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A Method for Quantitative Determination of Bile Acids in Human Feces

Bile Acids and Steroids 195

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Procedures for the extraction, saponification, and purification of human fecal bile acids have been studied and a method has been developed which permits the subsequent quantitative determination of different bile acids by gas-liquid chromatography. A fecal extract obtained by continuous extraction with hot chloroform-methanol is saponified with potassium hydroxide in aqueous dioxane. The acids are recovered by continuous ether extraction of the concentrated and acidified hydrolyzate. After group separation on silicic acid columns the bile acids are analyzed as methyl esters and methyl ester trifluoroacetates on QF-1 columns. The different steps have been quantitatively evaluated by analysis of feces from subjects on six different diets given either labeled cholic or chenodeoxycholic acid and by conventional recovery experiments with labeled and unlabeled compounds. The presence of non-bile acid contaminants has been studied and the possible interference of such compounds in dietary experiments is discussed.

In studies on the influence of diets and drugs on the metabolism and turnover of cholesterol, a method for the determination of fecal bile acids is of considerable value. Earlier studies involving determination of fecal bile acids were often based on the use of nonspecific methods (for a discussion of this subject see Refs. 1—3). The development of chromatographic techniques such as gas-liquid chromatography (GLC) and thin layer chromatography (TLC) has now made a more accurate analysis of fecal bile acids possible.

In a series of studies from this laboratory the detailed composition of human fecal bile acids has been elucidated.[4–7] In the course of this work various methods for quantitative extraction, saponification, purification, and GLC determinations have been studied.[4–9] The present paper describes a method for determination of fecal bile acids based on these qualitative and quantitative studies. While these investigations were in progress, Grundy, Ahrens and Miettinen [2] and Ali, Kuksis, Gordon and Beveridge [10,11] reported studies on the determination of human fecal bile acids by GLC.

MATERIALS AND METHODS

Solvents. Chloroform (stabilized with 1 % ethanol, ADA, Stockholm, Sweden), methanol and acetic acid (*p.a.* Merck AG, Darmstadt, Germany) were used as supplied. Diethyl ether, benzene, acetone and dioxane (purum grade, AB Kebo, Stockholm, Sweden) were purified according to standard procedures.[12] Dioxane was always tested for peroxides before use.

Reagents. Diazomethane, freshly prepared from a reaction mixture of nitrosomethylurea and 50 % KOH (w/v) was used. Trifluoroacetic acid anhydride was obtained from Eastman Kodak, Rochester, New York, USA.

*Reference substances.** Labeled bile acids (24-^{14}C-labeled cholic, deoxycholic, chenodeoxycholic, and lithocholic acids) were synthesized according to Bergström *et al.*[13] 24-^{14}C-3-Ketocholanoic acid was prepared by chromic acid oxidation of 24-^{14}C-3α-hydroxycholanoic acid in 95 % aqueous acetic acid. The labeled acids were analyzed for radiochemical homogeneity by autoradiography after thin layer chromatography in the systems previously described.[9] Unlabeled bile acids were those used in previous studies.[5,8,9]

Radioactivity determination. The method for wet combustion and subsequent radioactivity measurements described by Jeffay and Alvarez [14] as adapted for bile acids [15] was used. The trapped carbon dioxide was measured in a Packard Tri-Carb liquid scintillation spectrometer.

Homogenization of feces was done with an Ultra-Turrax homogenizer (Janke Kunkel AG, Staufen i. Bra., West Germany). Extraction of homogenates was done in paper thimbles in a Soxhlet extractor of the siphon type.

Continuous extraction of saponified lipids was made in a liquid extractor of the upward displacement type. The aqueous phase was stirred continuously with a magnetic stirrer.

Gas-liquid chromatography (GLC). Glass columns, 6 ft × 4 − 5 mm were packed with 3 % QF-1 coated on acid-washed, silanized Gas-Chrom P as described previously.[6] Approximate column conditions were (for bile acid methyl esters): flash heater temperature, 285°; column temperature, 235 − 245°. Carrier gas: argon; column inlet pressure: 1.6 − 2.0 kp/cm^2. For the analysis of trifluoroacetates [8] the flash heater was kept at 240 − 250° and the column at 225 − 235°. The argon ionization detector (Pye Ltd., Cambridge, England) was operated at a setting of 1500 V. Detector temperature was 240°.

Retention times were calculated relative to that of methyl deoxycholate (RRT values). Peak areas were measured with a planimeter.

Derivatives. Bile acids dissolved in diethyl ether-methanol, 9:1, were converted into methyl esters by the addition of freshly distilled diazomethane. The samples were left at 4° for 30 min, then taken to dryness under nitrogen. This procedure was repeated once. Trifluoroacetates (TFA) were prepared with trifluoroacetic anhydride as described previously.[8]

Silicic acid chromatography was carried out with silicic acid (Mallinckrodt, St. Louis, Mo.) which had been activated for 10 days at 120°. The adsorbent was rapidly weighed and allowed to settle in benzene in a chromatography tube. The column height was 4 − 10 times the diameter of the column.

EXPERIMENTAL PROCEDURE

Subjects maintained on standardized diets for two weeks were given about 5 μC of ^{14}C-labeled bile acid (sodium salts of cholic or chenodeoxycholic acid) orally. The diets were: regular solid diets cooked with corn oil (SC) or butter fat (SB);[16] formula type diets containing butter fat (FB) or corn oil (FC); mixed formula-solid diets without (FS) and with a supplement of egg yolk extract (FS + egg).[17]

* The following systematic names are given bile acids referred to by trivial names: cholanoic acid, 5β-cholan-24-oic acid; cholic acid, 3α,7α,12α-trihydroxy-5β-cholanoic acid; deoxycholic acid, 3α,12α-dihydroxy-5β-cholanoic acid; chenodeoxycholic acid, 3α,7α,-dihydroxy-5β-cholanoic acid; thocholic acid, 3α-hydroxy-5β-cholanoic acid.

Feces were collected in plastic bags and immediately stored at −20°. The contents of the bags were then transferred into 1000 ml plastic bottles, thawed in about 500 ml chloroform-methanol, 1:1 (v/v), at room temperature, and homogenized for 5 min. The bottles were sealed with a screw-cap through which the shaft of the homogenizer was introduced. When the amount of radioactivity in a fecal portion was expected to be low, a labeled bile acid (usually 3-keto- or 3β-hydroxycholanoic acid) was added prior to the homogenization. The plastic bags were cut into pieces and washed with chloroform-methanol. This solution and the fecal homogenate were transferred to the Soxhlet extractor and continuously extracted for 48 h at reflux temperature in a final volume of 1000—1500 ml of chloroform-methanol. The extract was stored at room temperature in dark, tightly stoppered bottles until analyzed (within 1—14 days). Duplicate aliquots were withdrawn for determination of total solids and activity of the extract.

An amount corresponding to 1/5—1/10 of a one day portion of feces was concentrated under reduced pressure until foaming began (usually when 20—50 ml were left). To this residue, peroxide-free dioxane (4 ml/100 mg solids) and 4 M potassium hydroxide (2 ml/100 mg) were added and the mixture was refluxed for 3 h. 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 h. The ether extract was washed 3 times with 0.05 volumes of a 0.08 M citrate-phosphate buffer solution, pH 5.8, and 3 times with 0.1 volumes of water. Each of the washings was re-extracted with ether. The combined ether extracts were reduced to dryness *in vacuo* and weighed. The residue was dissolved in benzene and transferred to a volumetric flask. Duplicate aliquots were withdrawn for radioactivity determination.

A known amount of the extract (usually 50 %) was applied to a silicic acid column in benzene, providing a 25-fold excess of silicic acid (w/w). Five fractions were collected: I. 10 ml benzene/g of silicic acid; II. 30 ml of 1 % acetic acid in benzene/g silicic acid; III. 40 ml of 25 % acetic acid in benzene/g silicic acid; IV. 10 ml of chloroform-methanol (1:1)/g silicic acid. A free solvent flow was used. From each fraction aliquots were withdrawn in duplicate for radioactivity determination and the remainder was taken to dryness and weighed.

Two samples from each of the radioactive fractions (III and IV) were methylated. The samples were then dissolved in a suitable volume of an acetone solution of an internal standard usually methyl 3-keto-12α-hydroxycholanoate. One of these samples was taken to dryness, trifluoroacetylated and redissolved in acetone to the previous volume.

GLC quantitations were made in the following way. A series of 6 samples containing increasing amounts (usually 0.5—4 mg) of methyl deoxycholate were dissolved in 4 ml of the internal standard solution (containing 1 mg/ml of internal standard). The linearity range of the GLC-system was tested by injections of 4 μl of each of these solutions. In this way a response factor between the internal standard and methyl deoxycholate was also obtained. Response factors and linearity range for the other bile acids to be determined were established with mixtures containing known proportions of bile acid methyl esters including methyl deoxycholate. Amounts giving peak heights between 5 and 80 % of full scale recorder deflection were injected.

The same procedure was used for trifluoroacetates. Since they do not separate on QF-1 columns, the TFA's of methyl 3α- and 3β-hydroxycholanoates were kept in different standard mixtures.

Different volumes of the fecal bile acid derivatives dissolved in the internal standard solution were injected so that each bile acid could be quantitated within its linear response range. The amounts of bile acid derivatives were calculated from the peak areas, the amount of internal standard added and the respective response factors.

RESULTS

Extraction of feces. To test the efficiency of the procedure for extraction of feces, the solid residue remaining after the initial chloroform/methanol extraction was combusted according to the method of Kelly *et al.*[18] or refluxed in 0.1 M hydrochloric acid in ethanol. These experiments showed that only

Table 1. Efficiency of the procedure for extraction of endogenously labeled fecal bile acids.

Sample	24-^{14}C-Bile acid administered to subject	Activity in $CHCl_3$ CH_3OH extract	Activity in fecal residue [a]	Activity in ethanolic HCl extract	Recovery % in $CHCl_3$/ CH_3OH extract
		cpm	cpm	cpm	
Sch 264	cholic acid	70 700	—	1078	98.5
Sch 294	»	153 000	—	2325	98.5
Sh 304	»	42 800	—	1660	96.1
Sel 35	»	376 000	—	3050	99.2
Sel 45	»	142 800	—	2600	98.2
Sel 75	»	50 600	—	3300	93.9
An 175	»	172 000	—	7190	96.0
Fr 246	»	278 000	11 400	—	96.2
We 16176	chenodeoxycholic acid	890 000	16 300	—	98.2
We 25266	»	362 000	1 700	—	99.5
We 22236	»	394 000	2 200	—	98.6
We 28296	»	157 000	5 000	—	96.8

[a] Determined by combustion to $^{14}CO_2$.

trace amounts of "endogenous" radioactive material was not extracted by chloroform-methanol (Table 1). The efficiency of the procedure has also been confirmed by wet combustion of aliquots of emulsified aqueous fecal homogenates. The same amount of ^{14}C was found in the homogenate as in the chloroform-methanol extract of the lyophilized homogenate (P. Eneroth, K. Hellström and S. Lindstedt, unpublished results).

The recovery of "non-endogenous" ^{14}C-labeled bile acids (*i.e.* bile acids added during the homogenization) is shown in Table 2. As might be expected the recovery was quantitative.

Table 2. Efficiency of the procedure for extraction of added labeled bile acids.

Sample	24-^{14}C-Bile acid added	Amount added	Amount recovered in $CHCl_3/CH_3OH$ extract	Recovery % of amount added
		cpm	cpm	
To 36	cholic acid	1.23×10^6	1.36×10^6	111
Tl 66	»	1.23×10^6	1.33×10^6	108
To 16	chenodeoxycholic acid	1.70×10^6	1.76×10^6	104
We 36	»	1.70×10^6	1.63×10^6	96
Li 265	lithocholic acid	1.40×10^6	1.39×10^6	99
Li 305	»	1.40×10^6	1.36×10^6	97
To 285	deoxycholic acid	1.76×10^6	1.70×10^6	97
To 16	»	3.40×10^6	3.33×10^6	98
He 225	3-ketocholanoic acid	6.46×10^5	6.41×10^5	99
He 235	»	6.46×10^5	6.75×10^5	105

Saponification of fecal lipids. The type of solvent used in the saponification of fecal lipids appeared to determine the yield of labeled material obtained in the subsequent diethyl ether extraction (Table 3). When aqueous methanol was used, the recovery of radioactivity in the ether extraction was often poor. When aqueous dioxane was used, the recovery averaged 100 %. Some purification was obtained in the hydrolysis step; an average of 87 % of the total solids were recovered (see Table 3).

Table 3. Influence of saponification conditions on recovery of endogenously labeled fecal bile acids.

Saponification conditions	24-^{14}C-labeled bile acid administered to subject	No. of samples	Recovery in ether extract	
			% of weight in original sample (range)	% of radioactivity in original sample (range)
1 M KOH/CH_3OH 1:1 (v/v)	chenodeoxycholic + cholic acids	7	81 (66—100)	86 (73—100)
2 M KOH/CH_3OH 1:1 (v/v)	»	2	86 (82—90)	69 (43—93)
4 M KOH/CH_3OH 1:1 (v/v)	»	8	80 (64—90)	73 (47—97)
4 M KOH/dioxane 1:2 (v/v)	chenodeoxycholic acid	4	83 (65—97)	99 (91—105)
4 M KOH/dioxane 1:2 (v/v)	cholic acid	5	87 (70—98)	100 (94—111)

The nature of the labeled material that was not extracted by ether after saponification in aqueous methanol was not studied in detail. However, a large part of this radioactivity could be extracted with chloroform. GLC of this extract gave essentially the same pattern of fecal bile acids as found in the diethyl ether extract.

Purification of the saponified extract by silicic acid chromatography. Chromatography of saponified extracts to which ^{14}C-labeled bile acids had been added showed that bile acids with a polarity ranging from that of 3-ketocholanoic acid to that of dihydroxycholanoic acids were eluted in fraction III. More polar bile acids, *e.g.* trihydroxycholanoic and dihydroxy-monoketocholanoic acids, appeared in fraction IV (see Fig. 1 and Ref. 7).

The purification obtained with the silicic acid chromatography was tested with saponified extracts of feces from subjects on different diets who had been given labeled cholic or chenodeoxycholic acid. The results are summarized in Fig. 2. An average of 14.8 % (range 3.5—33.9) and 3.4 % (range 1.3—6.5) of the weight of the material applied to the column were recovered in fractions III and IV, respectively. The recovery of radioactivity in these fractions was

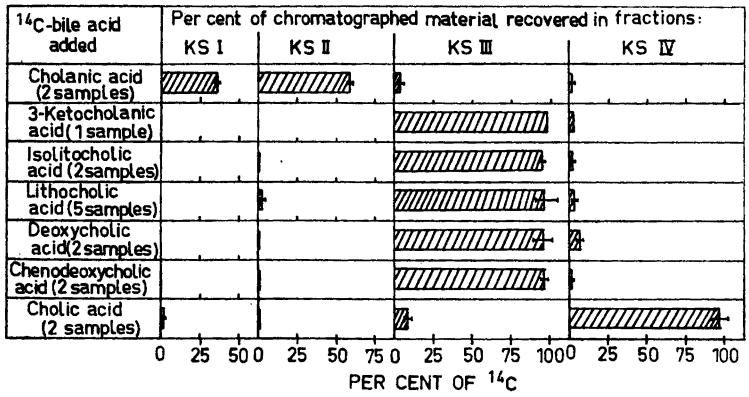

Fig. 1. Recovery of added labeled bile acids in the silicic acid fractionation procedure.

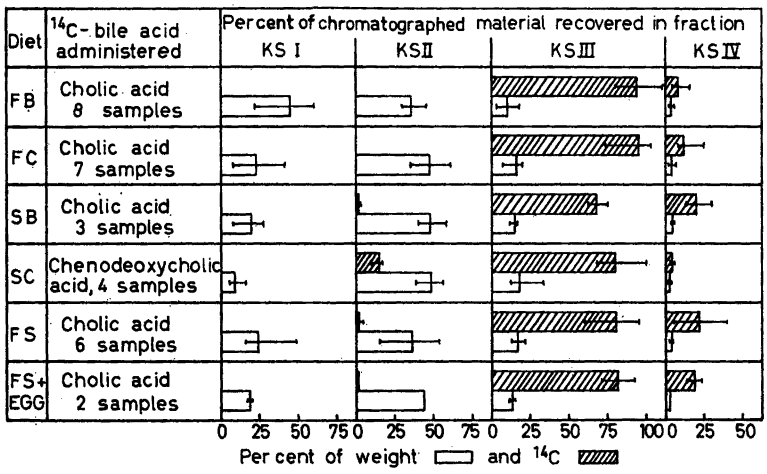

Fig. 2. Recovery of weight and endogenously labeled bile acids in the silicic acid fractionation procedure.

different depending on whether labeled cholic or chenodeoxycholic acid had been given. When 26 samples from subjects given cholic acid were chromatographed, 84.2 % (range 59.5—110.0) and 14.2 % (range 3.9—40.0) were recovered in fractions III and IV, respectively. When four samples from a subject given chenodeoxycholic acid were chromatographed, the values were 75.2 % (range 70.1—80.0) and 4.1 % (range 3.4—4.8) for fractions III and IV, respectively. The loss of activity in the latter chromatographies occurred in fraction II. Some of this activity might have been due to the presence of the cholenoic acid found by Norman and Palmer [19] (*cf.* the behaviour of

cholanoic acid, Fig. 1). GLC and TLC analyses of fraction II showed that some of the radioactivity was due to the presence of 3-ketocholanoic and 3β-hydroxycholanoic acids. Small amounts of these acids may therefore sometimes be lost from fraction III into fraction II. However, this is not usually the case (see Fig. 1). Another sample from the same subject was worked up and a 3-ketocholanoic acid fraction was isolated by silicic acid chromatography. Quantitative determinations by GLC showed that this acid made up about 3 % of the total bile acids in the sample.

Since acetic acid was used for the elution of bile acids from the silicic acid column, it could not be excluded that the reactive 3α-hydroxyl group of some bile acids might have been acetylated. However, 3α-acetoxy-substituted bile acids could not be demonstrated by GLC or TLC in any of the fractions from the silicic acid columns. Furthermore, synthetic lithocholic and deoxycholic acids did not form detectable amounts (<1 %) of acetylated derivatives when chromatographed under the same conditions as the fecal extracts.

The purification of the bile acids obtained by the silicic acid chromatography was sufficient for the subsequent GLC analysis in all cases studied. Usually about 10—30 % of the material in fraction III and 3—15 % of the

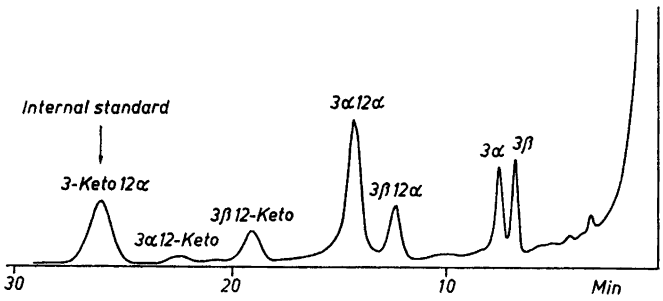

Fig. 3. Gas chromatographic analysis of methylated bile acids eluted in silicic acid fraction III (*cf.* Figs. 1 and 2). Tentative identifications are noted in the figure. For gas chromatographic conditions, see text.

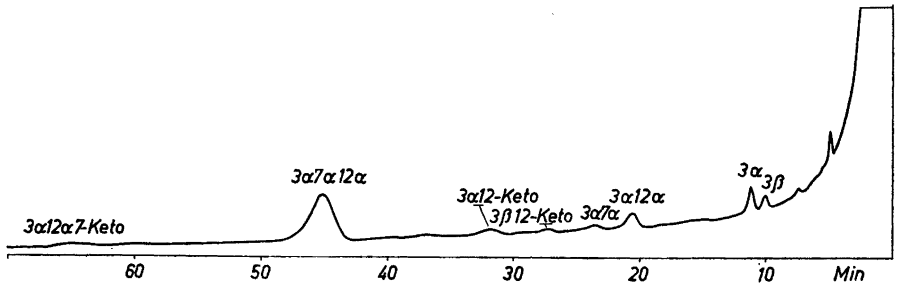

Fig. 4. Gas chromatographic analysis of methylated bile acids eluted in silicic acid fraction IV (*cf.* Figs. 1 and 2). Tentative identifications are noted in the figure. For gas chromatographic conditions, see text.

material in fraction IV consisted of bile acids. With these bile acid concentrations, overloading of the GLC-columns was not observed and the columns were not destroyed by the injections. Examples of GLC analyses of methylated bile acids from fractions III and IV are shown in Figs. 3 and 4, respectively.

GLC quantitation of bile acids. QF-1 was used as the stationary phase since this was the only phase found to give sufficiently large separation factors for the various bile acid derivatives to be determined. For quantitative work the columns should give only little tailing with polar bile acid methyl esters injected in amounts between 0.2 and 2 μg (depending on the retention time). At least 2000 theoretical plates were required for satisfactory separations under these conditions. Furthermore, the extent of degradation of the trifluoroacetates of the common bile acids should not be such as to give a drift of the base line before the appearance of a peak. The GLC system should also have a range where the peak area is linearly related to the amount of substance injected.

Columns meeting these requirements usually gave differences in the specific response (peak area/μg injected) for different bile acid methyl esters. These differences were related both to the retention times and to the structure of the compounds. The ratios between the specific response of various bile acid methyl esters and that of methyl deoxycholate usually ranged between 1.25 (*e.g.* for methyl 3β-hydroxycholanoate) and 0.90 (*e.g.* for methyl 3β-hydroxy-12-ketocholanoate). Trisubstituted methyl cholanoates gave a lower specific response in most cases (occasionally as low as 0.5 relative to that of methyl deoxycholate). Relative specific responses among the TFA derivatives usually ranged between 1.20 and 0.75 (TFA of methyl deoxycholate=1.0).

To test the accuracy of the GLC quantitations, an artificial mixture of bile acids, in the proportions usually found in fecal samples, was made. This mixture was analyzed with the GLC procedure. The agreement between the analyses of the methyl esters and the methyl esters trifluoroacetates is shown in Table 4.

When the purified fecal bile acids (silicic acid fractions III and IV) were analyzed as methyl esters and as methyl ester trifluoroacetates, a difference between the two determinations was often found (Table 5). In previous

Table 4. Quantitation of an artificial fecal bile acid mixture.

Methyl ester (mg)	3β [a]	3α	3β,12α	3α,12α	3β,12-keto	3α,12-keto	Total
Added:	4.30	13.4	5.70	25.2	3.82	2.62	55.0
Found: Me [b]	4.63	15.1	5.70	24.9	3.84	2.49	56.7
TFA [b]	— [c]	16.2	5.62	25.7	3.76	2.83	54.1

[a] Notations refer to substituents in 5β-cholanoic acid.
[b] Me: analyzed as methyl esters; TFA: analyzed as methyl ester trifluoroacetates.
[c] Included in the values for lithocholic acid (3α).

Table 5. Comparison between quantitations of fecal bile acid methyl esters before and after trifluoroacetylation.

Sample		Major bile acid determined [a] (mg/day)						
		3β	3α	3β,12α	3α,12α	3β,12-keto	3α,12-keto	Total
Fr 15246	Me [b]	8.60	51.6	3.20	24.6	2.20	5.80	96.0
	TFA [b]	—[c]	52.6	1.90	24.2	2.40	6.10	86.2
We 144	Me	2.90	11.0	0.70	5.90	0.30	1.60	22.4
	TFA	—[c]	13.3	0.80	6.00	0.80	2.50	23.4
To 23256	Me	8.40	17.2	23.1	26.2	2.20	3.70	80.8
	TFA	—[c]	25.6	20.3	16.8	1.60	1.80	66.1
We 16176	Me	18.4	80.6	5.50	121	9.80	—	235
	TFA	—[c]	101	5.50	105	8.20	—	220
Sp 192111	Me	2.40	14.5	12.6	39.7	6.80	4.40	80.4
	TFA	—[c]	16.8	14.8	43.8	3.40	3.80	82.6
Sp 232411	Me	7.00	15.9	8.00	25.6	3.30	—	58.8
	TFA	—[c]	24.7	9.50	28.0	3.50	—	65.7
Å 141511	Me	43.3	49.5	54.0	191	51.0	14.9	404
	TFA	—[c]	98.2	65.2	155	50.1	16.9	386
Å 2427113	Me	10.2	12.5	11.4	67.8	7.70	3.70	113
	TFA	—[c]	23.4	15.2	59.2	7.70	3.10	109
Å 161712	Me	13.9	40.9	30.6	127	31.7	17.0	261
	TFA	—[c]	56.8	43.7	106	26.8	18.4	251
Å 162012	Me	8.50	28.0	4.10	49.3	—	4.90	94.8
	TFA	—[c]	38.7	6.50	50.2	—	8.30	104

[a] Notations refer to substituents in 5β-cholanoic acid. Some GLC peaks probably contained minor amounts of bile acids other than those noted in the heading.
[b] Me: determined as methyl esters; TFA: determined as methyl ester trifluoroacetates.
[c] Included in the value for lithocholic acid (3α).

studies [5,6] the bile acids listed in Table 5 were found to be the major bile acids in human feces. Small amounts of a large number of other bile acid isomers were also identified.[6] The likely presence of isomeric dihydroxy bile acids with RRT's similar to those of the methyl esters and the TFA:s of 3β,12α-dihydroxy- and 3α,12α-dihydroxycholanoic acids might explain the lack of agreement between methyl ester and TFA quantitations.

However, in addition to bile acids, silicic acid fraction III was found to contain varying amounts of a compound previously shown not to be a bile acid.[6] The methylation product of this compound had an RRT of 1.13 (peak *x* in Fig. 5) and it was usually present together with smaller amounts of another non-bile acid contaminant with an RRT of 0.96.[6] These contaminants

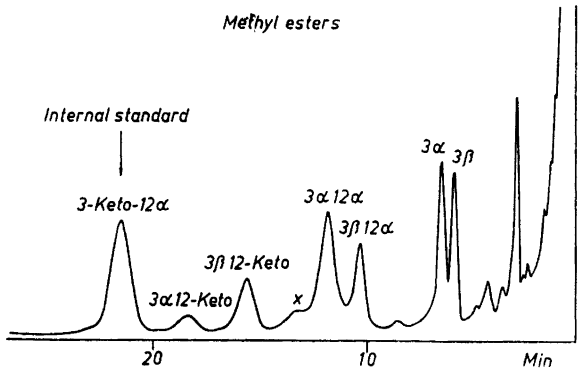

Fig. 5. Gas chromatographic analysis of methylated bile acids eluted in silicic acid fraction III (*cf.* Figs. 1 and 2). Tentative identifications are noted in the figure. Peak *x* is a non-bile acid contaminant. For gas chromatographic conditions, see text.

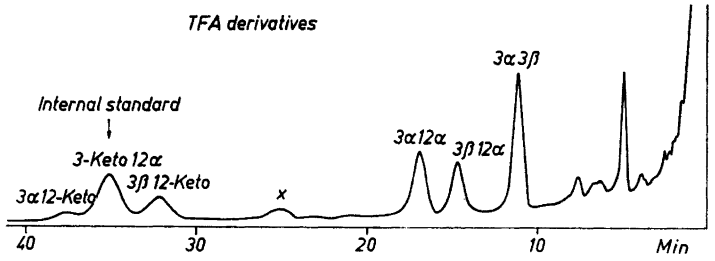

Fig. 6. Gas chromatographic analysis of methyl ester trifluoroacetates of bile acids in silicic acid fraction III. The sample was the same as that used for the analysis shown in Fig. 5. Tentative identifications are noted in the figure. Peak *x* is the TFA of the non-bile acid contaminant. For gas chromatographic conditions, see text.

are absent in the GLC analysis shown in Fig. 3 but can be seen in those shown in Figs. 5 and 6. The TFA's of the contaminants had RRT:s of 1.01 and 0.85, respectively (peaks *x* and *y* in Fig. 7). It is evident that the presence of these non-bile acid contaminants will decrease the accuracy of the dihydroxy bile acid determinations, particularly if a determination of chenodeoxycholic acid is attempted. An exceptional example of interference by the compounds with RRT's of 1.13 and 0.96 is shown in Fig. 7. Normally, the bulk of contaminants were eluted in fraction II from the silicic acid column.

Other contaminants of quantitative significance were not encountered. However, since different diets might introduce different contaminants the GLC procedure should always be based on determinations of both methyl esters and trifluoroacetates.

The quantitations were usually carried out using an internal standard of methyl 3-keto-12α-hydroxycholanoate (see Figs. 3, 5, and 6). When this

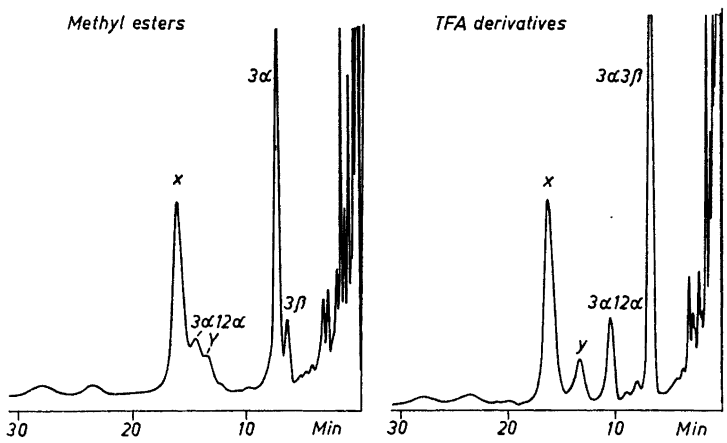

Fig. 7. Gas chromatographic analyses of methyl esters (left curve) and methyl ester trifluoroacetates (right curve) of bile acids eluted in silicic acid fraction III together with a large amount of non-bile acid contaminants (peaks *x* and *y*, see text). For gas chromatographic conditions, see text.

compound was already present in the fecal sample 3α-acetoxycholanoate was used as internal standard as shown in Fig. 8. It was also possible to make the GLC determinations without the use of internal standard (see Figs. 4 and 7). The peak areas were then compared with those of known amounts of appropriate reference compounds in the range of linear response. To minimize the injection error the same injection volume (4 μl) was always used both for the samples and for the dilution series of reference compounds. The variation between duplicate analyses was somewhat larger when internal standard was not used.

Recovery studies. To confirm the results of the recovery studies with ^{14}C-labeled bile acids, known amounts of the predominant bile acids were added to the fecal extracts before and after saponification. The samples were car-

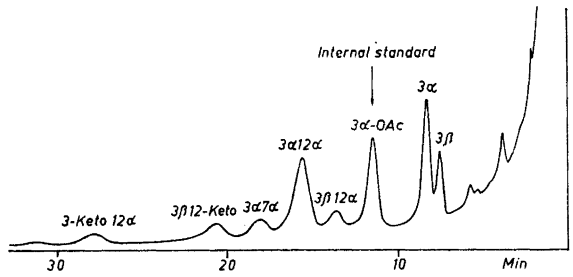

Fig. 8. Gas chromatographic analysis of methylated bile acids eluted in silicic acid fraction III (*cf.* Figs. 1 and 2). Tentative identifications are noted in the figure. For gas chromatographic conditions, see text.

Table 6. Recovery of bile acids added to the saponified fecal lipids after diethyl ether extraction.

Fecal sample[a]	Major bile acids determined[a]																	
	3β			3α			3β,12α	3α,12α			3β,12-keto	3α,12-keto			Total a-mount	Recovery per cent of		
	added (mg)	calc. (mg)	found (mg)	added (mg)	calc. (mg)	found (mg)	found (mg)	added (mg)	calc. (mg)	found (mg)	found (mg)	added (mg)	calc. (mg)	found (mg)	(mg)	added		calc.
S-3			1.7			4.8	1.2			6.4	1.7			0.8	16.6			
	1.4	3.1	3.2			4.7	1.3	4.7	11.1	11.5	1.3			0.8	22.8	107(3β)		103
																108(3α,12α)		104
S-5[c]	13.0	16.1	18.4			6.9	0.4			4.2	0.9			—	30.8	118		114
			2.7			6.9	0.9	6.3	10.6	11.0	0.4			0.1	22.0	106		104
			3.4			7.0	1.0			4.3	0.4	4.2	4.3	4.5	20.6	105		96
			3.1	12.0	18.9	19.8	0.3	6.3	10.6	10.5	0.7	4.2	4.3	4.2	38.6	108(3α)		105
																98(3α,12α)		99
																98(3α,12-keto)		98
S-2			2.3			6.6	0.9			5.6	0.7			0.8	16.9			
			2.3	3.6	9.8	10.7	1.1			5.8	0.6			0.6	21.1	125		109
			2.2			6.7	0.8	3.1	8.8	8.7	0.7			0.8	19.9	97		99
S-4			2.0			3.2	1.6			4.6	1.7			—	13.1			
			2.3			3.4	1.7			4.7	1.7			—	13.8			
			2.0			3.1	1.3			4.5	1.6			—	12.5			
			2.1	0.5	3.7	3.7	1.5			4.8	1.4			—	13.5	100		100
			2.1	0.5	3.7	3.5	1.5			4.9	1.7			—	13.7	60		95
			2.0			3.3	1.6	0.4	5.1	5.2	1.6			—	13.7	125		102
S-1			1.8			8.1	0.6			12.1	1.0			0.2	23.8			
			2.0			8.0	0.5			11.8	0.8			0.1	23.2			
			1.8			8.0	0.4			12.3	0.9			0.1	23.5			
			2.0			8.2	0.5	0.8	12.9	13.2	0.8			0.3	25.0	137		102
S-6			0.3			1.8	—			3.3	—			—	5.4			
			0.4			1.8	—	1.0	4.3	4.6	—			—	6.8	130		107

[a] Notations refer to substituents in 5β-cholanoic acid.

[b] In most cases the experiments were made with saponified fecal lipids corresponding to 10 % of the daily excretion. Samples S-5 and S-6 were analyzed without the use of internal standard.

[c] The bile acids were added to the chloroform-methanol prior to saponification.

ried through the standard procedure and the bile acids were quantitated by GLC. The results are summarized in Table 6. Both the recoveries and the reproducibility of the method were satisfactory.

In order to test the correlation between recovery of radioactivity and the GLC determinations, the following experiment was made. In a previous study [5] the specific activities of lithocholic and 3β-hydroxycholanoic acids in fecal samples of two subjects given chenodeoxycholic acid-24-^{14}C were determined. The amount of monohydroxycholanoic acids in two of these samples was determined by the present procedure. The total amount of radioactivity in silicic acid fraction III was determined. This value was compared with that obtained by multiplying the specific activity of the bile acids with the amount found in the GLC analysis. It was found that by GLC determination of lithocholic and 3β-hydroxycholanoic acids, 89 and 114 % of the activity in silicic acid III could be accounted for. This indicates that the major chenodeoxycholic acid metabolites in this fraction are included in the GLC quantitations. The turnover of chenodeoxycholic acid was previously found to be 1.5 and 2 times that of cholic acid in the two subjects.[5] Determinations by the present GLC procedure of the ratios between chenodeoxycholic and cholic acid metabolites in fecal samples yielded mean values of 1.5 and 1.4, respectively, for the two subjects. Taken together with the evidence for a quantitative determination of chenodeoxycholic acid metabolites, this indicates that the majority of cholic and chenodeoxycholic acid metabolites in feces are determined by the present procedure.

Composition and daily excretion of fecal bile acids. As seen in Tables 5 and 6 large individual variations were found both in the total daily excretion of bile acids and in the proportions of the individual bile acids. The daily excretion varied between 50 and 500 mg. In most samples analyzed 3β-hydroxy, 3α-hydroxy-, 3β,12α-dihydroxy, 3α,12α-dihydroxy, 3β-hydroxy-12-keto- and 3α-hydroxy-12-ketocholanoic acids were the six predominant bile acids but occasionally other bile acids were present in quantitatively important amounts. Samples from some subjects contained appreciable amounts of trisubstituted bile acids. In these cases cholic acid appeared to be the major bile acid in silicic acid fraction IV (see Fig. 4).

DISCUSSION

The development of the method described in this paper has required three major experimental conditions: (1) Subjects were given labeled bile acids so that the fecal samples contained endogenously labeled, naturally occurring bile acids of different structure. This is important since the physico-chemical state of different bile acids may vary [20] and studies of extraction recoveries cannot be made accurately by direct addition to feces of known bile acids. (2) The chemical nature of the fecal bile acids and the identity of the GLC peaks have been extensively studied using peak shift techniques and combined gas chromatography-mass spectrometry.[6,7] This is of importance for the establishment of specificity and for recognition of possible non-bile acid

contaminants. (3) Both solid and formula diets were given to the subjects to establish, as far as possible, that the method was unaffected by variations in the composition of feces.

Extraction of feces. Various procedures were tested for the extraction of fecal bile acids. Although 80 % aqueous ethanol can be used to extract bile acids in rat feces quantitatively [21] this solvent or ethanol did not extract bile acids in human or rabbit feces [15] quantitatively. Continuous Soxhlet extraction with hot choloroform-methanol (1:1) for 48 h, however, always gave quantitative recoveries. This finding has been confirmed by Norman [20] who also showed that acetone extraction was less efficient.

Saponification of fecal lipids. A saponification step had to be included since esters might be formed during the long extraction with hot chloroform-methanol. Furthermore, Norman [20] has shown that some of the bile acids in human feces occur as saponifiable derivatives.

The difficulties experienced in the extraction of bile acids from fecal lipids saponified in aqueous methanol might be due to poor solubility of the crude fecal lipids in this solvent.

The saponification conditions were not sufficient for hydrolysis of conjugated bile acids. These conditions were chosen, however, to avoid possible destruction of labile bile acids (see Ref. 1). Furthermore, none of the samples studied contained significant amounts of conjugated bile acids. Taurine conjugates, if present, would have been lost in the continuous ether extraction where, however, a quantitative recovery of radioactivity was obtained. Glycine conjugates, if present, would have appeared in silicic acid fraction IV (as studied by additions of glycocholic acid). Such fractions, containing proportionally large amounts of cholic acid metabolites, were analyzed by TLC.[22] Insignificant amounts of glycine conjugates were detected (estimated to less than 3 % of the bile acids in this fraction).

Although conjugated bile acids normally do not occur in human feces in appreciable amounts they may assume importance under pathological conditions. In such cases conditions suitable for the hydrolysis of conjugated bile acids may be used.

Silicic acid chromatography. The silicic acid chromatography system described was the simplest means found by which bile acid fractions could be obtained which were sufficiently pure for GLC on QF-1 and which contained minimal amounts of non-bile acid contaminants interfering with the peaks of the bile acid derivatives. The critical step in the chromatography was that involving the removal of contaminants by elution with 1 % acetic acid in benzene. Prolonged elution with this solvent could lead to loss of mono-substituted cholanoic acids. To control that such a loss does not occur ^{14}C-labeled 3-keto- or 3β-hydroxycholanoic acid is preferably added to unlabeled fecal extracts or fecal extracts which have not been endogenously labeled with chenodeoxycholic acid-24-^{14}C.

Recovery and specificity. The mean overall recovery of endogenously labeled cholic acid metabolites in the purification procedures was 95.6 %. The corresponding figure for chenodeoxycholic acid metabolites was 77.3 %. Specific losses were encountered only for the latter compounds. Not yet fully identified metabolites of chenodeoxycholic acid (*e.g.* the cholenoic acid described by

Norman and Palmer [19]) are lost with the nonpolar compounds eluted in fraction II from the silicic acid column.

Since feces contain bile acids differing considerably in polarity, the group purification of fecal bile acids cannot at present lead to fractions free from contaminants. It is therefore important to establish that the contaminants do not have a behaviour similar to that of the bile acids in the GLC system used. The nature and amount of contaminating compounds may show variations between different individuals. Each individual might also excrete different contaminants depending on, for instance, the nature of his diet.

When the present procedure was tested on feces from several subjects on six different diets only occasional interference with the determination of the major fecal bile acids occurred. To minimize the possible error caused by contaminants it is advisable to analyze bile acid containing fractions by two GLC procedures, *e.g.* analysis of methyl esters and methyl ester trifluoroacetates on QF-1 columns.

The complexity of the fecal bile acid mixture [6,7] makes an accurate determination of each bile acid difficult. The individual quantitation of the predominant fecal bile acids with the present method involves an error due to the presence of a number of isomeric compounds.[6] Thus the peaks of the predominant bile acids may contain other bile acids which might give a slightly different specific response. In our opinion, however, this error is small and can be accepted in view of the increased information obtained by the individual determination of the major fecal bile acids.

The values for total daily bile acid excretion, 50—500 mg, are in the same range as those reported by Grundy *et al.* (see discussion in Ref. 2) and by Ali *et al.*[11] who also used gas chromatographic methods for the quantitations. The values are lower than those obtained by several authors using less specific methods (see Refs. 2 and 3). Further studies are needed to establish correlation between GLC quantitations and values for bile acid production [5] obtained in studies of the turnover of labeled bile acids.

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