Identification of mono- and dihydroxy bile acids in human feces by gas–liquid chromatography and mass spectrometry

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ABSTRACT The mono- and disubstituted cholanoic acids present in human feces have been investigated. Extracts of feces were fractionated on silicic acid column and individual bile acids were isolated by preparative thin-layer chromatography. The isolated compounds were studied by gas–liquid chromatography of the methyl esters, partial trimethylsilyl ethers, oxidation products, and trifluoroacetates. The probable structures deduced were confirmed by gas chromatography–mass spectrometry and by comparisons with authentic compounds.

The following derivatives of 5β-cholanoic acid not previously isolated from human feces were identified: 3,12-diketo, 3-keto-12α-hydroxy, 3α,12β-dihydroxy, 3β,12β-dihydroxy, 3-keto-7α-hydroxy, 3α-hydroxy-7-keto, 3β,7α-dihydroxy, 3α,7α-dihydroxy, and 3α,7β-dihydroxy.

The presence of 3-keto-, 3β-hydroxy-, 3α-hydroxy-, 3β-hydroxy-12-keto-, 3α-hydroxy-12-keto-, 3β,12α-dihydroxy-, and 3α,12α-dihydroxy-5β-cholanoic acids was confirmed.

Evidence was obtained for the presence of two bile acids having at least one hydroxyl group at a carbon atom other than C_3, C_7, or C_{12}.

KEY WORDS bile acids · trifluoroacetates · partial trimethylsilyl ethers · dimethylhydrazones · thin-layer chromatography · gas–liquid chromatography · mass spectrometry · identification · feces · man

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Abbreviations: TLC, hin-layer chromatography; GLC, gas–liquid chromatography; TMSi, trimethylsilyl; TFA, trifluoroacetate(s); RRT, relative retention time(s) (referred to that of methyl deoxycholate); GC–MS, mass spectrometry of GLC effluents.

BILE ACIDS are excreted by the liver into the bile conjugated with either glycine or taurine. As a consequence of bacterial transformations in the intestine, the simple mixture of primary bile acids synthesized by the liver (cholic and chenodeoxycholic acids) is excreted as a complex mixture of primary and secondary bile acids in the feces. For a recent review of this subject see the paper by Danielsson (1).

Despite numerous attempts to quantify the bile acids excreted in human feces, in only a few studies have efforts been made to identify the components of this complex mixture. Carey and Watson described the isolation of deoxycholic acid from human feces (2). Heftmann, Weiss, Miller, and Mosettig (3) reported the occurrence of lithocholic, 3β-hydroxy-5β-cholanoic and 3α-hydroxy-12-keto-5β-cholanoic acids. Danielsson, Eneroth, Hellström, Lindstedt, and Sjövall confirmed the presence of the above acids and isolated 3β,12α-dihydroxy-5β-cholanoic and 3β-hydroxy-12-keto-5β-cholanoic acid. Evidence was obtained for the presence of chenodeoxycholic acid and 3-keto-5β-cholanoic acid (4). The latter acid was also identified by Norman and Palmer (5). Cholic acid has been identified by Jenke and Bandow (6) and evidence for the presence of 3α,12α-dihydroxy-7-keto-5β-cholanoic and 3α,7β,12α-trihydroxy-5β-cholanoic acids has been presented by Hamilton (7).

As a part of investigations concerning the influence of diet on bile acid excretion, a more complete characterization of the mixture of bile acids in feces was undertaken.

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The present paper describes the isolation and identification of a number of mono- and disubstituted cholanoic acids. The characterization of trisubstituted cholanoic acids in human feces is reported in the following paper (8).

MATERIALS AND METHODS

Preparation of Crude Fecal Extract

Feces collected in 2-day periods were homogenized in 1.5 liters of chloroform–methanol 1:1 with an Ultra-Turrax homogenizer (Janke and Kunkel, K. G. Staufen i. Br., West Germany). The homogenate was then transferred to a paper thimble in a jacketed Soxhlet extractor of the siphon type and continuously extracted with hot chloroform–methanol 1:1 for 48 hr.[1] An aliquot of the extract was withdrawn for determination of total solids. The extract was then concentrated in vacuo until foaming began. Redistilled, peroxide-free dioxane (10 ml/100 mg) and 4 N potassium hydroxide (5 ml/100 mg) were added and the mixture was refluxed for 3 hr. The hydrolyzate was neutralized and a volume of the solvent equal to that of the dioxane added was distilled off. The concentrated hydrolyzate was acidified to pH 3 and extracted continuously with peroxide-free diethyl ether for 16 hr. The ether extract was washed 3 times with 0.05 volume of a 0.08 M citrate–phosphate buffer solution, pH 5.8, and 3 times with 0.1 volume of water. Each of the washings was reextracted with 3–5 volumes of ether. The combined ether extracts were reduced to dryness in vacuo.[1]

Chromatography

Silicic acid chromatography was carried out with Mallinckrodt silicic acid (100 mesh) activated for 6 days at 120°C. All solvents used were distilled.

Thin-layer chromatography was carried out as described by Eneroth (9). Some modifications in this procedure were made for preparative work. To eliminate substances which gave rise to extraneous peaks on gas chromatography we refluxed the Silica Gel G (E. Merck AG, Darmstadt, Germany) with acetone for 30 min. The solvent was filtered off and the extraction was repeated once with acetone and twice with methanol. The silica gel was finally dried overnight at 80°C. The chromatoplates were spread with a layer of adsorbent 0.25–1.0 mm in thickness depending on the load to be applied. To obtain quantitative recoveries of the compounds from the plates, we used methyl esters of the bile acids. The separated zones were located with iodine vapor and extracted, after sublimation of the iodine, with 10 ml of methanol as described by Matthews, Pereda, and Aguilera (10). The solvent systems used are listed in Table 1.

TABLE 1 SOLVENT SYSTEMS FOR TLC OF BILE ACIDS

System	Components	Ratio
N 1	Diethyl oxalate–dioxane	40:10
S 1	Benzene–dioxane–acetic acid	75:20:2.0
S 9	Trimethylpentane–isopropanol–acetic acid	30:10:1.0
S 10	" " "	60:20:0.5
S 11	Trimethylpentane–ethyl acetate–acetic acid	10:10:2.0
S 12	" " "	5:25:0.2
S 13	" " "	50:50:0.7
S 14	" " "	10:10:0.25
S 15	" " "	10:10:0.1

Gas–Liquid Chromatography. Some modifications of the procedure previously described (11) were introduced. Thus, the acid-washed Gas-Chrom P (100–120 mesh, Applied Science Laboratories, Inc., State College, Pa.) was silanized by passing nitrogen containing dimethyldichlorosilane vapor through a gas washing bottle containing the support. Both the flask with dimethyldichlorosilane and the one containing the support were kept at −70°C. Nitrogen was blown through the system for 3 hr. The support was then washed with methanol and coated with 3% QF-1 (methyl fluoroalkyl silicone, Dow Corning Corp., Midland, Mich.). The packed columns were conditioned for 72 hr at 230°C without carrier gas and then for another day with a slow argon flow. With this technique for silanization and conditioning, columns with 2500–4000 theoretical plates for methyl deoxycholate (retention time about 20 min) could be obtained. Only minor tailing of polar methyl cholanoates was observed. The column was operated at a temperature of about 230°C. Argon inlet pressure was kept at 1.5–2.0 kg/cm², giving a flow rate of about 60 ml/min. Retention times were calculated relative to the retention time of methyl deoxycholate.

Preparation of Derivatives for GLC. The methyl esters of the bile acids were prepared by adding diazomethane in ether to the solution of free bile acids in ether–methanol 9:1.

Methyl esters of bile acids were oxidized as described by Djerassi, Engle, and Bowers (12). A solution of the bile acid ester, 10–100 μg in 1 ml of acetone, was cooled in an ice bath. Ten microliters of oxidizing reagent (26.72 g of CrO_3 + 23 ml of concd H_2SO_4 diluted to 100 ml with water) was added and the mixture was left for 10 min in an atmosphere of nitrogen. The reaction was terminated by the addition of 5 ml of water and the oxidation product was extracted with ethyl acetate.

Partial trimethylsilyl (TMSi) ether derivatives were prepared by dissolving 10–100 μg of the bile acid methyl ester in 0.06 ml of dimethylformamide (purified by refluxing with calcium carbide, distilled, and stored over

[1] Eneroth, P., K. Hellström, and J. Sjövall, to be published.

neutral aluminum oxide, Woelm grade I) and 0.03 ml of hexamethyldisilazane (Fluka AG, Buchs, Switzerland). After being heated at 50°C for 3 hr, the reaction mixture was directly analyzed by GLC. Under these conditions selective reactions with 3α-, 3β-, 6α-, 7β- and 12β-hydroxyl groups were obtained, whereas axially oriented 7α- and 12α-hydroxyl groups did not react.

Trifluoroacetates (TFA) were prepared as described previously (11). TMSi derivatives were dissolved in trifluoroacetic anhydride and heated for 15 min at 35°C (13).

1,1-Dimethylhydrazones (14) of 3-ketocholanoates were prepared in screw-capped vials under nitrogen by reacting 50–100 μg of the bile acid methyl esters with 0.1 ml of 1,1-dimethylhydrazine (Fluka AG, Buchs, Switzerland) at room temperature for 16 hr. After evaporation of the dimethylhydrazine, the residue was dissolved in 2 ml of dry acetone and left at room temperature for 1 hr prior to GLC.

Gas Chromatography–Mass Spectrometry

A description of the instrument employed has recently been presented (15). The outlet of the GLC column was connected directly to a two-stage molecule separator attached to a direct inlet system of an Atlas CH 4 mass spectrometer. A glass column (2 m × 4 mm) filled with 3% QF-1 on Gas-Chrom P was used. The column temperature was 238°C. The helium inlet pressure was 0.6 kg/cm^2, giving an outlet flow rate of 30 ml/min. The carrier gas was partially removed by the molecule separators, giving an approximate flow rate of 0.1–0.2 ml/min at the entrance to the ion source. The molecule separators, inlet connection, and ion source were kept at 250°C. The accelerating voltage was 3000 v. The energy of the bombarding electrons was 22.5 ev and the ionizing current was 40 or 60 μamp.

Most samples were analyzed as trifluoroacetates; in some cases TMSi ethers or methyl esters were used. Background mass spectra (derived mainly from the liquid phase) were always recorded and subtracted from the sample spectra. To reduce the contribution of background peaks to insignificant levels, we usually analyzed about 10 μg of each bile acid trifluoroacetate. Detailed mass spectra could, however, also be obtained from 1–2 μg amounts. Spectra were recorded from m/e 0–500 in 6 sec. A detailed description of the GC–MS analysis of bile acid derivatives will be published separately.[2]

Reference Compounds. The reference bile acids were those used in our previous studies of fecal bile acids (4, 16). 3β,12β-Dihydroxycholanoic acid was synthesized as described by Chang, Wood, and Holton (26).

[2] Eneroth, P., B. Gordon, R. Ryhage, and J. Sjövall, to be published. See also reference 17.

EXPERIMENTAL PROCEDURE AND RESULTS

The bile acids were isolated from feces collected from a single subject during two consecutive periods, each of 2 days' duration. Feces from this subject, one of a number participating in dietary experiments, were chosen for investigation because of the relatively high content of trisubstituted bile acids. For 14 days prior to the collection of feces the subject was maintained on a standardized solid diet providing 56% of calories from butter fat (17). She received 5 μc of cholic acid-24-^{14}C 6 days before the collection of feces was started.

Fractionation of Fecal Lipids

The crude fecal extract, 4.03 g, was dissolved in benzene and applied to a 100 g silicic acid column. The column was developed with benzene, 40 ml/g of silicic acid, which yielded a fraction containing primarily fatty acids and sterols. Acetone–benzene 1:3 (20 ml/g), eluted most of the mono- and disubstituted cholanoic acids. Acetone (10 ml/g) eluted most of the trisubstituted acids. Development was completed with methanol–chloroform 1:1 (10 ml/g). The four fractions contained 60, 25, 11, and 2% respectively of the material applied to the column. All the radioactivity was found in the second and third fractions (68 and 34%, respectively, of the activity applied).

Fig. 1 shows a gas–liquid chromatogram of the methylated material eluted with acetone–benzene 1:3. The relative retention times (RRT) of the more significant peaks are indicated in the figure.

The fraction eluted by acetone–benzene 1:3 was dissolved in 2% acetone in benzene and applied to a column providing a 50-fold excess of silicic acid (w/w). Development was commenced with 2% acetone in benzene, and continued with progressive increments in the acetone concentration. The composition of each fraction eluted was assessed by GLC and TLC. Table 2 shows the composition of the solvents, the weight and activity of material eluted with each solvent, and the RRT of the compounds eluted. A partial separation of the bile acids according to the number and nature of the functional groups was obtained. Most of the radioactivity was eluted with 24–50% acetone in benzene. Only traces of radioactivity appeared in the earlier fractions containing monosubstituted cholanoic acids.

After methylation the individual bile acids were purified by preparative TLC. The efficiency of this procedure was tested with labeled cholic and deoxycholic acid methyl esters. Recoveries ranged between 88 and 109% (mean 98%), whereas with the free bile acids somewhat lower yields were obtained.

When a band from the preparative thin-layer plate contained more than one component, as analyzed by

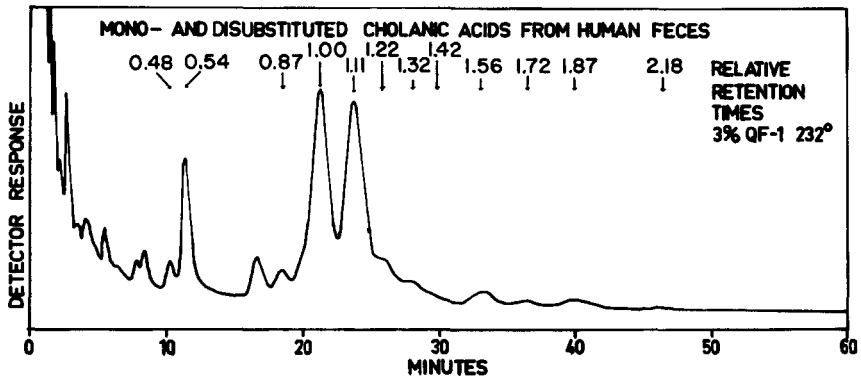

FIG. 1. Gas chromatographic analysis of methylated fecal bile acids obtained by eluting the initial silicic acid column with acetone–benzene 1:3. Retention timescal culated relative to that of methyl deoxycholate (largest peak shown). For subsequent identifications see Table 3.

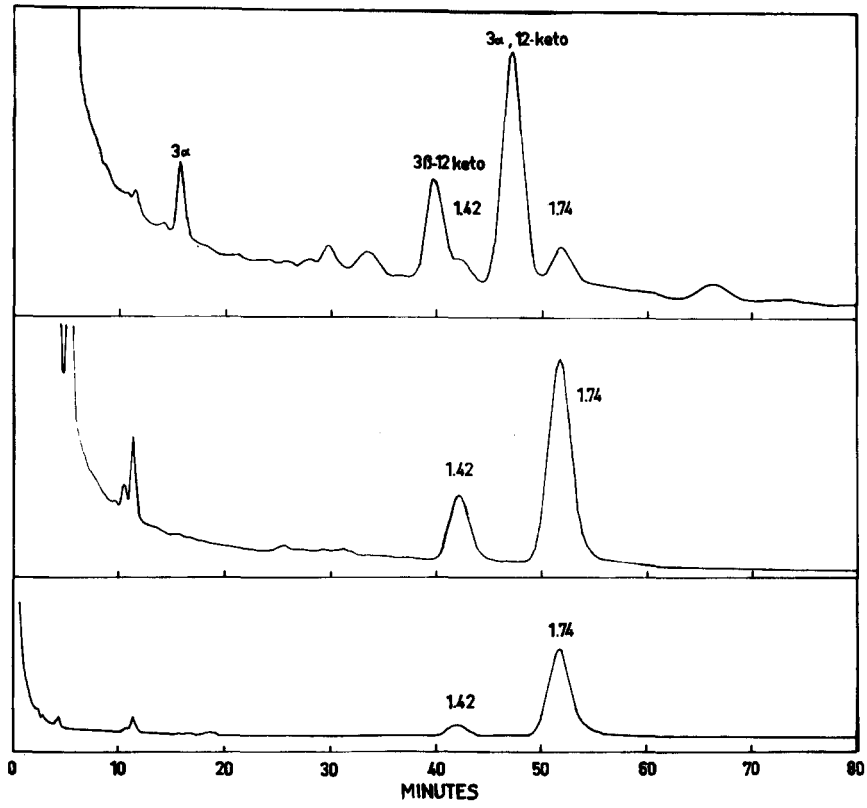

FIG. 2. Gas chromatographic analysis of methylated bile acids obtained by eluting the second silicic acid column with 24% acetone in benzene (upper curve, see also Table 1). The other two gas chromatograms show the enrichment of the compound with an RRT of 1.74 after one (middle curve) and three (lower curve) preparative thin-layer chromatographies (see Table 3).

GLC, the compounds could usually be separated by a second TLC in other solvent systems (see reference 9). Fig. 2 shows the use of this procedure in the isolation of the compound with an RRT of 1.74 (1.72 in Fig. 1) present in the 24% acetone fraction from the silicic acid column (Table 2).

Several substances had similar retention times on GLC (RRT of methyl esters: 0.96 A,B,C; 1.00 A,B; 1.13 A,B; 1.85 A,B; 2.22 A,B; Table 2). These substances were distinguished as different compounds by their behavior on the silicic acid column and (or) their mobility on TLC.

The solvent systems used for the isolation of the different compounds by TLC are listed in Table 3. The following pairs of compounds could not be separated (RRT of the methyl esters): 0.96 A and 1.13 A; 1.85 A and 2.22 A.

On the basis of the TLC behavior and the RRT of the compounds isolated from the thin-layer plates, appropriate reference compounds were chosen for further comparisons of the GLC behavior of different derivatives. As evident from the chromatogram shown in Fig. 1, a complex mixture of compounds was eluted from the silicic acid column. The detailed study was limited to compounds with an RRT greater than 0.45 (Fig. 1), but this included the bulk of the mono- and disubstituted cholanoic acids. The retention times of the trifluoroacetates, the partial trimethylsilyl ethers, and the oxidation products were determined. The results are summarized in Table 3. The compound with an RRT of 2.53 (Table 2) is not included since it was found to be a trisubstituted bile acid and is described in the accompanying paper (8).

Monosubstituted Cholanoic Acids

The identity of 3-keto-5β-cholanoic acid (1.00 A) was indicated by RRT values of the methyl ester and of the dimethylhydrazone (0.50) and by TLC mobility data. The mass spectrum showed the same fragmentation pattern as that of authentic methyl 3-keto-5β-cholanoate (Fig. 3). The differences observed in the relative intensities of the peaks are probably due to a difference in the concentration of the samples in the ion source.[2] The pronounced peak at m/e 318 (M − 70) indicates an A/B *cis* structure (18) which is also supported by the GLC data (methyl 3-keto-5α-cholanoates have longer retention times than the corresponding 5β-isomers).

The presence of lithocholic and 3β-hydroxy-5β-cholanoic acids has been shown previously (3, 4). Monosubstituted 5α-cholanoic acids could not be identified. No attempt was made to isolate cholanoic acids monosubstituted in rings B or C. However, there was no indication for the presence of significant amounts of such acids in the fractions containing the less polar bile acids.

Cholanoic Acids Substituted Both at C_3 and C_{12}

As previously shown (4), deoxycholic, 3β,12α-dihydroxy-, 3α-hydroxy-12-keto-, and 3β-hydroxy-12-keto-5β-cholanoic acids were found to be the predominant bile acids in this group (see Table 3). The dihydroxycholanoic acid fractions contained two other acids that could be identified as 3,12-dihydroxy-5β-cholanoic acids.

TABLE 2 SILICIC ACID CHROMATOGRAPHY OF THE PARTIALLY PURIFIED MONO- AND DISUBSTITUTED CHOLANOIC ACIDS FROM FECES
The notations + and tr. refer to a rough estimation of the relative proportions of the compounds in each fraction as judged from GLC.

Eluting Solvent (% Acetone in Benzene)	2	6	12	18	24	30	50	75	100
Fraction: Weight, *mg*	136	128	245	75	70	66	178	32	18
Radioactivity, *cpm*	0	1,000	1,200	5,600	29,400	21,600	110,000	12,000	100
GLC Retention Times Relative to Methyl Deoxycholate									
0.48	tr.	tr.	++	tr.					
0.53	tr.	tr.	+++	++	+	tr.			
0.68							tr.		
0.72	++			tr.					
0.87		tr.			tr.	++	+	tr.	tr.
0.96, A, B, C	tr.	++	tr.			+			
1.00, A, B		tr.	++		+	tr.	+++	+	tr.
1.06						+			
1.13, A, B	+	+++	+++	tr.	+	+	+	tr.	
1.23							+	tr.	
1.30	+								
1.34				tr.	++				
1.41					+				
1.57				tr.	+++	+			
1.74					+	+			
1.85, A, B				+	tr.	+			
1.93			++						
1.99					tr.				
2.22, A, B				+	+				
2.42				tr.	tr.				
2.53								tr.	
2.76				tr.	tr.				
4.21							tr.		
4.30				tr.					

TABLE 3 RESULTS OF GLC AND GC–MS ANALYSES OF MONO- AND DISUBSTITUTED CHOLANOIC ACIDS ISOLATED FROM HUMAN FECES
(Data in parentheses refer to known reference compounds)

Silicic Acid Fraction (% Acetone in Benzene)	Solvent Systems Used in TLC (Table 1)	Retention Times of Different Derivatives, All Relative to That of Methyl Deoxycholate*				GC–MS Results	Identification (Derivative of 5β-Cholanoic Acid)
		Methyl Ester	TFA	TMSi	Oxidation Product		
2	S 15	0.72†	—	—	—	—	See text
2	S 15	1.30	1.29	1.33	1.29	—	See text
6	S 15	0.96 A	0.85	0.65	1.32	See text	See text
6	S 15	1.13 A	1.01	0.76	1.57	See text	See text
12	S 15, S 15	0.48	0.46	0.29	1.00	—	3β
		(0.49)	(0.45)	(0.29)	(1.00)	—	(reference compound 3β)
12	S 15, S 15	0.53	0.47	0.30	1.00	—	3α
		(0.53)	(0.48)	(0.30)	(1.00)	—	(ref. 3α)
12	S 15	1.00 A	—	—	—	Identical	3-keto
		(1.00)	—	—	—		(ref. 3-keto)
12	S 15	1.93	—	—	—	—	See text
18	S 14, S 13, S 1	1.85 A	1.35	—	Degr.	See text	See text
18	S 14, S 13, S 1	2.22 A	1.65	—	Degr.	See text	See text
18	S 14, S 10, N 1	2.76	—	—	—	Identical	3,12-diketo
		(2.71)	—	—	—		(ref. 3,12-diketo)
18	S 14, S 10, N 1	4.30	—	—	—	See text	See text
24	S 12	1.34	1.23	0.86	2.74	—	3β,12-keto
		(1.34)	(1.23)	(0.85)	(2.71)	—	(ref. 3β,12-keto)
24	S 13	1.41	1.18	0.78	Degr.	See text	See text
24	S 12	1.57	1.42	0.98	2.70	—	3α,12-keto
		(1.57)	(1.38)	(0.98)	(2.71)	—	(ref. 3α,12-keto)
24	S 15, S 13, S 11	1.74	1.50	1.03	2.73	Identical	3α,7-keto
		(1.73)	(1.52)	(1.01)	(2.69)		(ref. 3α,7-keto)
24	S 15, S 12, S 9	1.85 B	1.35	1.82	2.68	Identical	3-keto,12α
		(1.84)	(1.36)	(1.84)	(2.71)		(ref. 3-keto,12α)
24	S 15, S 12, S 9	1.99	—	—	—	—	See text
24	S 15, S 10	2.22 B	1.62	2.22	2.68	Identical	3-keto,7α
		(2.22)	(1.61)	(2.16)	(2.69)		(ref. 3-keto,7α)
24	S 15, S 15	2.42	—	—	—	—	See text
30	S 12, S 1	0.87	0.60	0.53	2.70	Identical	3β,12α
		(0.88)	(0.59)	(0.53)	(2.71)		(ref. 3β,12α)
30	S 12, S 11	0.96 B	0.69	0.58	2.69	Identical	3β,7α
		(0.96)	(0.69)	(0.58)	(2.69)		(ref. 3β,7α)
30	S 12, S 15	0.96 C	0.72‡	0.31	2.72	See text	3β,12β
30	S 12, S 1	1.06	0.78	0.30	2.72	Identical	3α,12β
		(1.06)	(0.78)	(0.30)	(2.71)		(ref. 3α,12β)
50	S 12, S 15	0.68	0.44	0.70	Degr.	See text	See text
		(0.78)	(0.43)	(0.73)	(1.41)	—	(ref. 7α,12α)
50	S 12	1.00 B	0.68	0.56	2.78	—	3α,12α
		(1.00)	(0.68)	(0.56)	(2.71)	—	(ref. 3α,12α)
50	S 11, S 1	1.13 B	0.83	0.65	2.70	Identical	3α,7α
		(1.15)	(0.84)	(0.64)	(2.69)		(ref. 3α,7α)
50	S 11, S 1, S 1	1.23	0.95	0.37	2.69	Identical	3α,7β
		(1.22)	(0.95)	(0.37)	(2.69)		(ref. 3α,7β)
50	S 11, S 1	4.21	—	2.80	Degr.	—	See text

TFA, trifluoroacetate; TMSi, partial trimethylsilyl ether. GLC was on 3% QF-1 at 232°C, argon 60 ml/min.

* Methyl deoxycholate was, of course, a component of the fecal bile acids and is listed as part of silicic acid fraction 50 in the table, where the GLC characteristics of other derivatives of this bile acid are compared with those of the reference compound. The relative retention time is given as 1.00 B because another bile acid methyl ester has the same retention time, methyl 3-ketocholanoate, in silicic acid fraction 12. The latter compound was referred to, before identification, as the one with an RRT of 1.00 A; and a similar system is used throughout when two methyl esters have the same RRT.

† Present prior to methylation.

‡ This TFA was degraded extensively on the GLC column.

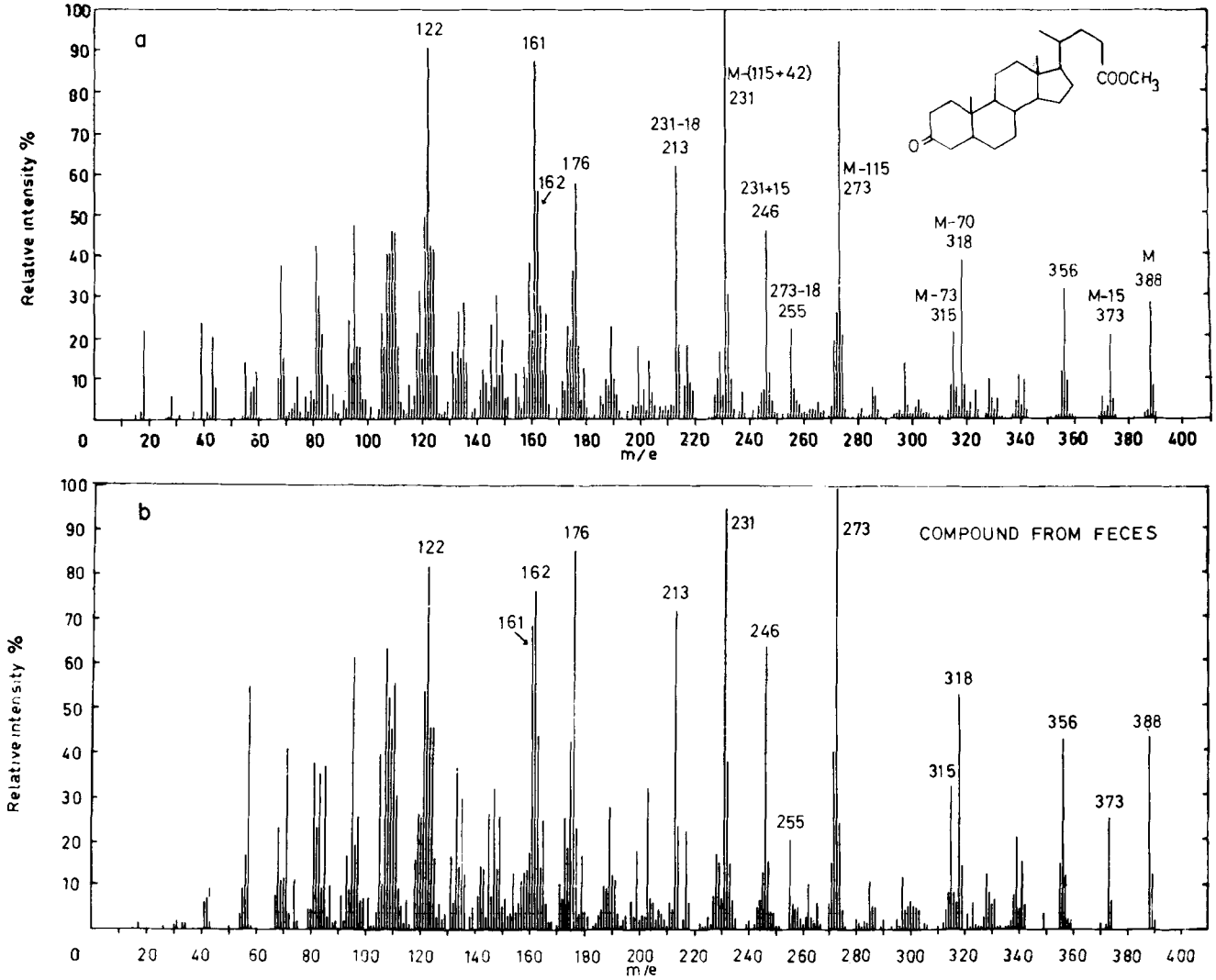

FIG. 3. Comparison between the mass spectra of authentic methyl 3-keto-5β-cholanoate (Fig. 3a) and of the same compound isolated from feces (Fig. 3b).

With solvent systems S 12 and S 1, a compound with the mobility of methyl 3α,12β-dihydroxy-5β-cholanoate was isolated from the thin-layer plates. The GLC behavior of this compound and its derivatives was identical with that of the corresponding derivatives of authentic 3α,12β-dihydroxy-5β-cholanoic acid. The mass spectra of the TFA derivatives were also identical (Fig. 4). The fragmentation pattern differed from those of the TFA of the other methyl dihydroxycholanoates by the lack of a peak at m/e 411 [M − (114 + 73)] and by the low intensity of the peak at m/e 154. The peak at m/e 411 present in the spectra of other dihydroxycholanoates is due to the loss of trifluoroacetic acid and carbons 23 and 24 with the ester group.[2] The ion m/e 154, which gave a pronounced peak in all other TFA of methyl dihydroxycholanoates, has been found to contain the side chain.[2]

The fourth methyl 3,12-dihydroxycholanoate isolated had an RRT of 0.96, i.e., the same as methyl 3β,7α-dihydroxycholanoate, from which it was separated, however, by TLC. In contrast to 3β,7α-dihydroxycholanoate it formed a di-TMSi derivative. In order that the position of the hydroxyl groups and the stereochemistry at C_5 might be studied, the compound was oxidized. The RRT of the oxidation product, 2.72, indicated that a 3,12- or 3,7-diketocholanoate of the 5β series had been formed. To differentiate between these two possibilities we allowed the compound(s) to react with dimethylhydrazine. The product had an RRT of 1.34, corresponding to the 3-dimethylhydrazone of methyl 3,12-diketo-5β-cholanoate. These data taken together could be explained only by a 3β,12β-dihydroxy-5β-cholanoic acid structure. The TFA of the methyl ester was extensively degraded on the GLC column, as was the corresponding synthetic compound. This prevented the analysis of the TFA in the GC–MS instrument. The mass spectrum of the di-TMSi derivative was typical for a ditrimethyl-

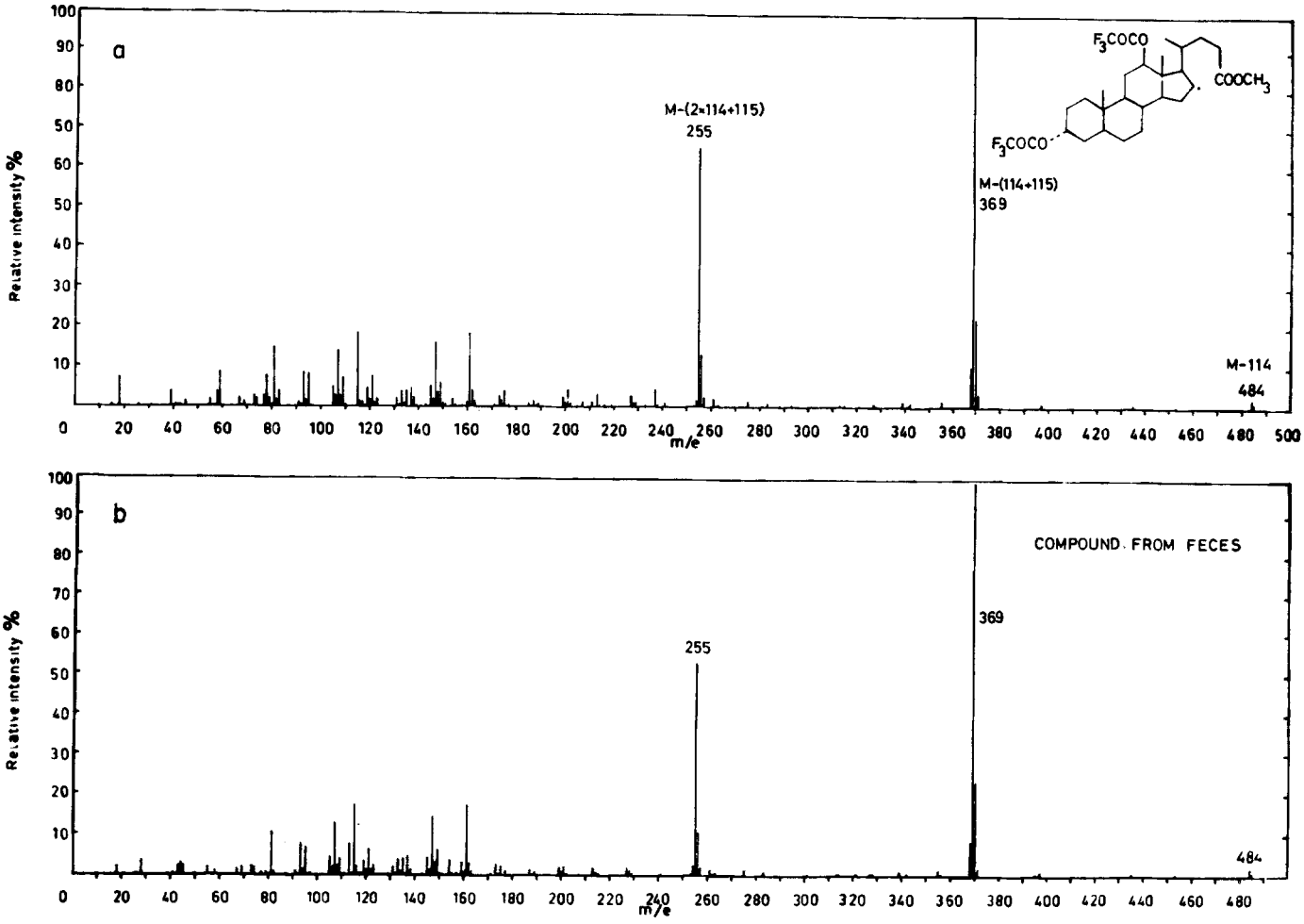

FIG. 4. Comparison between the mass spectra of the trifluoroacetates of authentic methyl 3α,12β-dihydroxy-5β-cholanoate (Fig. 4a) and of the compound isolated from feces (Fig. 4b).

silyl ether of a methyl 3,12-dihydroxycholanoate [main peak above m/e 75 at m/e 255, i.e., M − (2 × 90 + 115)] and different from the mass spectrum of the di-trimethylsilyl ether of a 3,7-dihydroxycholanoate (base peak at m/e 370, i.e., M − 2 × 90).[2]

In a separate experiment, an attempt was made to find allodeoxycholic acid in the material eluted with acetone–benzene 1:3 from the first silicic acid column. Oxidation of a fraction eluted from the zone of a preparative thin-layer plate where methyl allodeoxycholate would be found yielded no compound with the RRT of methyl 3,12-diketo-5α-cholanoate (RRT 3.18).

The silicic acid column fraction obtained with 24% acetone in benzene contained a compound with an RRT of 1.85. Its mobility on TLC was that of 3-keto-12α-hydroxy-5β-cholanoic acid. It did not give a TMSi derivative, which showed the absence of a hydroxyl group at C_3. The GLC data excluded a 3-keto-7-hydroxycholanoate, since methyl esters of such compounds have longer retention times than the compound from feces. A 12β-hydroxyl group could also be excluded since it would give a TMSi derivative. The mass spectra of the TFA derivatives of authentic methyl 3-keto-12α-hydroxy-5β-cholanoate and the compound from feces were identical. The base peak in the mass spectra was found at m/e 271 [M − (114 + 115)]. Loss of trifluoroacetic acid (114), side chain (115), and the A-ring with the keto group (70) resulted in a peak at m/e 201.

The identification of methyl 3,12-diketo-5β-cholanoate was based on the formation of a dimethylhydrazone with an RRT of 1.34 (see above) and on the results of the GC–MS analysis. Fragmentation through the D-ring, typical for 12-ketones (18), yielded a base peak at m/e 247 [M − 115(side chain) + 40 (D-ring)]. The molecular ion (m/e 402) had a relative intensity of 44% as compared with 43% for the reference compound. The 3,7-diketocholanoate by contrast had a base peak at m/e 287 (M − 115) and the relative intensity of the molecule ion was only 13.5%.

Cholanoic Acids Substituted Both at C_3 and C_7

Three 3,7-dihydroxycholanoic acids were isolated. The methyl ester of one of these had the RRT of methyl chenodeoxycholate (1.13). TLC, GLC, and GC–MS analyses all confirmed the identity of this compound with chenodeoxycholic acid. Another compound in this group had the same RRT as methyl 3β,12β-dihydroxy-5β-cholanoate (0.96). Upon oxidation, a compound with the RRT of a 3,12- or 3,7-diketo-5β-cholanoate was formed. The mass spectrum of the TFA was typical for that of a 3,7-dihydroxycholanoate and identical with the mass spectrum of methyl 3β,7α-di(trifluoroacetoxy)-5β-cholanoate (Fig. 5).

As is evident from Table 3, the GLC behavior of one of the compounds (RRT 1.23) emerging from the silicic acid column with 50% acetone in benzene was the same as that of methyl 3α,7β-dihydroxy-5β-cholanoate. The mass spectrum was identical with that of the reference compound and similar to the spectra shown in Fig. 5.

The presence of two 3,7-monohydroxy-monoketocholanoates was established on the basis of GLC and TLC data. The TFA of one of these compounds (having an RRT of 2.22 as the methyl ester; Table 3) showed signs of degradation on the GLC column. The degradation was more pronounced at the higher column temperature used in the GC–MS instrument. This precluded mass spectrometric analysis of this derivative. The mass spectrum of the unacylated methyl ester was therefore recorded and found to be identical with that of methyl 3-keto-7α-hydroxy-5β-cholanoate (Fig. 6). The base peak was found at m/e 271 [M − (115 + 18)], which is typical for 3,7- and 3,12-monohydroxy-monoketocholanoates except for those containing a 12-keto group.

The compound identified as 3α-hydroxy-7-keto-5β-cholanoic acid could be analyzed in the GC–MS instrument as the TFA. Its mass spectral fragmentation pattern was identical with that of the reference compound, but the relative intensities were different.

Unidentified Compounds

A compound with an RRT of 1.99 was found in trace amounts in the same silicic acid fraction as methyl

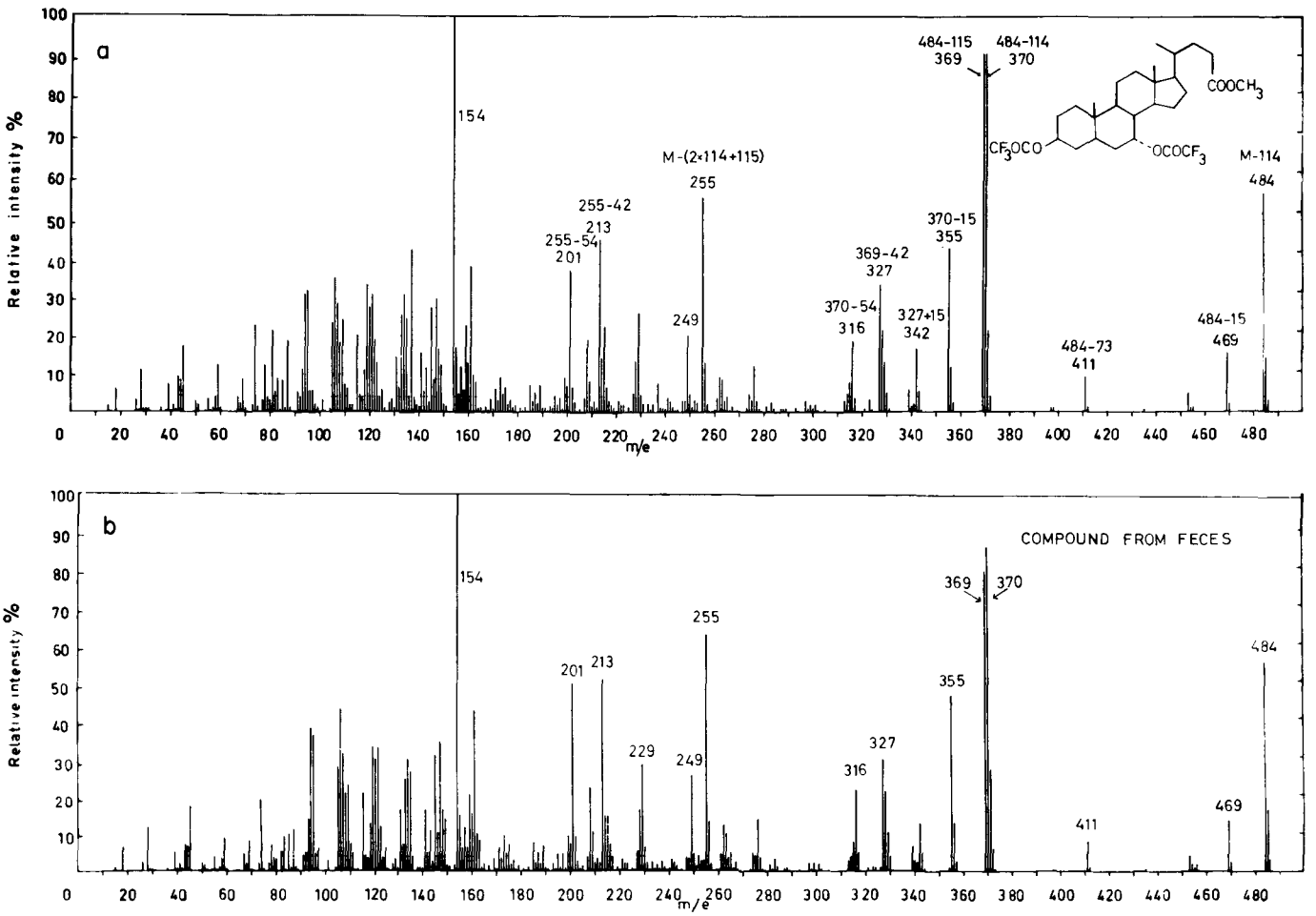

FIG. 5. Comparison between the mass spectra of the trifluoroacetates of authentic methyl 3β,7α-dihydroxy-5β-cholanoate (Fig. 5a) and of the compound from feces (Fig. 5b).

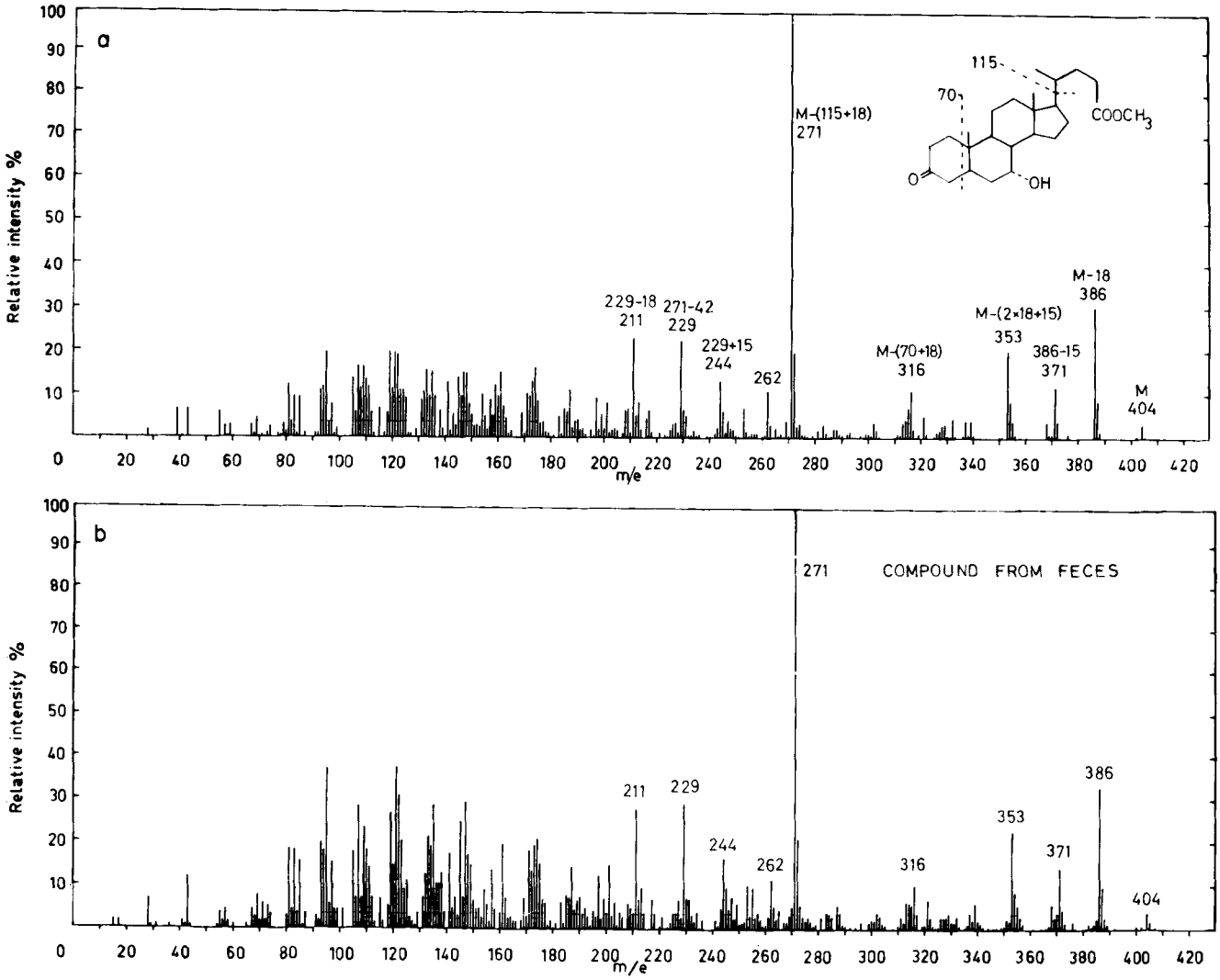

FIG. 6. Comparison between the mass spectra of authentic methyl 3-keto-7α-hydroxy-5β-cholanate (Fig. 6a) and of the compound isolated from feces (Fig. 6b).

3-keto-12α-hydroxycholanoate. It could not be obtained free from contaminating 3-keto-12α-hydroxycholanoate by TLC. If this compound was a methyl cholanoate, its TLC and GLC behavior suggested that it could have been 3-keto-12β-hydroxy-5β-cholanoate, the retention time of which could be calculated to be about 1.96 with the column used (19). Because of the limited amount of material available, this compound could not be further characterized.

Methyl 3-keto-7β-hydroxy-5β-cholanoate was calculated to have an RRT of 2.42 on the GLC column used. Trace amounts of a compound with this RRT, and the TLC behavior expected for this compound, were found in the monohydroxy-monoketo fraction from the silicic acid column.

The same fraction also contained a compound with an RRT of 1.41. From the GLC data it was concluded that the substance was probably disubstituted and that it contained only one hydroxyl group that could be converted to a trimethylsilyl ether under the selective conditions used. No uniform product could be obtained on oxidation. The mass spectrum of the TFA was similar to those of dihydroxycholanoates (see Figs. 4 and 5). The following important peaks were found (interpretation based on an assumed molecular weight of 598): m/e 484 (M − 114), m/e 370 (M − 2 × 114), m/e 369 [M − (114 + 115); 97.7% relative intensity], m/e 316 (M − 2 × 114 + 54), m/e 255 [M − (2 × 114 + 115); base peak], m/e 213 [M − (2 × 114 + 157)], m/e 201 [M − (2 × 114 + 115 + 54)], m/e 154 (containing the side chain). A spectrum of this type would also be obtained with a monounsaturated-monohydroxylated cholanoic acid, but the GLC data render this structure highly unlikely. The following conclusions may be drawn from the mass spec-

trum. The side chain is the same as in methyl cholanoate (peak at m/e 154 and loss of a fragment with mass 115), as is the D-ring [loss of a fragment with mass 157 (side chain + D-ring)]. The A-ring contains one hydroxyl group, as evidenced by the loss of a fragment of mass 54.

The compound with an RRT of 0.68 (as the methyl ester), although eluted from the silicic acid column with the dihydroxy acids, was similar in its mobility on TLC to the monohydroxy acids. No defined oxidation product was obtained. Since no TMSi derivative was formed, it may be concluded that there is no hydroxyl group at C_3. The mass spectrum of the TFA was indistinguishable from that of the corresponding derivative of 7α,12α-dihydroxy-5β-cholanoic acid. The most prominent peaks were found at m/e 370, m/e 255 (base peak), and m/e 213. This shows that the compound is a methyl cholanoate[2] either with two hydroxyl groups or with one hydroxyl group and one double bond. The possibility of a diene structure is excluded by the GLC data. The conclusion that there is no hydroxyl group at C_3 is supported by the absence of a peak at m/e 201 (255 − 54) in the mass spectrum. However, the possibility that this was 7α,12α-dihydroxy-5β-cholanoic acid could be ruled out by comparisons with the reference compound on TLC and GLC.

Several compounds were found that did not appear to be bile acids. The compound with an RRT of 0.72 not did not require treatment with CH_2N_2 to give a peak in the GLC. No peak shift was observed when the methylated compound with RRT 1.30 was treated with hexamethyldisilazane, trifluoroacetic anhydride, or chromic acid. The mass spectrum of the compound with an RRT of 4.30 (methyl ester) indicated a molecular weight of 484. Although the spectrum was not that of a methyl cholanoate, a steroid structure could not be ruled out with certainty. Four compounds (RRT 0.96 A, 1.13 A, 1.85 A, and 2.22 A, see Table 3) had very similar mass spectra. Mass spectra of the TMSi derivatives showed prominent peaks at m/e 262 (base peak for compounds with RRT 0.96 A and 1.13 A), m/e 203, m/e 189, and m/e 133 (base peak of compounds with RRT 1.85 A and 2.22 A). It was evident that these compounds belonged to the same class of substances. The mass spectra of the methylated product, the TMSi derivative, and the TFA of the compound with RRT 1.13 A all had peaks at m/e 452 and 437. In addition, the TMSi derivative yielded peaks at m/e 542 and 527. This indicates that the peak at m/e 452 is formed by the loss of trimethylsilanol (542 − 90), trifluoroacetic acid, and water from the respective derivatives. The mass spectra indicate that the compounds are not cholanoic acids. This conclusion is supported by the fact that although treatment with diazomethane was needed to give peaks on GLC, the compounds thus formed were unchanged after treatment with 2 N KOH in 50% water–dioxane at 60°C for 3 hr. The oxidation products formed from the compounds with RRT values of 1.13 A and 0.96 A did not correspond to any known ketocholanoates and did not form a dimethylhydrazone even when acetic acid was used as a catalyst.

The nature of the compounds with RRT 1.93 and 4.21 was not investigated.

Proportions between Compounds Isolated

A detailed study of the quantitative relationship between the different compounds was not made. Figure 1 and Table 2 provide some information on the relative quantity of each compound. It is seen that 3α,12α-dihydroxy- and 3α-hydroxy-5β-cholanoic acids are the main compounds.

The 3,7-disubstituted acids were present in smaller amounts than the 3,12-disubstituted ones. Chenodeoxycholic acid was a minor component of the peak with RRT 1.11 in Fig. 1. Most of this peak was due to a compound which was not a bile acid; it had an RRT of 1.13 (Table 3).

DISCUSSION

The very small amounts of many bile acids found in human feces make their identification by conventional methods difficult. In the present study the characterization of unknown compounds has been based on chromatographic methods and mass spectrometry.

A tentative identification was achieved by GLC analysis. Studies were made of the changes in RRT that occurred when the unknown substances were treated with various reagents. Such peak shift studies are preferably carried out with single compounds, since it is difficult to interpret the results if there are several peaks in the chromatogram. The compounds were therefore purified by TLC prior to derivative formation.

As has been shown for other groups of compounds, the RRT of methyl cholanoates give valuable information regarding the number, nature, and configuration of the substituents in the steroid nucleus. Each substituent adds a logarithmic factor to the retention time of the parent compound (methyl cholanoate) provided there is no interaction between different substituents (19).

Oxidation of a sample establishes whether a compound with the RRT of methyl 3-keto-, 3,6-diketo-, 3,7-diketo-, 3,12-diketo-, or 3,7,12-triketocholanoate of the 5α or 5β series is formed. The oxidation product should be analyzed on QF-1 columns since this phase differentially retains compounds with carbonyl groups. Because of the interaction between the 3-keto and 7-keto groups in methyl 3,7-diketocholanoate (see references 19 and 20) this compound is not separated from methyl 3,12-diketocholanoate. When 3-dimethylhydrazones are

prepared the interaction is reduced and the compound having a 12-keto group will have a shorter retention time than that having a 7-keto group (19, 20). Since mild conditions are used for the preparation of dimethylhydrazones only the 3-keto group reacts (14) and the formation of 3-dimethylhydrazones results in an approximately twofold reduction of the RRT.

Evidence regarding the orientation of hydroxyl groups is obtained by a reaction with hexamethyldisilazane in dimethylformamide, which selectively leads to the formation of trimethylsilyl ethers of equatorial hydroxyl groups at C_3, C_6, C_7, and C_{12}, and an axial hydroxyl group at C_3 (i.e., 3α-, 3β-, 6α-, 7β-, and 12β-hydroxyl groups). Only trace amounts of 7α-, 6β-, or 12α-trimethylsilyl ethers are formed under the conditions used. Each hydroxyl of the substituted methyl cholanoate converted to a TMSi ether will contribute to a reduction in the RRT of that compound when chromatographed on QF-1. On this basis it is possible to estimate the number of reacting groups. Recently Briggs and Lipsky (21) have studied the kinetics of TMSi ether formation using hexamethyldisilazane and trimethylchlorosilane in dioxane for the formation of partial TMSi derivatives of bile acids. Exposure of bile acid methyl esters to a silylating reagent containing pyridine, hexamethyldisilazane, and trimethylchlorosilane leads to complete conversion of all hydroxyl groups into trimethylsilyl ethers. This reaction has been used in the gas chromatographic analysis of fecal bile acids (22, 23).

Trifluoroacetylation of hydroxyl groups on QF-1 columns results in a decreased RRT of a hydroxylated compound. In compounds containing one hydroxyl group, the decrease is smaller when the hydroxyl group is at C_3 (4–17% decrease) than when it is at C_7 or C_{12} (22–41%) (Table 3 and reference 11). When two hydroxyl groups are present, the decrease in RRT is about the same as that of compounds having one hydroxyl group at C_7 or C_{12}. In the case of dihydroxycholanoates, the trifluoroacetates are useful mainly for comparisons with reference compounds. This derivative has recently been used by Kuksis in studies of human fecal bile acids (24).

By using the above-mentioned reactions it is possible to deduce a tentative structure for an unknown substituted cholanoic acid isolated from biological materials. For a confirmation of the proposed structure, the authentic compound should be synthesized so that the GLC and TLC behavior of the compounds can be compared. The identification of 3β,12β-dihydroxy-5β-cholanoic acid clearly shows the usefulness of the peak shift technique on QF-1 columns for the identification of bile acids. It should be stressed that the main structural information is obtained from the oxidation experiment (including dimethylhydrazone formation) and the selective reaction with hexamethyldisilazane.

Direct mass spectrometry of the compounds emerging from the gas chromatography column yields important information regarding the nature and position of functional groups. As shown by Budzikiewicz and Djerassi (18), mass spectrometry can be used to establish the position of keto groups. Spectra of trifluoroacetylated methyl 3,7- and 3,12-dihydroxycholanoates show significant differences in the fragmentation pattern (see Figs. 4 and 5). The configuration of hydroxyl groups usually cannot be ascertained by mass spectrometry although in the case of methyl 3α,12β-di-(trifluoroacetoxy)-5β-cholanoate distinct differences from the 12α-substituted dihydroxycholanoates were observed. It may therefore be possible to distinguish 12α- and 12β-hydroxyl groups by mass spectrometry, but reference compounds should be analyzed under identical conditions.

To achieve good resolution on the GLC column and to avoid memory effects of polar substances in the ion source, we usually analyzed trifluoroacetoxy derivatives in the GC–MS instrument. Molecular ion peaks were seen in the mass spectra of monotrifluoroacetoxy and monotrifluoroacetoxy-monoketocholanoates, whereas with ditrifluoroacetoxycholanoates no such peak could be recorded with the amounts analyzed. The peak with the highest mass number in the spectra of the latter compounds was usually found at m/e 484 (M − 114; loss of trifluoroacetic acid) and these spectra were similar to those obtained with monotrifluoroacetoxycholanoates having one double bond (tested for Δ^5 and Δ^{11}). However, monohydroxy-monoenoic cholanoates are separated from dihydroxycholanoates by GLC. Since the completion of the present work, we have found that trimethylsilyl ethers will give a molecular ion peak if a sufficiently large sample is analyzed (about 10 μg).

The combined data from GLC and MS analyses are usually sufficient for the identification of methyl cholanoates isolated from biological materials. In the present investigation comparisons with synthetic compounds were carried out to confirm the proposed structures. The following bile acids not previously isolated from human feces were identified: 3,12-diketo-, 3-keto-12α-hydroxy-, 3α,12β-dihydroxy-, 3β,12β-dihydroxy-, 3-keto-7α-hydroxy-, 3α-hydroxy-7-keto-, 3α,7α-dihydroxy-, 3β,7α-dihydroxy-, and 3α,7β-dihydroxy-5β-cholanoic acids. The presence in human feces of 3α-hydroxy-, 3β-hydroxy-, 3α,12α-dihydroxy-, 3β,12α-dihydroxy-, 3α-hydroxy-12-keto-, and 3β-hydroxy-12-keto- and 3-keto-5β-cholanoic acids was confirmed. In the subject studied 3α-hydroxy- and 3α,12α-dihydroxy-5β-cholanoic acids were quantitatively the most important compounds. The large number of minor components, although individually present in small amounts, contributed significantly to the total amount of fecal bile acids. Qualitatively, the fecal bile acid pattern found in this investigation was

similar to that found by GLC analysis of a large number of samples from other subjects. However, considerable individual differences in the proportions of the various bile acids have been noted.[1]

In addition to the cholanoic acids described above, at least two other compounds showing the GLC and GC–MS characteristics of dihydroxycholanoates were isolated. Since they gave no uniform oxidation product, at least one hydroxyl group must have been in a position other than C_3, C_7, or C_{12}. Indications were obtained for the absence of a hydroxyl group at C_3 in one of these compounds.

Several peaks in the gas chromatograms were not caused by bile acids. One of the major peaks in Fig. 1 (RRT 1.11) represents such a compound and substances with RRT 0.72, 0.96, 1.85, and 2.22 are other examples of such compounds. Since, on GLC, these compounds will contribute to bile acid peaks, it is necessary to identify all compounds contributing to the individual GLC peaks before quantitative studies are made.

The possibility that some bile acids, isolated in very small amounts, have been formed by isomerization of the major bile acids during the isolation procedure has to be considered. However, isomerization of synthetic bile acids carried through the saponification and the silicic acid chromatography has not been observed. Detection of compounds on TLC plates by means of iodine vapor has not been found to change the TLC or GLC behavior of synthetic bile acids and this technique has also been applied to cholesterol and stigmastérol (unpublished data) without production of artifacts. Gas chromatograms of fecal bile acids from samples not carried through the hydrolysis have shown peaks with the retention times of the bile acids now isolated.

The present results emphasize that the bile acids are metabolized extensively in the intestinal tract (see reference 1). It is apparent that factors which influence the composition of the intestinal flora and the conditions for the reactions catalyzed by the microbial enzymes will determine the relative proportions between oxidized and reduced forms of the bile acids (25) and probably also the ratios between epimeric hydroxycholanoic acids.

In studies of fecal bile acid excretion it is important, therefore, to have detailed knowledge of the composition of the bile acid mixture, since under certain conditions the minor components now identified may be of considerable quantitative importance.

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