Gas–liquid chromatographic determination of human fecal bile acids

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ABSTRACT A method for the determination of total bile acids in human feces that is suitable for routine application is described and discussed. Bile acids are extracted from freeze-dried feces with acetic acid and toluene, in the presence of the internal standard 23-nordeoxycholic acid. After saponification of the extract, bile acids and the internal standard are methylated and converted by mild chromic acid oxidation into their ketonic derivatives. The resultant mixture of a few stable compounds can be separated and measured quantitatively by gas–liquid chromatography on a methylsiloxane polymer. A reference bile acid mixture including the internal standard is also taken through the entire procedure with each series of samples. It has been demonstrated that, in spite of the omission of the usual purification steps, the method is specific for bile acids.

KEY WORDS fecal bile acids · man · extraction · hydrolysis · keto derivatives · gas–liquid chromatography · temperature programmed · reproducibility · antibiotics

A RELIABLE PROCEDURE for determining the excretion of bile acids is desirable in studies of cholesterol metabolism in man. The trend of most recently developed methods (1–5) is to apply quantitative GLC to certain derivatives of the fecal bile acids. The most elaborate of these methods, developed by Grundy, Ahrens, and Miettinen (5), integrates individual or overlapping peak areas under conditions that insure a constant instrument response factor. Because fecal bile acids are numerous (6–8) and their composition may vary with changes of the intestinal flora (8), interference from nonbile acid contaminants is probably best avoided when several purification steps are included in the preparation of samples (5) and identification work is carried out with each type of pattern encountered (6, 7, 9–11).

Abbreviation: GLC, gas–liquid chromatography.

The feasibility of determining the total human feca bile acids in a few steps seemed, in our judgment, greater with a method that includes mild chromic acid oxidation of hydroxyl groups carried out with the methyl esters, after extraction of the samples and alkaline hydrolysis. Early experiments in this laboratory indicated the formation, under these conditions, of a few ketonic esters whose behavior on several gas-chromatographic phases was identical with that of the methyl esters of 3-keto-5β-cholanoic, 3,12-(or 3,7-)diketo-5β-cholanoic, and 3,7,12-triketo-5β-cholanoic acid. The last-named compound was a minor component in normal subjects. These findings were in line with the recent reports by Eneroth, Gordon, Ryhage, and Sjövall (6, 7) who identified in human feces a number of saturated 5β-cholanoic acids that were substituted mainly by hydroxyl groups, and to a lesser extent by keto groups exclusively in the 3, 7, and 12 positions, but found that trisubstituted bile acids commonly constituted a minor fraction. The present paper suggests a simplified procedure for quantitative analysis based on these considerations.

MATERIALS AND APPARATUS

Solvents and reagents were all of analytical grade. Solutions (20% w/v) of KOH in ethylene glycol were discarded if unused after 2 days. Ethereal diazomethane (distilled) was freshly prepared.

Chromic acid in acetic acid (12) was made by dissolving chromium trioxide (20 g) in a minimum of water, and making the solution up to 100 ml with acetic acid.

Bile Acid Standards

The internal standard (used for extraction of feces) was a solution of 23-nordeoxycholic acid (concentration range, 80–300 μg/ml) in acetic acid. The diacetate may

also be used. We prepared this compound by Barbier–Wieland degradation of deoxycholic acid (13).

The solution of reference bile acids (used for calibration) consisted of a solution in acetone or in methanol (concentration range, 100–300 μg/ml for each of the bile acids) of lithocholic, 23-nordeoxycholic, deoxycholic and cholic acids. Acetates or methyl esters are equally convenient. We purified cholic acid and deoxycholic acid (the latter through the diacetate methyl ester) as described by Hofmann (14). Commercial lithocholic acid was purified through silicic acid chromatography of the methyl ester and saponification (mp 184.5–185.5°C, from aqueous methanol).

Glassware and Apparatus

Stoppered, round-bottom, Pyrex test tubes, 16 × 160 mm, were heated, in different parts of the procedure, in drilled aluminum blocks (holes at least 5 cm deep) at 120 ± 5 and 220 ± 5°C.

The gas chromatograph was a Pye (series 104, Model 64) temperature-programmed, flame ionization instrument with an integrating amplifier for measurement of peak areas (Pye Instruments, Cambridge, England). The glass column, 140 cm × 4 mm I.D., was packed with 1% JXR (a recently introduced methylsiloxane polymer similar to SE-30) on Gas-Chrom Q, 100–120 mesh (factory-pretested packing, Applied Science Laboratories Inc., State College, Pa.). The flash heater was set at 270–280°C, the detector at 275°C. The column was maintained at 175–180°C during injection of the samples and for 15–20 min thereafter. The temperature was then rapidly programmed (e.g., at 10°C/min) up to 250°C for chromatography of the bile acid derivatives.

METHOD OF ANALYSIS

Collection and Preparation of Feces

Feces are collected quantitatively over 3–4 days in plastic bags and then frozen. The collections detached from the bags are pooled in a new, large, weighed bag; the content is weighed and filter-aid Celite is added (10% by weight of the feces). The mass is defrosted and made homogeneous inside the bag by externally kneading with the fingers. Unless feces are diarrheic, a known weight of water is added to give a uniform soft paste. A large aliquot (e.g. 50 g) is weighed in a crystallizing dish and the sample is freeze-dried overnight. The dry mass is again weighed, and then blended by grinding in a mortar. The resulting powder is stored in a dark bottle.

Extraction of Bile Acids from Feces

An aliquot of the fecal powder (range 100–300 mg) is weighed in a test tube. A known amount of internal standard is added in glacial acetic acid (3 ml pipetted exactly; 1 mg/250 mg dry feces is convenient for most normal subjects). The solids are extracted by heating (1 hr at 120°C) with frequent mixing of the stoppered tube. The tube is cooled, toluene (6 ml) is added, and the contents are vigorously mixed. A deep yellow-brown, clear supernatant portion separates immediately upon standing. An aliquot (e.g. 3 ml; precision pipetting is not requisite) is removed; if desired, the procedure may be interrupted at this step for an unlimited amount of time.

Saponification

The acetic acid–toluene extract (e.g. 3 ml) is evaporated to dryness under reduced pressure on a hot water bath. The distillation of such a mixture is considerably easier than that of the individual solvents (caution: any rubber used in the vacuum line must be Teflon-lined). 1 ml of 20 % KOH in ethylene glycol is added to the residue, which is dissolved by progressive heating with mixing. Hydrolysis is then carried out under reflux for 20 min (dry bath at 220°C); for this purpose the upper part of the open test tube (over 10 cm) serves as an air condenser for glycol vapors. 1 ml of 20% aqueous NaCl and 1 ml of methanol are added to the cooled hydrolysate.

Extraction of Neutral Sterols

From the alkaline mixture containing salt, water, glycol, and methanol the unsaponifiable matter is extracted by vigorous shaking with petroleum ether (4 × 5 ml). In most instances a clear petroleum ether solution separates immediately upon standing. However, if the amount of soaps in the aqueous phase is large, the latter may form a gel, which disappears after gentle warming or addition of 20% NaCl. The petroleum ether extract, which contains the neutral sterols, is discarded.

Extraction of Bile Acids; Methylation

The extracted hydrolysate is diluted with 20% NaCl (8 ml), acidified with 6 N HCl (approximately 10 drops; thymol blue to red), and extracted with diethyl ether (3 × 5 ml). The ether extract containing the bile acids is reduced to a small volume under reduced pressure; evaporation to dryness is then completed in the hot water bath with successive additions of toluene (3 × 2 ml). The dry residue (a brown oil) is redissolved in a mixture of diethyl ether (2 ml) and methanol (5 drops); an excess of ethereal diazomethane is added to the cool solution. After 15 min at room temperature, the mixture is evaporated to dryness in vacuo.

Oxidation

The residue of methyl esters is redissolved in 1 ml of 90% acetic acid. The solution is cooled with tap water,

and an excess (5 drops) of 20 % chromic acid in acetic acid is added dropwise with mixing. After 15 min, 10 ml of 20% NaCl is added, and the ketonic esters are extracted with diethyl ether (3 × 5 ml). The combined extracts are reduced to a small volume in vacuo; evaporation is continued to dryness in the hot water bath after the addition of toluene (2 ml). The residue (usually a colorless wax; sometimes beautiful needles from diethyl ether) is dissolved in a volume of acetone suitable for gas chromatography of a portion (1–5% of the sample). Since these solutions (or the dry residues) keep very well upon storage, they can be chromatographed at the experimenter's convenience.

Bile Acid Standards

With each series of fecal samples analyzed, duplicate portions (1 ml) of the solution of reference bile acids are evaporated to dryness in test tubes, and starting from the saponification step, are prepared for chromatography of the derived ketonic esters exactly like the fecal samples.

Gas Chromatography

Instrumental details given under Materials and Apparatus include suggested operating conditions which may serve as a guide. A minimum requirement is that the operator has a working knowledge of the pitfalls of high-temperature gas chromatography. We do not recommend using the same column for both fecal bile acid analysis by the present method and for any analysis requiring the injection of silylating mixtures containing pyridine. This results in a rapid loss of efficiency for the bile acid ketonic esters.

Calculations

The results of the analysis are calculated from the gas chromatograms of both the sample and the bile acid standards. The peak at the location of 3-ketocholanoate for the fecal sample (peak *1* on Fig. 1) includes those bile acids that upon oxidation yield a 3-ketone; the peak with the retention time of 3,12-(or 3,7-)diketocholanoate[1] (peak *3*, Fig. 1) includes those fecal bile acids that yield a diketone; finally a peak at the location of methyl dehydrocholate, if detectable (arrow, Fig. 1), includes the trisubstituted bile acids that yield a triketone. Peak *2*, Fig. 1, arises from the internal standard (3,12-diketo-23-norcholanoate). The corresponding peaks on the chromatogram of the bile acid standards arise from known amounts of lithocholic acid, deoxycholic acid, cholic acid, and the internal standard, respectively.

[1] These compounds are not separated.

The ratio of the peak area for each of the three peaks to that for the internal standard on the chromatogram from feces is related to the amount of bile acid present in the sample. Although it might be possible under certain conditions (see Results and Discussion) to calculate the amounts of bile acids from those ratios alone, we attempt here to take into account possible fluctuations of the relative responses for the different bile acids due either to specific losses during the preparation of the derivatives, to specific losses on the column, or to both causes. Therefore the peak-area ratios observed for the bile acid standards (run on the same GLC column at almost the same time) serve as experimental correction factors by means of the following relation:

$$M_x = \frac{M_{\text{int.st.sample}} \times M_{\text{ref.}} \times P_{\text{int.st.ref.}} \times P_x}{M_{\text{int.st.ref.}} \times P_{\text{ref.}} \times P_{\text{int.st.sample}}}$$

where

$M_{\text{int.st.sample}}$ and $M_{\text{int.st.ref.}}$ = amounts (μg or μeq) of internal standard added to feces and used in the standard mixture, respectively;

M_x and $M_{\text{ref.}}$ = amount of bile acid (μeq) in the fecal sample (the unknown), and amount of bile acid in the standard mixture giving rise to the same ketonic ester;

$P_{\text{int.st.ref.}}$ and $P_{\text{int.st.sample}}$ = peak areas (arbitrary units) of the internal standard in both chromatograms (reference and sample);

P_x = peak area of the unknown;

and $P_{\text{ref.}}$ = peak area for the reference bile acid.

Peak areas from duplicate analyses are first normalized relative to the internal standard, and then averaged. Since all the bile acids are measured as dehydro derivatives, the expression of the results as equivalents avoids the use of misleading conventions as to the average molecular weight of the parent fecal bile acids. However, a dihydroxy and a monohydroxy–monoketo bile acid differ in this respect by only two hydrogen atoms (i.e. 0.5% of the average mol wt); the error thus would be negligible if the three peaks in the relation above were calculated directly as lithocholic, deoxycholic, and cholic acids, respectively. Alternatively, the data may be transformed as follows: 1 μeq = 0.377 mg (as lithocholic acid); 0.393 mg (as deoxycholic acid); 0.409 mg (as cholic acid).

For the relation above to be applicable, certain conditions must be fulfilled. The sample chromatogram should be clean in the region of bile acids, except for the expected peaks (28–50 min portion on Fig. 1). A peak of coprostanone (at 29 min on Fig. 1) would indicate unsatisfactory removal of the neutral sterols.

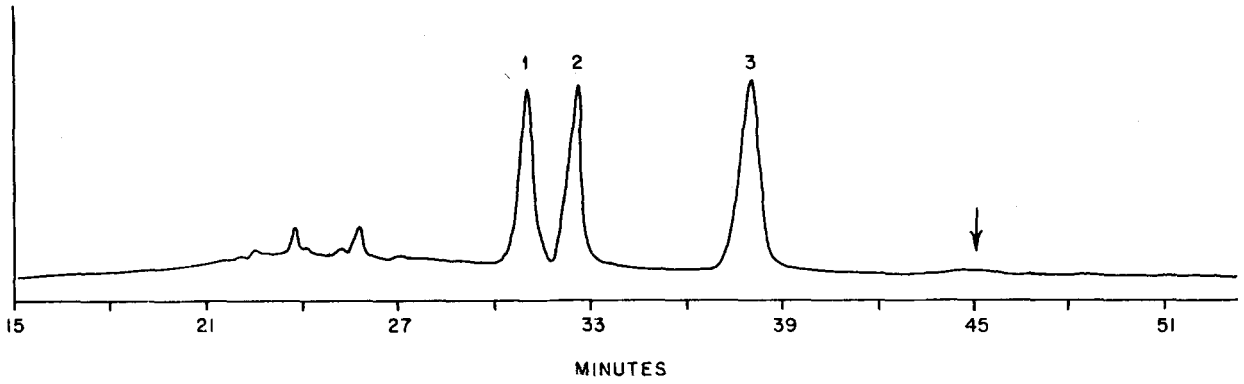

FIG. 1. Gas chromatogram of oxidation products from normal human feces. Numbered peaks correspond in position to the following methyl esters: *1*, 3-ketocholanoate; *2*, 3,12-diketo-23-norcholanoate (internal standard); *3*, 3,12-(or 3,7-)diketocholanoate. The arrow indicates the location of methyl dehydrocholate. Column load approximately 7 μg for the internal standard. Conditions: 1% JXR on Gas-Chrom Q, 100–120 mesh, 150 cm × 4 mm I.D. Nitrogen flow, 30 ml/min; flame ionization detector. Temperature, isothermal at 180°C during 15 min; then programmed up to 250°C at 12°C/min; finally held isothermal at 250°C.

The separation of the pair 3-ketone and internal standard (peaks *1* and *2* on Fig. 1) must be satisfactory (this requires 1600–1800 theoretical plates); the use of lower efficiencies would unduly impair the specificity of the method. The peak areas for both the sample and the standard must each fall within the linear range of the instrument. Since with normal samples the amount of triketone is often very small and hardly detectable (see arrow, Fig. 1), and the latter component is the more likely to exhibit a narrow linear range (see Results), it might be advisable for the operator to provide and comment on a possibly underestimated figure, rather than resorting to calibration techniques of doubtful value. Finally, the systematic inspection of reference chromatograms is essential (15) to validate day-to-day results in respect to both the technique of sample treatment and the general condition of the gas chromatograph. It is particularly important that the peak-area ratio, in the reference mixture, for the homologous pair internal standard and diketone (peaks *2* and *3*, Fig. 1) remains unchanged (within a few per cent of experimental error) at all times.

Daily Excretion of Bile Acids

The daily excretion figure is obtained by multiplying the total of bile acids found in the sample (see above) by the factor relating the size of the sample to the total fecal collection averaged for 24 hr. For example, 250-mg aliquots (dry powder) were taken for analysis from a subject who excreted 100 g of feces daily (averaged for the total collection period; wet weight, including water and Celite added). The moisture content was 75% (from the weights before and after freeze-drying); the total excretion was thus 25 g (dry weight) daily. The analytical result was 6 μeq of bile acids (in the sample). The 24 hr figure was therefore 600 μeq (roughly 240 mg).

VALIDATION PROCEDURES

Extraction of Bile Acids from Feces

Three different methods of preparation of the extract were used for comparison purposes.

Procedure A. The fecal powder (about 250 mg) was extracted (dry bath at 120°C) with acetic acid (3 ml) that contained the internal standard. After cooling, 6 ml of toluene was added and vigorously mixed. The tube was centrifuged and the liquid decanted. The solids were then washed with acetic acid–toluene 1:2 (3 × 5 ml, with repeated centrifugation). The extract and washings were combined; the residue eventually was kept for radioactivity measurements.

Procedure B. After identical extraction in acetic acid, the cooled tube was centrifuged. A portion (1 ml) of the supernatant acetic acid solution was removed and vigorously mixed with toluene (2 ml). After separation of a precipitate the supernatant was decanted and taken for analysis.

Procedure C. The procedure was that described above, under Method of Analysis, in which toluene is added directly to the mixture of extract and solids. The initial steps were thus as in Procedure A, but no attempt was made to isolate completely the solution from the solid residue.

In Vivo Labeling and Preparation of Labeled Feces

Chenodeoxycholic- or cholic acid-24-^{14}C (Philips-Duphar, Amsterdam, The Netherlands) was given by mouth (3–5 μc with lactose capsules). Fecal collections within 48–72 hr after administration of the label were prepared for analysis and described above.

Radioactivity Measurements

Fecal powders (250-mg portions), their acetic acid–toluene extracts (procedure A) after removal of the

solvents, or the residues after extraction (procedure A) were counted by liquid scintillation after wet ashing and absorption of carbon dioxide in ethanolamine. The general procedure was that described by Jeffay and Alvarez (16). For our purposes the capacity of the apparatus was enlarged (oxidation flask, 250 ml; volumes of washing and absorbing fluids, 20 ml each). The oxidizing mixture was a saturated solution of potassium dichromate in concentrated sulfuric acid (100 ml; potassium dichromate present in excess). As a consequence of the increased size of the system, only 3/20 aliquots of the ethanolamine–methyl Cellosolve mixture containing the labeled carbon dioxide were taken for scintillation. The lack of interference by nitrogen oxides or by inactive carbon dioxide was demonstrated by counting small amounts of deoxycholic acid-24-^{14}C (counting range, 100–2000 cpm) added to inactivate 250-mg fecal samples, to their extracts, or to various loads of cellulose. Quenching effects were examined in the vials by the internal standard technique, using hexadecane-1-^{14}C.

Reference Bile Acids

Taurolithocholic acid, glycodeoxycholic acid, and sodium taurocholate were prepared by synthesis, following the modifications by Hofmann (14) of Norman's method (17). Methyl 3α-acetoxy-12-keto-5β-cholanoate was a gift of Professor H. Vanderhaeghe. 3α-Hydroxy-7-keto-5β-cholanoic acid was prepared according to Samuelsson (18). Methyl 5β-cholanoate was obtained from Steraloids, Inc., Flushing, N.J. Samples of 3β-hydroxy-5β-cholanoic and 3β,12α-dihydroxy-5β-cholanoic acids were gifts of Dr. J. Sjövall.

Reference Ketonic Esters

Methyl 3-keto-5β-cholanoate, 3,12-diketo-5β-cholanoate, 3,12-diketo-23-nor-5β-cholanoate (Analysis: $C_{24}H_{36}O_4$. Calculated: C, 74.18; H, 9.34. Found: C, 74.36; H, 9.07), and 3,7,12-triketo-5β-cholanoate were prepared by chromic acid oxidation (19) of the corresponding hydroxy compounds.

Gas–Liquid Chromatography

n-Alkanes used to check the general performance of the instrument (tetracosane, octacosane, dotriacontane, and hexatriacontane) were obtained from Fluka AG, Buchs, Switzerland.

Bile acid recovery studies were carried out with a 1% JXR column. Unsubstituted methyl cholanoates were analyzed on 1% JXR at 210–220°C. Methyl esters of hydroxy bile acids were analyzed on 1% JXR at 250°C; responses were corrected for nonlinearity by using calibration graphs within a narrow range of sample loads. For chromatography of ketonic esters on selective columns, XE-60 (methyl cyanoethyl silicone) and QF-1 (methyl fluoroalkyl silicone) were used (both 1% on Gas-Chrom Q, 100–120 mesh, Applied Science Laboratories Inc., State College, Pa.; 5-ft columns).

Wolff–Kishner Reduction of Bile Acid Ketonic Esters

The procedure of Huang-Minlon (20) was adapted for the submilligram scale. The ketonic esters were brought to dryness in a test tube. Hydrazine hydrate in ethylene glycol (25% v/v solution, 0.5 ml) was added; the stoppered tube was heated (30 min at 110°C). After cooling, a fresh solution (0.5 ml) of KOH in ethylene glycol (37.5% w/v) was added; the mixture was then refluxed (dry bath at 220°C) for 90 min after removal of the hydrazine and water vapors. The cooled mixture was diluted with water (1 ml) and methanol (1 ml), and extracted with petroleum ether (3 × 5 ml; to discard). The aqueous phase was then acidified with 6 N HCl, and the unsubstituted cholanoic acids were extracted with petroleum ether (3 × 5 ml). The acids were methylated with diazomethane prior to GLC.

RESULTS

Preparation of Feces

The large daily fluctuations of fecal excretion justified the use of pooled collections; a 3–4 day period was considered a minimum. The addition of Celite ensured sufficient channelling in the paste for smooth and efficient removal of water during freeze-drying. Homogeneity of the final dry powder for weighing small aliquots is demonstrated by the reproducibility of the analysis of replicates (see below, Table 4).

Extraction of Bile Acids from Feces

The efficiency of extraction of 250-mg samples (procedure A) with acetic acid (3 ml), including the effect of adding toluene to the extract, was tested by measuring recoveries of ^{14}C from feces obtained from three normal subjects after the feeding of labeled bile acids. The results (Table 1) indicate satisfactory recovery (85–105%) even with brief extraction periods (subject 1). In line with the latter finding, brief or prolonged extraction (15–360 min range) resulted in identical figures (Table 2) in the analysis of fecal bile acids as the derived ketonic esters.

Addition of toluene after the primary extraction step was found to be important as it removed by precipitation some nonlipid contaminants that are soluble in acetic acid alone (e.g. sugars; the mixture was not favorable for deproteinization). A toluene–acetic acid ratio of 2:1 in the final extract was adopted as it was sufficiently selective yet adequate to keep most of the more polar conjugated bile acids (e.g. taurocholic) in solution.

TABLE 1 RECOVERY OF ^{14}C IN ACETIC ACID–TOLUENE EXTRACTS (PROCEDURE A) FROM FECES LABELED IN VIVO

Subject	Labeled Bile Acid Given by Mouth	Dose	Period of Extraction	Radioactivity Found: Intact Feces	Radioactivity Found: Extract	Radioactivity Found: Residue	Recovery in Extract
		μc	min		cpm		%
1	Chenodeoxycholic	4	5	470	405	30	85
			10	455	420	—*	93
			20	460	485	—	105
			30	470	420	20	89
2	Cholic	5	60	410	390	—	95
3	Cholic	3	15	860	730	—	85

250 mg of lyophilized feces was extracted with 3 ml of hot acetic acid.
* Not significantly different from background counts.

TABLE 2 EFFECT OF DURATION OF EXTRACTION ON THE GAS-CHROMATOGRAPHIC YIELD OF FECAL BILE ACIDS

Time of Extraction*	Found as Ketonic Esters
min	μeq/g dry feces
15	17.5
30	16.9
60	17.3
120	16.7
240	17.3
360	17.6

Feces were from a normal subject; single determination for each time point.
* With 3 ml of acetic acid per 250 mg of feces. Further treatment according to procedure C (see *Validation Procedures*).

The latter feature is illustrated in the results of recovery experiments in which sodium taurocholate was added to feces containing no detectable amount of trisubstituted bile acids (see Table 3).

Toluene may be added at a number of stages. For the tests with radioactive feces, toluene was added to the mixture of extract and solids: and to separate the extract from the residue, the latter was washed (procedure A). Alternatively, toluene may be added to a portion of the extract (procedure B); or simply to the mixture of extract and solids, followed by vigorous mixing and by the removal of an aliquot for analysis (procedure C, used in our proposed method of analysis). In recovery experiments where bile acids were added to feces (Table 3) the results seemed to indicate that bile acids were not absorbed onto the solids with any of the procedures.

Acetic acid–toluene extracts of bile acids were found to be stable at room temperature over long periods; Table 4 shows identical results obtained at 14-month intervals with aliquots of the same extract.

Alkaline Hydrolysis

Bile salts normally appear in feces in the unconjugated form (8). However, after certain modification of the intestinal flora, excretion of glycine- or taurine-conjugated bile acids might occur. Therefore a step of rigorous hydrolysis is considered requisite (5) for a method of analysis intended to be of general application. Prolonged autoclaving with aqueous or aqueous alcoholic alkali is the most common method (5); the idea of speeding up deconjugation by raising the temperature was suggested to us by Dr. A. F. Hofmann. The course

TABLE 3 RECOVERY OF BILE ACIDS ADDED TO FECES

Subject	Compound Added	Amount Added	Extraction Procedure*	Ketones Found: Mono-	Ketones Found: Di-	Ketones Found: Tri-	Recovery of Added Bile Acid
		μeq			μeq/250 mg feces		%
1	—	—	A	1.52	1.82	tr.	—
	—	—	B	1.47	1.85	tr.	—
	—	—	C	1.50	1.78	tr.	—
	Lithocholic acid	1.04	C	2.65	1.85	tr.	90
	Deoxycholic acid	1.10	C	1.48	3.10	tr.	120
	Sodium taurocholate	1.32	A	1.49	1.80	1.25	95
	Sodium taurocholate	1.32	C	1.53	1.82	1.15	88
	Corn oil	(50 mg)	C	1.48	1.83	tr.	—
2	—	—	C	none	none	none	—
	Sodium taurocholate	1.32	C	none	none	1.27	96
	Lithocholic acid	1.04	C	1.15	none	none	110
	Deoxycholic acid	2.20	C	none	1.85	none	84

Values are averages of duplicates. 250-mg aliquots of fecal powder were used. Subject 1 was normal; subject 2 had complete biliary obstruction, with colorless feces and steatorrhea.
* See *Validation Procedures*.

TABLE 4 REPRODUCIBILITY OF ANALYSIS AND EFFECT OF SAMPLE STORAGE; INFLUENCE OF ANTIBIOTIC TREATMENT UPON THE FECAL BILE ACID PATTERN

Subject Condition*	Date of Feces Collection	Sample Taken for Analysis	Date of Analysis	Replicates	Ketones Found		
					Mono-	Di-	Tri-
					μeq/g dry feces		
Normal	Mar. '66		Mar. '66	1	4.20	13.10	tr.
				2	4.00	13.30	tr.
		Dry feces	May '67	1	3.80	13.15	tr.
				2	4.05	13.25	tr.
		Stored extract†	May '67	1	4.15	13.25	tr.
				2	4.00	13.40	tr.
		Stored ketonic esters‡	July '67	—	4.05	12.90	tr.
Normal	May '67		May '67	1	6.15	12.4	tr.
				2	6.30	12.7	tr.
				3	6.45	12.3	tr.
				4	6.10	12.5	tr.
				5	6.15	12.4	tr.
				6	6.10	12.5	tr.
After tetracycline	July '67		July '67	1	none	2.15	6.25
				2	none	1.85	6.40
				3	none	2.05	6.30
				4	none	1.75	6.25
				5	none	1.90	6.15
				6	none	2.15	6.15

* Subject A.V., both in the normal condition (fecal collections at 14-month intervals), and after tetracycline treatment (1 g daily for 4 days prior to analysis).
† Acetic acid–toluene extract, prepared Mar. '66.
‡ In acetone; prepared May '67.

of hydrolysis with KOH in ethylene glycol (20% w/v) at reflux temperature is shown in Fig. 2 for different conjugates. Cleavage was complete after less than 15 min; the $t^1/_2$ (hydrolysis with a large excess of alkali is a degraded-second order reaction) is apparently about 3 min for each type of conjugate. The reaction was followed by GLC of the methyl esters of free bile acids released, after addition of a known amount of 23-nordeoxycholic acid to the hydrolysates.

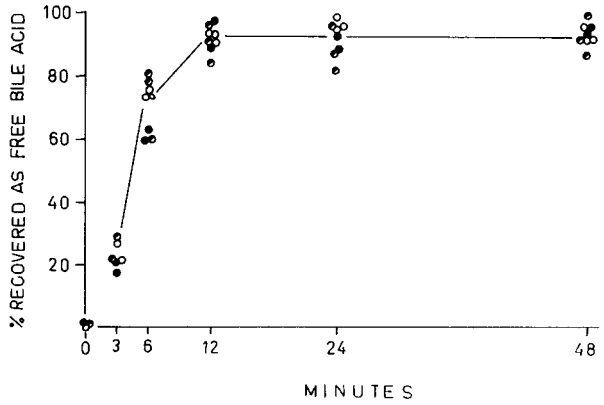

FIG. 2. Hydrolysis of conjugated bile acids (1 μeq each) with KOH in ethylene glycol (20% w/v, 1 ml) at reflux temperature. The time of reaction was measured from the insertion of the tubes in the dry bath, and thus includes the warm-up period. Closed circles, taurolithocholic acid; open circles, glycodeoxycholic acid; half-closed circles, sodium taurocholate.

The effect of the same reagent (1 ml; 20 min at reflux temperature) upon the recoveries of free bile acids (10–400 μg range) is shown in Table 5 (same internal standard technique). With the exception of somewhat low recoveries with the smaller samples of cholic acid (10 and 25 μg), the figures were satisfactory for each bile acid.

Liquid–Liquid Extractions

Our method includes three steps of liquid–liquid partition. The unsaponifiable matter (including the coprosterols) is first removed from the hydrolysate by conventional extraction with petroleum ether. The original viscous glycolic mixture is unsuitable for this procedure and requires additives (methanol and 20% NaCl). Efficient removal of neutral sterols with this system was demonstrated by the complete absence from the chromatogram of a peak corresponding to 3-keto-coprostane. Coprostanol is commonly present in large amounts in feces; it yields, on chromic acid oxidation, coprostanone (retention time relative to 3-ketocholanoate, 0.85; separation factor 1.18 on JXR at 250°C). A major product from dietary vegetable sterols, 24-ethylcoprostanol, would also yield a ketone distinguishable from the bile acids (retention time relative to 3,12-diketocholanoate, 0.89; separation factor 1.12).

Bile acids are extracted with diethyl ether. Although the solubility of cholic acid in diethyl ether is not high, recovery of the latter acid was adequate when extraction

TABLE 5 RECOVERY* OF HYDROXY BILE ACIDS AFTER TREATMENT WITH KOH IN ETHYLENE GLYCOL†

Added	Recovered		
	Lithocholic Acid	Deoxycholic Acid	Cholic Acid
		μg	
10.0	10.0	9.5	8.4
25.0	28.1	24.5	21.3
100	104	105	101
200	191	216	210
400	391	376	379

* Determined by GLC after methylation.
† 20% w/v, 1 ml; 20 min at reflux temperature.

was carried out in the presence of salt. The recoveries shown in Table 5 (and Fig. 2) include the effect of the extraction step.

A significant amount of glycol is carried over in the ether extract. Glycol does not interfere with the methylation, but is undesirable at the oxidation step, as it would consume a large portion of chromic acid. Therefore evaporation to dryness of the ethereal extract includes three successive additions of toluene to induce vacuum distillation of azeotropic mixtures.

Ether extraction of the products of chromic acid oxidation is discussed below.

Oxidation

The conditions adopted for nonselective chromic acid oxidation of hydroxyl groups were not found to be critical for routine work. As judged from gas chromatograms carried out at different reaction times with a column selective for keto groups (QF-1), oxidation was very rapid in 90% acetic acid; methyl dehydrocholate was formed from methyl cholate (10 μg–1 mg range) in less than 1 min, with no evidence of partial oxidation products. Recoveries of ketonic esters from the common bile acid esters were practically quantitative (internal standard technique). With the time of reaction selected for routine oxidations (15 min), wide fluctuations of temperature made little difference (10–25°C). The products were easily extracted with diethyl ether after the oxidation mixture had been quenched with 20% NaCl. A single addition of toluene to effect complete evaporation of solvents from the extract was necessary for the removal of the acetic acid. Reduction of excess chromic acid prior to extraction afforded no obvious advantage (at least when peroxide-free diethyl ether was used for extraction), and was therefore omitted. If preferred, the ether extract might be washed once with 20% NaCl (1 ml) containing 1% ascorbic acid.

Bile acid ketonic esters in the oxidation products from fecal samples, either dissolved in acetone or as the dry residue, kept well on storage at room temperature; identical analytical results were obtained after several months (see an example in Table 4).

Gas–Liquid Chromatography

Steroid ketones, in contrast to the corresponding hydroxy compounds, are known (15) to exhibit good GLC characteristics on the thin-film columns available to date. We determined on the flame instrument the relative responses (on a weight basis) for several bile acid ketonic esters including unsubstituted methyl cholanoate (Table 6); a commercially available, pretested packing (1% JXR) was used. Under the working conditions, long-chain *n*-alkanes (C_{24} to C_{36}) exhibited within a wide range of column loads (1 – 100 μg) practically identical responses on a weight basis. A simple relation was not, however, confirmed for the responses of the bile acid derivatives (Table 6). The response for the unsubstituted cholanoate was higher than expected (5) and varied in an apparently unpredictable fashion during column aging or with different columns. More consistent were the relative responses of the monoketone, diketone, and 23-nordiketone. The trend for the triketone was a lower and un-

TABLE 6 RELATIVE RESPONSES OF KETONIC BILE ACID METHYL ESTERS ON GLC

Methyl 5β-Cholanoate Derivative	Column Load	Relative responses*			Linear Range‡
		Column† A	Column B	Column C	
	μg				μg
Unsubstituted	2.5	1.41	1.30	1.23	n.d.
3-Keto	5.0	1.06	1.05	1.07	0.5–25
3,12-Diketo-23-nor	5.0	(1.00)	(1.00)	(1.00)	0.5–15
3,12-Diketo	10.0	1.02	1.00	0.99	0.5–15
3,7,12-Triketo	10.0	0.86	0.94	0.92	1–12

* On a weight basis, relative to methyl 3,12-diketo-23-nor-5β-cholanoate = 1.00; hydrogen flame detection; column temperature 250°C. The instrument showed, under the conditions used, a linear range (at least 0.5–30 μg) and identical responses for four reference *n*-alkanes.
† Glass columns, 150 cm × 4 mm I.D., 1 % JXR on Gas-Chrom Q, 100–120 mesh. A, recently installed, flow-conditioned; B, the same column after 3 months of use (about 400 injections of fecal products); C, different column, same packing batch, not flow-conditioned (10), after 3 wk of use.
‡ Determined with column A. The upper limit of range was taken as the load leading to unsymmetrical peaks.

stable response. However, the linear range for the latter was limited (1–12 μg). The linear range observed for the three former compounds might be considered satisfactory (15), if one takes into account that the upper limit (about 15 μg) is inherent in the low capacity of methylsiloxane-type columns with polar steroids.

The quality of chromatograms from fecal products was found to depend upon the mode of operation of the column. Ordinary isothermal chromatograms exhibited a slow return to the base line after the solvent peak, obviously an effect of the sample contaminants; the chromatograms, however, were utilizable. Tracings were considerably improved (Fig. 1) by the adoption of an initial lower isothermal period (175–180°C during 15–20 min) followed by temperature programming; this technique led to a total base line drift in the bile acid region of $< 5\%$ of the recorder span.

Specificity for Bile Acids

Peaks distinct from the four peaks of bile acid ketonic esters (Fig. 1) already mentioned, with the fecal specimens we have analyzed to date, accounted for less than 5% of peak areas in the bile acid region of the chromatogram. The fecal samples came from healthy volunteers on various ad lib. diets, and from hospital patients who were generally receiving drugs.

Nonbile acid compounds overlapping with the ketonic esters on the JXR column apparently did not occur, since similar chromatograms were regularly obtained on phases moderately (XE-60) or highly (QF-1) selective for keto groups. An assay of specificity, including the identification of the cholanoic acid skeleton, was based on observations that yields of unsubstituted cholanoic acid after Wolff–Kishner reduction of keto groups from different ketonic esters were similar. The reaction products from fecal samples were analyzed by GLC after methylation. Two peaks were recorded, with the retention times of methyl 23-nor-5β-cholanoate (from the internal standard) and of methyl 5β-cholanoate (derived from the total fecal bile acids). It could be calculated from peak-area ratios in both types of chromatograms available (ketonic esters and unsubstituted cholanoates), that the peaks measured as ketonic esters were derived solely from bile acids.

These findings, taken together, justify the omission of the classical steps of purification (5) of the bile acid fraction, including the removal of fatty acids. Chromatograms obtained after complete treatment, in the same way as feces, of saturated or unsaturated fats (20–50 mg samples) revealed no peaks in the bile acid region. Addition of corn oil to fecal samples did not affect the analytical results; two pertinent examples of the absence of interference from fats are shown in Table 3. We believe that both the last step of liquid–liquid extraction after oxidation, and the mode of operation of the chromatographic column may together explain the observed adequacy of an unpurified extract.

Over-All and Relative Recoveries of Bile Acids

For the three common bile acids and the internal standard used in the reference mixture (100–300 μg range), the recoveries as ketonic esters after complete processing averaged 80% of the theory; this was determined by precision injection of aliquots of the products and of reference ketonic esters, and comparison of the chromatograms.

Small, nonspecific losses are to be expected, in view of the relative complexity of manipulations. The method was, however, designed to correct automatically for such losses, starting from the early step of extraction with acetic acid, where an exactly known amount of internal standard is added. Volumetric work (except for the addition of the internal standard) is completely avoided by making all the determinations relative to the internal standard. The recovery figures that are of practical importance for a discussion of analytical results are thus those expressed (e.g. in percentages) relative to the recovery of the internal standard. Such figures for several individual bile acids are given in Table 7. The recoveries were generally satisfactory. Deoxycholic acid, as a true homologue of the internal standard, is the least likely to be affected by specific losses; recoveries significantly different from 100% would indicate that the considered bile acids are more or less affected by specific losses than is the internal standard. For moderate losses of this type, a correction factor was provided, as far as possible, in the calculation of the results (see Method of Analysis, $P_{\text{int.st.ref.}}/P_{\text{ref.}}$) by the use of peak ratios from

TABLE 7 RECOVERY, RELATIVE TO THE INTERNAL STANDARD OF BILE ACIDS LIKELY TO OCCUR IN FECAL SAMPLES

Bile Acid	Amount Treated	Methyl Cholanoate Derivative Formed	Relative Recovery
	μeq		%
5β-Cholanoic acid	0.23	Unsubstituted	120
Lithocholic acid	0.52	3-Keto	105
3β-Hydroxy-5β-cholanoic acid*	—	3-Keto	—
Deoxycholic acid	0.55	3,12-Diketo	102
Glycodeoxycholic acid	1.12	3,12-Diketo	105
3β,12α-Dihydroxy-5β-cholanoic acid*	—	3,12-Diketo	—
Methyl 3α-acetoxy-12-keto-5β-cholanoate	1.15	3,12-Diketo	112
3α-Hydroxy-7-keto-5β-cholanoic acid	1.25	3,7-Diketo	115
Cholic acid	1.50	3,7,12-Triketo	95
Sodium taurocholate	1.32	3,7,12-Triketo	92

* Qualitative analysis; indicated the formation of the expected ketone.

reference mixtures treated exactly like the sample by the same operator and on the same day. Of course, the reference standards must consist of only a few selected bile acids, which imperfectly represent the actual composition of a fecal sample.

Recoveries from Feces

We did not attempt in the present study to isolate quantitatively the individual fecal bile acids. Validity of the method is thus dependent upon the correctness of assumptions that in our judgment were reasonable. Fecal bile acids should be stable to alkaline treatment under our conditions; further, they should each yield upon oxidation one of the few ketones mentioned. This assumption seems reasonable for most of the compounds recently reported to occur in human specimens (6, 7).

A point of importance is that the yields of ketonic esters must be similar to amounts handled in model mixtures and must not vary with the presence of nonbile acid material extracted from feces in the acetic acid–toluene system. This was tested by determining the recovery of bile acids added to fecal samples (Table 3); the results appeared satisfactory.

Reproducibility; Daily Excretion Data

Table 4 reports analyses of specimens from a single subject (A. V., normal male adult, ad lib. diet). Two collections in the normal condition were obtained at 14-month intervals. A collection was also made after the subject had been given tetracycline by mouth (1 g daily during 5 days; feces collected the 4th day) for treatment of a local infection; the excretion of trisubstituted bile acids was obviously an effect of the treatment. From the early collection, samples were stored (dry powder and acetic acid–toluene extract) and again analyzed 14 months later; from the latter analysis a sample of the ketonic esters in acetone was stored and again chromatographed two months later. The good reproducibility of the method is evident in Table 4; we prefer not to make a statistical evaluation before the more important investigation of interlaboratory comparison has been undertaken. It may be mentioned, for the sake of comparison with other workers' methods and results, that the average daily excretion figure found within a group of five healthy adults (ad lib. diets), was 218 mg/24 hr of bile acids (range, 127–290 mg).

DISCUSSION

The unusual procedure for extraction of bile acids from feces is not considered an essential feature of the present method. However, we found it inconvenient to carry crude feces (5) through alkaline hydrolysis, as this invariably resulted in the formation of dark products (probably partly from degradation of carbohydrates). The observed efficiency of nonaqueous extraction of bile acids with acetic acid was not unexpected since the latter is a powerful general solvent although a mild reagent (it may be noted that cholesterol is partly acetylated by the procedure). With such a solvent one can avoid acidifying the feces with mineral acids (for instance, to liberate bile acids from their calcium salts). Finally, the virtually complete suppression of dissociation of taurine-conjugated bile acids in acetic acid favors their solubility in nonaqueous mixtures.

Brief hydrolysis of the extract with glycolic alkali at high temperature is not claimed to be superior over prolonged autoclaving (5), although it is more convenient and may suppress the release of silicate from glass vessels (9).

Removal of neutral sterols from the hydrolysate appeared satisfactory and was tested in each determination by examination of the coprostanone area on the chromatogram.

Extraction of bile acids with diethyl ether has been widely used in the past (9); faulty technique at this step (e.g., insufficient shaking during extraction), which causes low general recoveries or specific losses, can be detected on the reference chromatograms.

Oxidation of fecal bile acids disguises the occurrence of epimers and the diversity of functional groups. The accompanying loss of resolution turns out to be an advantage, since routine quantification of the complex mixture by high-resolution GLC (4) would, in view of the complexity of the pattern, be a laborious task. A few stable oxidation products, including that formed from a homologous internal standard, are separated on the chromatogram with high efficiency, which allows for the detection of any nonbile acid contaminants. By contrast, rather poor information on whether bile acids specifically are being determined can be obtained from the chromatogram of the trimethylsilyl ethers of the intact bile acids (5).

The use of 23-nordeoxycholic acid as internal standard offers further advantages. Apart from the elimination of volumetric work during routine analysis, it is possible to carry out identification tests with the oxidation products while preserving the original relationship between the internal standard and the fecal bile acids. The reduction of keto groups has been mentioned; in this test the difference by one methylene unit in the side-chain between the internal standard and the natural bile acids was fully exploited. The use of an internal standard that is practically indistinguishable from the sample through both the physical separations and chemical transformations of the procedure is also a valuable feature.

GLC of bile acid ketonic esters on the selective phase QF-1 has proved useful for identification work (10). Quantification of mixtures of these compounds has been approached by Grundy et al. (5), though in a different context. The only documented report, to our knowledge, mentioning a very small column adsorption effect with steroid ketones was that of Horning, Maddock, Anthony, and Vanden Heuvel (15), but their discussion was restricted to the case of cholestanone. Menini and Norymberski (21) have determined quantitatively di- and triketones of the androstane series and 5β-pregnane-3,20-dione on an SE-30 (1%) column; they reported an accuracy of 5% within the range 0.5–1.5 μg, using peak heights. Our results concerning the responses and linear ranges apparently have no counterpart in the available literature; they indicate the feasibility of quantitative GLC of bile acid ketonic esters, perhaps with reservations concerning the triketone.

A satisfactory separation of the products from human feces on the JXR phase was illustrated in Fig. 1. On the selective phases (XE-60 and QF-1) the C_{23} internal standard shifts on the chromatogram in the direction of the C_{24} diketone, while the triketone is considerably retarded. It was possible by temperature programming a compound column (25 cm of XE-60 following 135 cm of JXR, both 1%) to achieve nearly equal spacing of the four peaks and very rapid analysis. However, we preferred to use a JXR column with high column efficiency.

The failure to separate the 3,7-diketone from the 3,12-diketone (10) might be overcome after formation of partial dimethyl hydrazones (10) from the crude oxidation products; the reaction was found to be specific and suitable for quantification. However, such complication of the method seemed unnecessary. Conventional relative retention times were not determined in the present study, because some kind of temperature programming was used in all separations.

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References

1. Bloomfield, D. K. 1962. *Anal. Chem.* **34:** 737.
2. Makita, M., and W. W. Wells. 1963. *Anal. Biochem.* **5:** 523.
3. Eneroth, P., K. Hellström, and J. Sjövall. Cited by H. Danielsson. 1963. *Advan. Lipid Res.* **1:** 359.
4. Kuksis, A. 1965. *J. Am. Oil Chemists' Soc.* **42:** 276.
5. Grundy, S. M., E. H. Ahrens, Jr., and T. A. Miettinen. 1965. *J. Lipid Res.* **6:** 397.
6. Eneroth, P., B. Gordon, R. Ryhage, and J. Sjövall. 1966. *J. Lipid Res.* **7:** 511.
7. Eneroth, P., B. Gordon, and J. Sjövall. 1966. *J. Lipid Res.* **7:** 524.
8. Danielsson, H. 1963. *Advan. Lipid Res.* **1:** 335.
9. Sjövall, J. 1964. *Methods Biochem. Analy.* **12:** 97.
10. Sjövall, J. 1964. *In* Biochemical Applications of Gas Chromatography. H. A. Szymanski, editor. Plenum Press, New York. 151–167.
11. Kuksis, A. 1966. *Methods Biochem. Analy.* **14:** 325.
12. Anderson, I. G., G. A. D. Haslewood, and I. D. P. Wootton, 1957. *Biochem. J.* **67:** 323.
13. *Organic Syntheses*. 1955. John Wiley & Sons Inc., New York. 1st edition Collect. **3:** 234.
14. Hofmann, A. F. 1963. *Biochem. J.* **89:** 57.
15. Horning, E. C., K. C. Maddock, K. V. Anthony, and W. J. A. VandenHeuvel. 1963. *Anal. Chem.* **35:** 526.
16. Jeffay, H., and J. Alvarez. 1961. *Anal. Chem.* **33:** 612.
17. Norman, A. 1955. *Arkiv Kemi.* **8:** 331.
18. Samuelsson, B. 1959. *Acta Chem. Scand.* **13:** 236.
19. Reichstein, T., and M. Sorkin. 1942. *Helv. Chim. Acta.* **25:** 797.
20. Huang-Minlon. 1949. *J. Am. Chem. Soc.* **71:** 3301.
21. Menini, E., and J. K. Norymberski. 1965. *Biochem. J.* **96:** 1.