CDC-gly-Na CDC-tau-Na	CDC-HFB	36 ± 5
C-gly-Na C-tau-Na	C-HFB	45 ± 4

[a] A mixture of the six bile conjugates was used in each determination. Reactions were carried out over 0.1–10.0 μg range of substrates. The glycine–taurine ratio of each bile conjugate was varied from 0.5 to 5.0, without effect on the results.

[b] Analyses were carried out on 3% OV-225/Gas Chrom Q at 220°C oven temperature; flow rate, 27 ml/min; quantitation by external standards.

[c] Each yield (mean ± SD) is the mean of nine determinations.

TABLE 6. GLC analysis of bile extracts

Bile Sample[a]	HFB-products[b], ng/nl bile			HFB-products[c], ng/nl bile		
	C	CDC	DC	C	CDC	DC
1	14.8	9.16	7.16	32.9	25.4	21.0
2	4.32	3.49	5.66	9.60	9.69	16.6
3	14.8	8.72	2.63	32.9	24.2	7.74
4	26.3	17.3	3.50	58.5	47.9	10.3
5	2.25	1.52	1.25	5.01	4.22	3.68
6	3.24	1.45	0.424	7.20	4.03	1.25
7	7.94	2.06	1.01	17.6	5.71	2.97
8	1.84	1.23	0.197	4.08	3.41	0.579
9	11.4	6.26	1.44	25.3	17.4	4.22
10	9.99	3.26	1.52	22.2	9.06	4.47
11	4.46	1.61	2.41	9.90	4.48	7.08
12	7.05	3.16	1.23	15.7	8.78	3.62

[a] Bile samples were analyzed as crude extracts, without prior thin-layer chromatographic separation.

[b] Product values are absolute amounts obtained by quantitation with external standards and reflect the average of triplicate runs.

[c] Product values are corrected for derivatization yields listed in Table 5.

applicable to bile where all the bile acids are conjugated and where G:T values range from 0.5 to 5.0. However it is expected to be more useful for fecal bile acid analysis where conjugated bile acids are not present. In a sample where bile acids are present as free acids and conjugates, then absolute values of individual bile acids cannot be obtained using this single GLC method.

The formation of HFB-derivatives of bile acids is somewhat analogous to that described previously in our laboratory (31) using acetic anhydride, although deconjugation did not occur in the latter case. Acetic anhydride and Azure A ion-pair complexes were used to measure the sulphur component in taurine conjugated and sulphated bile acids. This has recently been applied (32) to differentiate bile of normal subjects and patients with chronic liver disease from that of patients with Crohn's disease and small bowel involvement.

We are aware that the structures presented in Fig. 7 may not be correct, in view of the unusual stability of these derivatives. However, a more complete chemical identification of the products is under study, as well as isotope studies to further characterize the derivatization reaction.

The use of high-resolution mass spectrometry for identification of the bile acid HFB-derivatives is necessary due to the high molecular weights of these compounds. This may be a disadvantage for those using GLC–MS facilities which usually consist of low-resolution chemical ionization mass spectrometers (33). The simplicity and convenience of derivatizing and deconjugating in one step before chromatographic analyses indicate that this procedure should be of most interest to those desiring rapid routine assays of bile acids.

TABLE 7. Comparison of bile acid analysis by GLC and by 3α- and 7α-HSDH assay

Bile Sample[a]	GLC analysis[b]			Enzyme analysis[c]		
	%C	%CDC	%DC	%C	%CDC	%DC
1	38	34	28	41	36	23
2	24	28	48	27	25	48
3	47	40	13	52	38	10
4	46	44	10	54	40	6
5	35	35	30	37	35	28
6	54	35	11	55	34	11
7	63	24	13	59	26	15
8	47	46	7	49	45	6
9	50	40	10	51	42	7
10	58	28	14	60	27	13
11	42	23	35	39	25	36
12	52	34	14	55	32	13

[a] Samples with widely differing bile acid ratios were chosen to compare the correlation of the two procedures.

[b] The quantities of HFB-products (corrected for reaction yield) from Table 6 were adjusted to correspond to free acid values, using molecular weight ratios between starting material and derivative.

[c] The percentages were calculated from duplicate assay of bile acid conjugates after thin-layer chromatographic separation. The correlation coefficients between the two methods for C, CDC, and DC, respectively, are 0.954, 0.960, and 0.988.

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