General methods for the analysis of metabolic profiles of bile acids and related compounds in feces

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Abstract A general method is described for the detailed qualitative and quantitative analysis of bile acids and related compounds from feces. The technique utilizes a novel combination of liquid–gel and liquid–solid extraction, lipophilic ion exchange chromatography, and capillary column gas–liquid chromatography coupled to mass spectrometry, which permits the detailed composition of bile acids in feces in terms of both the individual bile acids present and their mode of conjugation in the original fecal sample. The extraction, purification, and isolation procedures have been evaluated using fecal samples containing endogenous radioactive bile acid metabolites and from the addition of radiolabeled standards to fecal homogenates. The applicability of the general procedure is illustrated with examples from the analysis of bile acids and sterols in the feces collected from normal healthy subjects, patients with chronic diarrhea, and an adult female Sprague-Dawley rat. The flexibility of the method, and the general problems encountered in the extraction, purification, and isolation of bile acids and related classes of compounds from feces for subsequent analysis of gas–liquid chromatography are discussed in detail.—**Setchell, K. D. R., A. M. Lawson, N. Tanida, and J. Sjövall.** General methods for the analysis of metabolic profiles of bile acids and related compounds in feces. *J. Lipid Res.* 1983. **24:** 1085–1100.

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The literature relating to the measurement of bile acids in feces is vast, yet in spite of recent advances in the use of lipophilic gel chromatography, liquid–solid extraction techniques, and high resolution glass capillary column gas–liquid chromatography (GLC), many of the methods currently in use are relatively crude and represent very little advance upon the techniques developed in the 1960's (1–6). Moreover it is clear from several studies (3, 4, 7) that the composition of fecal bile acids and sterols is by no means as simple as many investigations seem to imply.

In recent years there has been a renewed interest in the measurement of bile acids in feces. This is due to the possible influence of cholesterol and bile acid metabolism on diseases such as atherosclerosis, colo-rectal cancer, and disorders associated with a changed intestinal microflora. In these conditions and others that have an effect upon the enterohepatic circulation, changes may occur in the qualitative and quantitative composition of bile acids excreted in the feces. We have therefore evaluated the application of more recent methodology to the analysis of bile acids and related compounds with the aim of providing generally applicable and convenient methods for detailed analysis of a variety of lipophilic compounds in feces. Outlined in this communication is the analytical procedure that has been developed, a discussion of the problems associated with the analysis of fecal samples, and examples of the application of the techniques.

Abbreviations and trivial names: coprostanol, 5β-cholestane-3β-ol; β-sitosterol, 5-stigmastene-3β-ol; β-sitostanol, 5α-stigmastane-3β-ol; lithocholic, 3α-hydroxy-5β-cholanoic; chenodeoxycholic, 3α,7α-dihydroxy-5β-cholanoic; ursodeoxycholic, 3α,7β-dihydroxy-5β-cholanoic; deoxycholic, 3α,12α,dihydroxy-5β-cholanoic; cholic, 3α,7α,12α-trihydroxy-5β-cholanoic; α-muricholic, 3α,6β,7α-trihydroxy-5β-cholanoic; β-muricholic, 3α,6β,7β-trihydroxy-5β-cholanoic; ω-muricholic, 3α,6α,7β-trihydroxy-5β-cholanoic. Prefixes glyco and tauro are used for bile acids having glycine and taurine in amide linkage at C-24. TMS, trimethylsilyl; GLC, gas–liquid chromatography; MS, mass spectrometry.

MATERIALS AND METHODS

Solvents and reagents

All solvents were of analytical grade and distilled twice. Water was distilled twice in an all-glass distillation apparatus. Cholylglycine hydrolase from *Clostridium perfringens* (Welchii) 250 units/mg protein (Biuret) was purchased from Sigma, St. Louis, MO.

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Reference bile acids

The following radiolabeled compounds were obtained from the Radiochemical Centre, Amersham, UK: [24-^{14}C]lithocholic acid 59 mCi/mmol, [24-^{14}C]cholic acid 52 mCi/mmol, [24-^{14}C]taurocholate 59 mCi/mmol, [1-^{14}C]glycocholate 51 mCi/mmol, [1-^{14}C]palmitic acid 57 mCi/mmol, and [4-^{14}C]cholesterol 57.8 mCi/mmol. The methyl and ethyl esters of [24-^{14}C]lithocholic acid were prepared from the parent radiolabeled bile acid (8). [^{3}H]Chenodeoxycholate-3-sulfate was a gift from Dr. T. C. Bartholomew (Royal Free Hospital, London, UK).

Column materials

Lipidex-DEAP (diethylaminohydroxypropyl Sephadex LH-20) was purchased from Packard-Becker, Groningen, Netherlands or synthesized from Sephadex LH-20 (9). The ion exchanger (100 g) was transferred to a sintered funnel and washed with 500-ml volumes of methanol followed by 72% ethanol and converted to the acetate form by washing with 0.1 M acetic acid in 72% ethanol followed by 72% ethanol until neutral.

SP-Sephadex was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden) and 50-g quantities were successively washed with 500-ml volumes of 72% methanol, 0.1 M HCl in 72% methanol, and 72% methanol to convert it to the [H^+] form prior to use.

Lipidex 1000 was purchased from Packard-Becker (Groningen, Netherlands) and washed with methanol prior to use. The gel was allowed to swell in methanol and slurried into glass columns. A bed size of 5 × 1 cm was prepared and the gel was washed with distilled water (20 ml) before application of the sample.

Bond Elut cartridges (reversed phase octadecylsilane bonded silica) were obtained from Analytichem International (Harbor City, CA) and from Jones Chromatography Ltd., (Llanbradach, Mid Glamorgan, Wales); they were washed with methanol (5 ml) and distilled water (5 ml) prior to use.

Collection and homogenization of stools

Stools were collected for 4 consecutive days from healthy hospital staff members and two patients (J and A) with chronic diarrhea of unknown etiology. Collections were also made from several patients with gallstones who had been given orally 5 μCi each of [^{14}C]cholic acid and [^{14}C]chenodeoxycholic acid.

Stools were collected directly into preweighed polythene bags and stored at −20°C until required for analysis. The samples were pooled, allowed to thaw at 4°C, and homogenized within 2 hr with cold distilled water. Homogenization was carried out in steps using a Stomacher 3500 (Colworth, Sharnbrook, Bedfordshire, UK) (10). First, an equal volume of water was added to the stools and the sample was homogenized for 3–5 min. The volume was then made up to approximately 500 ml and homogenized for a further 3 min, after which 500 ml of water was added and the homogenization was repeated. The homogenate was finally made up to 2500 ml with water and homogenized for 3 min. Immediately following homogenization, 25-ml volumes (equivalent to 1/100th samples) were removed and either analyzed directly or stored at −20°C until required for analysis.

Analytical procedures

The general scheme of the method employed for the analysis of fecal bile acids is illustrated in **Fig. 1.**

Extraction of bile acids. The homogenized fecal sample (5 ml), equivalent to 1/500th of a daily collection, was added dropwise to 45 ml of ethanol in a round-bottomed flask and held in an ultrasonic bath. Following sonication for 30 min the sample was refluxed for 2 hr. The sample was cooled, transferred to tubes, and centrifuged at 3500 rpm for 10 min. The supernatant was removed and retained. The pellet was resuspended in 80% aqueous ethanol (50 ml), transferred to the original flask, and refluxed for a further 2 hr. The sample was again centrifuged and the supernatant was removed and retained. The pellet was finally resuspended in 50 ml of chloroform–methanol 1:1 (by vol) and refluxed for 1 hr. The sample was centrifuged and the supernatant was removed. The residue was then transferred to a filter paper and washed with a small volume of chloroform–methanol 1:1 (by vol) to minimize any losses. The chloroform–methanol extract was transferred to a clean flask and taken to dryness on a rotary evaporator. The ethanolic supernatants were then added to the same flask and the pH, which is sometimes slightly acidic, was adjusted to neutrality with a few drops of 4 N sodium hydroxide and the combined extracts were evaporated to dryness on a rotary evaporator.

Liquid–gel/liquid–solid extraction. The dried extract was resuspended in 5 ml of 0.01 N HCl by sonication for 5 min. The suspension was filtered through the bed of Lipidex 1000 and the effluent was collected. The flask was washed three times with 0.01 N HCl (5 ml) and the washings were passed through the gel bed which was then washed with distilled water (20 ml). The effluent and washings were pooled and passed through a Bond Elut cartridge and discarded. Bile acids and sterols were recovered by elution of the Lipidex gel bed with 20 ml of methanol and of the Bond Elut cartridge with 5 ml of methanol and these extracts were combined.

Cation exchange chromatography. The methanolic extract was passed through a column of SP-Sephadex prepared in the[H^+] form and packed in 72% methanol

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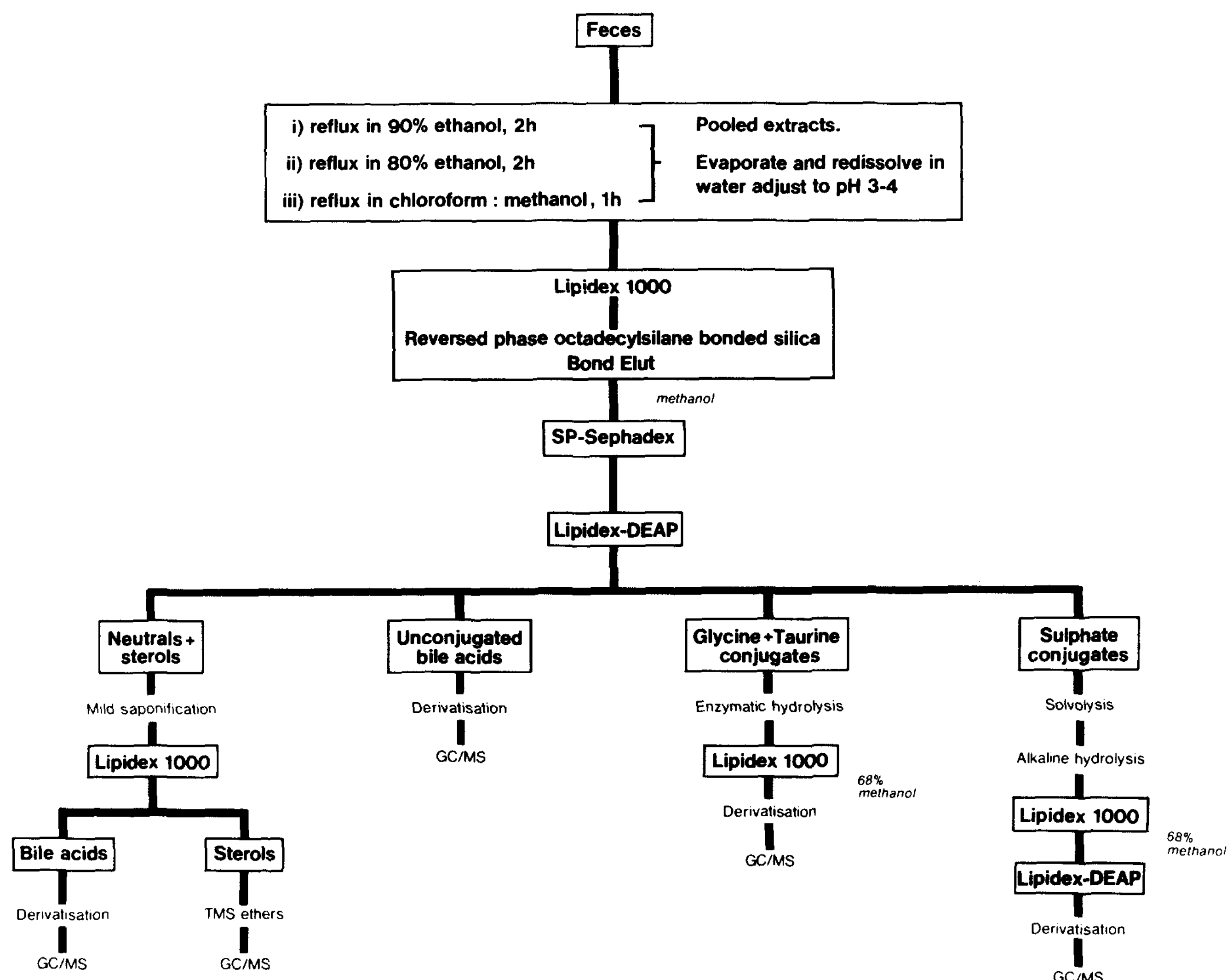

Fig. 1. Scheme of method for detailed analysis of the composition of bile acids, neutral steroids, and related compounds in feces.

(bed size 5 × 1 cm). The column was washed with 20 ml of 72% methanol which was added to the sample effluent. When necessary, the pH was brought to neutrality with sodium hydroxide.

Group separation of bile acids by anion exchange chromatography on Lipidex-DEAP. Bile acids were fractionated into groups, based upon their mode of conjugation, using a lipophilic anion exchanger (9). The combined neutralized effluent from the cation exchanger was applied directly to a column of Lipidex-DEAP (0.6 g) prepared in the acetate form and packed in 72% methanol (bed size 13 × 0.4 cm). The sample was eluted under a pressure of nitrogen gas (0.5 $kg \cdot cm^{-2}$) which gave a flow rate of ca. 25 $ml \cdot hr^{-1}$. The flask and Lipidex-DEAP column were washed with 2 × 5 ml of 72% ethanol and the sample and washings were combined to give a fraction comprising neutral compounds including sterols and any esterified (C-24) bile acids. The stepwise elution of *i*) unconjugated bile acids, *ii*) glycine conjugates, *iii*) taurine conjugates, and *iv*) total sulfates, was achieved essentially as described by Almé et al. (9). Alternatively, after collection of the neutral compounds, the fecal bile acids may be fractionated into groups: unconjugated ones, the glycine + taurine, and sulfate conjugates using the solvents listed in **Table 1.** Since conjugated bile acids normally comprise only a small proportion of the total bile acids present in feces, this alternative fractionation considerably reduces the time required for the analysis.

Saponification of neutral fraction. The neutral fraction, which may contain bile acids with an esterified carboxyl group, was subjected to mild saponification. Sodium hydroxide pellets (2.5 g) were dissolved in the neutral extract using a sonic bath to give a final concentration

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TABLE 1. Solvent system for the fractionation of bile acids on Lipidex-DEAP

Fraction	Acetate Concentration[a]	'Apparent'[b] pH	Applied[c] Volume
Neutral compounds and sterols	0	neutral	(25 ml)[d] 9 ml
Unconjugated bile acids	0.1M	4.0	6.5 ml
Glycine and taurine conjugates	0.15M	6.4	6.5 ml
Sulfate conjugates	0.3M	9.6	10 ml

[a] The buffers were made from 72% (v/v) aqueous ethanol to which acetic acid was added to give the appropriate molarity.
[b] Measured with a glass electrode. The pH was adjusted by addition of concentrated ammonium hydroxide to the solution of acetic acid in ethanol.
[c] Between each change of solvent, 2 ml of 72% ethanol was added to wash out residual buffer from the gel.
[d] The total sample was applied directly following the cation exchange chromatography and the column was washed with 72% ethanol.

of 5% in 50 ml of 72% methanol. Hydrolysis was carried out at 60°C for 3 hr after which the solution was cooled in an ice bath and the pH was adjusted to neutral by the dropwise addition of concentrated HCl (approx 5.2 ml). The methanol was removed using a rotary evaporator. Distilled water (20 ml) was added, the pH was adjusted to 3–4 with HCl, and the solution was passed through a small column of Lipidex 1000 prepared in water as described above. The column was washed with 0.01 N HCl (20 ml) followed by distilled water (20 ml). Bile acids were recovered from the gel by elution with 68% methanol (20 ml), and neutral sterols by elution with methanol (20 ml). The fractions were taken to dryness, redissolved in methanol (2 ml), and transferred to a small vial to be derivatized for GLC and GLC–MS analysis.

Hydrolysis of glycine and taurine conjugates. The glycine and taurine conjugated fractions were hydrolyzed enzymatically using cholylglycine hydrolase. Each fraction was taken to dryness and lyophilized overnight. The extract was redissolved in 0.1 M phosphate buffer, pH 5.6 (5 ml), 25 units of enzyme were added, and the sample was incubated at either 37°C overnight or 55°C for 4 hr. Following hydrolysis, the pH was adjusted to 3–4 and the solution was passed through a column of Lipidex 1000. The sample tube and column were washed with 3 × 5 ml 0.01 N HCl followed by distilled water (20 ml). Bile acids were recovered by elution with methanol (20 ml). The extracts were derivatized and analyzed by GLC and GLC–MS.

Solvolysis and hydrolysis of bile acid sulfates. Sulfated bile acids were first solvolyzed and then hydrolyzed using strong alkaline conditions. The sulfate fraction was taken to dryness using a rotary evaporator and the residue was lyophilized overnight to remove any traces of ammonium acetate. Two solvolytic procedures were employed *1)* methanol–acetone–hydrochloric acid (11), or *2)* ethyl acetate–ethanol–sulfuric acid (12). In the former case the residue was dissolved in anhydrous methanol (1 ml) and anhydrous acetone (9 ml) to which three drops of 6 N HCl were added. The sample was incubated at 39°C for 16 hr (overnight). The solution was then neutralized by addition of 6 N sodium hydroxide and taken to dryness on a rotary evaporator. In the second method, the residue was dissolved in 2 ml of ethanol, and ethyl acetate (18 ml) and 4 N H_2SO_4 (0.5 ml) was added. After mixing in a sonic bath, the sample was left at 39°C for 16 hr. The solution was neutralized by addition of approximately 2.5 ml of 4 N sodium hydroxide and taken to dryness using a rotary evaporator.

Irrespective of the method of solvolysis, the dried extract was then redissolved in 3 × 5 ml of 4.5 N sodium hydroxide, transferred to a Teflon-lined stainless steel digestion vessel, and hydrolyzed at 130°C for 4 hr. After cooling, the hydrolysate was neutralized with 4 N HCl, diluted with distilled water (20 ml), and the pH was adjusted to 3–4. The solution was then passed through Lipidex 1000 as described to extract bile acids, which were then recovered with methanol (20 ml). The extract was dried and transferred in methanol (2 ml) to a small vial for derivatization.

Derivatization. After addition of the internal standard, 5β-cholestane-3β-ol (1–30 μg depending upon the fraction being analyzed), to the bile acid fractions and 5α-cholestane (20 μg) to the neutral fraction, the samples were taken to dryness under nitrogen. Methyl ester–trimethylsilyl ether derivatives were prepared essentially as described by Fales, Jaouni, and Babashak (8) using diazomethane freshly prepared from the reaction

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between sodium hydroxide and N-methyl-N-nitroso-*p*-toluenesulfonamide (Diazald, Aldridge Chemical Co., Gillingham, UK). Excess reagents were removed under nitrogen and the trimethylsilyl ether derivative was prepared by the addition of 50 μl of a mixture of pyridine–hexamethyldisilazane–trimethylchlorosilane 3:2:1 (by vol). The derivatized sample may be further purified if necessary by passage through a small column of Lipidex 5000 as described elsewhere (13).

Gas–liquid chromatography (GLC) was performed on two instruments: *i)* a Pye 106 gas chromatograph modified to accept a 25-metre Silicone OV-1 wall-coated open tubular glass capillary column (Jaeggi, Trogen, Switzerland), and *ii)* a Carlo Erba 2960 gas chromatograph having an identical column. Both were equipped with an all-glass solid injection device of the type described by Van den Berg and Cox (14). Helium was used as the carrier gas and the flow rate through the column was approx 2 ml $\cdot$ min^{-1}. After an initial isothermal period of 5 min at 220°C, temperature-programmed operation from 220°C to 285°C with increments of 2°C/min was carried out.

Gas–liquid chromatography–mass spectrometry (GLC–MS) was carried out using two types of systems and the conditions have been reported elsewhere (9, 15).

RESULTS AND DISCUSSION

Collection and homogenization of stools

While many methods have been described for the estimation of bile acids in feces, the choice of procedures employed for the collection, storage, sampling, and the initial treatment of the fecal extract will always be important to the meaningful interpretation of the final data. To minimize possible bacterial degradation of bile acid structures following collection, the stools were immediately stored at −20°C. It is recommended that their homogenization be carried out as quickly as possible at low temperature, using cold distilled water.

Since only 1/500th of the daily fecal collection, and in some instances less, was taken for analysis, the method of homogenization becomes important and will have a bearing on the precision and accuracy. Based on experience in measuring the radioactivity in fecal homogenates after the [1-^{14}C]cholylglycine breath test (16), homogenization is best achieved in a stepwise manner by diluting with water. After rapidly removing the required proportion of the homogenate, it was either analyzed immediately or stored at −20°C. The storage of fecal samples, although often unavoidable, leads to questions concerning the stability of endogenous compounds. Fecal fat and lipids have been shown to be stable in feces for up to 48 hr when stored at 4°C, however, the hydrolysis of fatty acid esters will occur at this temperature. Thus, if free fatty acids or lipid classes are to be quantified, samples should be frozen immediately (17). In a limited study it has been shown that the composition of bile acids in feces stored at −20°C was similar to that for feces that were homogenized in alcohol and stored at 0°C (18). In cases where fecal samples are to be stored for long periods before analysis, it may be preferable to perform the initial extraction step and store the dried extract.

Extractions

To meet our objective of an "unbiased" technique, an initial extraction method was required that would quantitatively extract bile acids and related compounds and at the same time produce little or no artificial transformation of the compounds present. Many methods have been described for the extraction of bile acids from fecal samples; however some of these have the disadvantage that either artefacts may be formed during the procedure, or that a modification of the initial composition of the bile acid profile takes place.

The alkaline ethanol extraction described by Grundy, Ahrens, and Miettinen (1), and modifications of this method, leads to hydrolysis of bile acid esters, which have been shown to comprise a small but significant proportion of the bile acid metabolites in feces (19–21). Acetic acid–toluene has also been employed (2), however, the formation of acetoxy derivatives and partial solvolysis of sulfated bile acids cannot be excluded when this method is used. Prolonged refluxing with chloroform–methanol may also lead to esterification of bile acids (5), as does extraction with methanol–hydrochloric acid (22).

Although several unidentified esterified bile acids were shown to be present in fecal extracts (19, 20), in these studies the bulk of the bile acids were extracted first with acetone, to minimize the possibility of these occurring artificially in the prolonged reflux with chloroform–methanol which followed.

Based upon the above observations, bile acids and related compounds were extracted from small proportions (1/500th–1/1000th) of aqueous homogenates of feces by sequential refluxing in 90% ethanol, 80% ethanol, and chloroform–methanol 1:1 (by vol), the whole procedure requiring approximately 1 day to complete. The efficiency of the extraction method was assessed from recoveries of radiolabeled bile acids and their conjugates that were added to the fecal homogenates and from the analysis of feces that contained "endogenous" radioactive bile acid metabolites derived from administered radiolabeled cholic and chenodeoxycholic acids. As seen in **Table 2,** and confirming previous observations (5), the recovery of exogenous trac-

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TABLE 2. Recovery of radioactivity by the initial extraction procedure

Samples	Percent of Added Radioactivity in Extract			
	90% Ethanol	80% Ethanol	Chloroform–Methanol (1:1)	Total[a]
Feces containing endogenous radiolabeled bile acids				
Subject 1	76.0	11.1	11.8	98.9
Subject 2	78.5	7.9	11.1	97.5
Subject 3	89.9	5.9	1.6	97.4
Subject 4	85.0	6.2	8.4	99.6
Radiolabeled compounds added to fecal homogenate[b]				
Lithocholic acid	100.1	4.1	0.1	104.3
Cholic acid	89.8	5.9	0.7	96.4
Glycocholic acid	100.5	3.9	0.7	105.1
Taurocholic acid	94.7	2.5	0.1	97.3
Chenodeoxycholic acid-3-sulphate	94.0	4.2	0.2	98.4
Cholesterol	91.1	8.0	1.4	100.5

[a] Remaining radioactivity was found in the residue after chloroform–methanol reflux.
[b] Mean of two experiments.

ers does not give a true reflection of the efficiency of the extraction procedure for endogenous bile acids. To facilitate a complete recovery of the latter, the fecal residue was finally refluxed for 1 hr in chloroform–methanol, and this extract yielded a variable but significant amount (1.6–11.8%) of radioactivity (Table 2). A similar, but more time-consuming procedure, was reported for the extraction of radiolabeled bile acids from rat feces by Gustafsson and Norman (23). In their study the chloroform–methanol extract was found to contain 2.6–6.7% of the radioactivity from the feces.

The quantitative recovery of radioactivity from fecal samples from subjects given labeled cholic and chenodeoxycholic acid indicates that the extraction procedure is satisfactory for metabolites of these bile acids. Although the assessment of the efficiency of the initial extraction procedure was limited to C-24 bile acids and cholesterol, the procedure would also be expected to be satisfactory for related compounds such as bile alcohols, as well as other neutral and acidic steroids. The possible formation of small amounts of bile acid esters is discussed below. Finally, it should be mentioned that if bile acid sequestrants, such as ion exchangers, have been given to the subjects studied, the extraction method has to be modified accordingly.

Desalting and purification of the extract by Lipidex 1000 and reversed phase octadecylsilane-bonded silica (Bond Elut)

The objective of this stage of the method was to provide a rapid means of recovering the wide range of nonpolar and polar lipophilic compounds from the fecal extract, while at the same time eliminating inorganic salts, water-soluble polar metabolites, and some contaminants such as pigments. The lipophilic gel, Lipidex 1000, has previously been shown to extract nonpolar and medium polarity steroids and bile acids from aqueous solutions (24, 25). Reversed phase octadecylsilane-bonded silica cartridges, which are not suitable for the extraction of nonpolar lipids from aqueous solutions, efficiently extract more polar amphiphilic compounds from biological fluids (26–31). In the present procedure the two materials are used in series so that the polar lipophilic compounds that escape extraction by Lipidex 1000 are trapped by the Bond Elut cartridge. The complete removal of all compounds of interest is not possible by either technique alone (**Table 3**).

Following the initial solvent extractions, therefore, the residue is resuspended in 0.01 N HCl using an ultrasonic bath to obtain adequate dispersion. The aqueous suspension is then rapidly percolated through a small column of Lipidex 1000 and the effluent is passed through a Bond Elut cartridge. Only trace quantities of radioactivity remain in the flask after reconstitution of the dried extract in acidic water (Table 3), and bile acids and sterols are recovered from the adsorbents by elution with methanol.

The recoveries of radiolabeled bile acids added to fecal extracts were quantitative and the distribution of the different compounds between the two adsorbents is indicated in Table 3. With the exception of taurine-conjugated bile acids and occasionally a proportion of glycine conjugates that remain in the aqueous phase, Lipidex 1000 efficiently extracts most of the groups of compounds of interest from the acidified aqueous ex-

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TABLE 3. Distribution of radiolabeled compounds added to fecal extracts and extracted using Lipidex 1000 and Bond Elut

	Percent of Added Radioactivity[a] Recovered in:				
		Lipidex 1000		Bond Elut	
Compound	Residue[b]	Aqueous	Methanol	Aqueous	Methanol
Cholesterol	3.2		95.5		
Palmitic acid	1.5		95.8		
Ethyl lithocholate	1.0		98.7		
Lithocholic acid	2.0		98.4		
Cholic acid	1.0	1.0	100.3		1.0
Glycocholic acid	1.0	3.0[c]	93.0		2.5
Taurocholic acid		98.2			98.9
Lithocholic acid sulfate			105.5		

[a] Mean values from six separate analyses.
[b] The radioactivity (%) remaining following resuspension of the dried extract in acidic water.
[c] This value was occasionally as high as 23%; glycocholic acid was then extracted by the Bond Elut cartridge.

tract and these data are in agreement with those reported previously (25). Surprisingly, lithocholic acid sulfate was retained by Lipidex 1000. In agreement with previous reports (30, 31), taurine-conjugated bile acids are quantitatively extracted by Bond Elut. Thus, unless unknown forms of highly polar bile acid derivatives exist, the aqueous effluent that is finally discarded contains only salts and unwanted contaminants.

The application of the method to the analysis of fecal extracts containing a large amount of fat, e.g., from patients with steatorrhea, has not yet been studied. Problems may arise in obtaining an adequate dispersion of the extract in water. In such cases is may be possible to add Lipidex 1000 directly to the aqueous suspension in the flask and then pour the mixture onto a column of Lipidex 1000 (32). Since triglycerides and cholesteryl esters are not eluted from Lipidex with methanol, their presence in large amounts may affect the quantitative elution of bile acids. This should be checked in the individual case. Elution of total lipids is achieved however with 20 ml of chloroform–methanol (32).

Ion exchange chromatography

Bile acids were separated into groups according to mode of conjugation using the lipophilic anion exchange diethylaminohydroxypropyl Sephadex LH-20, (Lipidex-DEAP) (9). The sample is first passed through SP-Sephadex in $[H^+]$ form in order to remove organic cations and to obtain the free bile acids. This ion exchanger appears to effect a greater purification of the sample, removing a higher proportion of coloration from the extract than Amberlyst A-15 previously used. It should be stressed that the effluent from the cation exchanger must not be too acidic when applied to the anion exchanger, since this will prevent uptake of unconjugated bile acids and affect the separation of the conjugates. The pH should therefore be checked and adjusted to neutrality before application of the sample.

The capacity of the anion exchange column was 0.8 mEq. The major anions present in the desalted fecal extracts will be those of fatty acids accounting for ca. 2.5 mEq per day (assuming fecal fat of 7 g/day and mol wt of 280) and unconjugated bile acids. Part of the fatty acids will be present as esters. Thus, the capacity of the column should be sufficient to handle 1/100th daily fecal samples from normal subjects. In cases of patients with steatorrhea or diarrhea however, it may be necessary to analyze a smaller proportion of feces or to use a larger ion exchange column.

The recovery of a number of selected radiolabeled bile acids, cholesterol, and palmitic acid added to fecal homogenates and separated on Lipidex-DEAP was found to be satisfactory (**Table 4**). Cholesterol, representative of a neutral sterol, was quantitatively recovered in the neutral fraction while palmitic acid appeared in the fraction of unconjugated bile acids. These data provide evidence that bile acid conjugates were unchanged after the initial extraction and chromatographic procedures. The small percentage of radioactivity recovered in the neutral fraction when cholic acid was added to fecal homogenates might indicate esterification of the carboxyl group during the initial extraction. Radiolabeled ethyl lithocholate added to the fecal homogenate was quantitatively recovered in the neutral fraction.

Neutral fraction. This fraction will contain many classes of compounds, e.g., glycerolipids, sterols and their esters, and glycosides and bile acids, having an esterified carboxyl group (21). Although the latter may normally be present only in small amounts, mild sapon-

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TABLE 4. Recovery and distribution of radiolabeled compounds added to feces, extracted, and separated according to their charge and state of conjugation

Compound	Extract[a]	Percent of Added Radioactivity Recovered in Lipidex-DEAP fractions			
		Neutral	Unconjugated	Glyco + Tauro	Sulfates
Lithocholic acid	98.4	2.1	96.7		
Cholic acid	98.2	5.3	93.7	1.0	
Glycocholic acid	95.5			98.3	1.1
Taurocholic acid	98.9		0.9	96.4	1.4
Chenodeoxycholic acid 3-sulfate	96.9				95.2
Ethyl lithocholate	94.0	92.1			
Cholesterol	95.1	92.1			
Palmitic acid	96.8		97.1		

[a] Recovery of radiolabeled compounds after the Lipidex/Bond Elut stage and prior to ion exchange chromatography. Mean of three experiments for each radiolabeled compound.

ification will permit their separate analysis following isolation from the bulk of monohydroxylated sterols using Lipidex 1000 in a reversed phase mode. By the appropriate choice of aqueous methanol for elution of the gel, separate fractions containing bile acids, fatty acids, and monohydroxylated sterols can be obtained (**Fig. 2**). However, bile acids without hydroxyl groups, such as the cholenoic acid formed from lithocholic acid sulfate (33), will co-elute in the region of fatty acids. Furthermore, dihydroxylated sterols will be eluted prior to monohydroxylated ones, since the separation is based on reverse phase partition (Fig. 2). In spite of these limitations, the method will permit a separate analysis of sterols and bile acids in the neutral fraction. Alternatively, a more specific separation of neutral and acidic compounds may be attained by rechromatography on Lipidex-DEAP as used in analyses of urinary steroids (34) and bile acids (9).

When there is no interest in esterified bile acids and particularly when routine measurements of sterols are required, the neutral fraction may be directly trimethylsilylated and analyzed by capillary column GLC (**Fig. 3**). The possibility of interference by TMS ethers of mono- and diglycerides should be considered, and it is advisable to carry out mild saponification in situations where the amounts of glycerolipids are expected to be large.

Unconjugated fraction. In most instances, this fraction will contain the bulk of the bile acids excreted in the feces and, although not indicated in the scheme (Fig. 1), fatty acids will also be present in this fraction. (Table 4). The two groups of compounds are effectively separated by GLC of the methyl ester TMS ether derivatives; the fatty acid esters are eluted rapidly and are in the solvent front at the high temperature conditions employed. The derivatives of normally occurring hydroxy fatty acids are also eluted before the bile acid derivatives. In most cases, the unconjugated bile acid

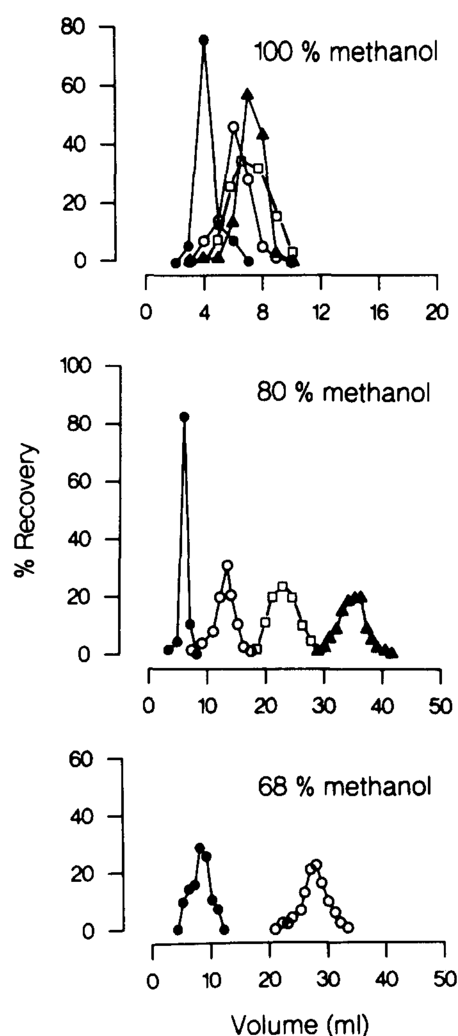

Fig. 2. Separation of radiolabeled lithocholic acid (●), methyl lithocholate (○), 5β-cholestane-3α,7α-diol (□), and cholesterol (▲) which were added to fecal extracts and extracted from the reconstituted aqueous acidic solution by Lipidex 1000 and eluted using methanol, 80% methanol, or 68% methanol. Cholesterol and 5β-cholestane-3α,7α-diol are retained on the column (eluted after 80 ml) when the most polar solvent is used.

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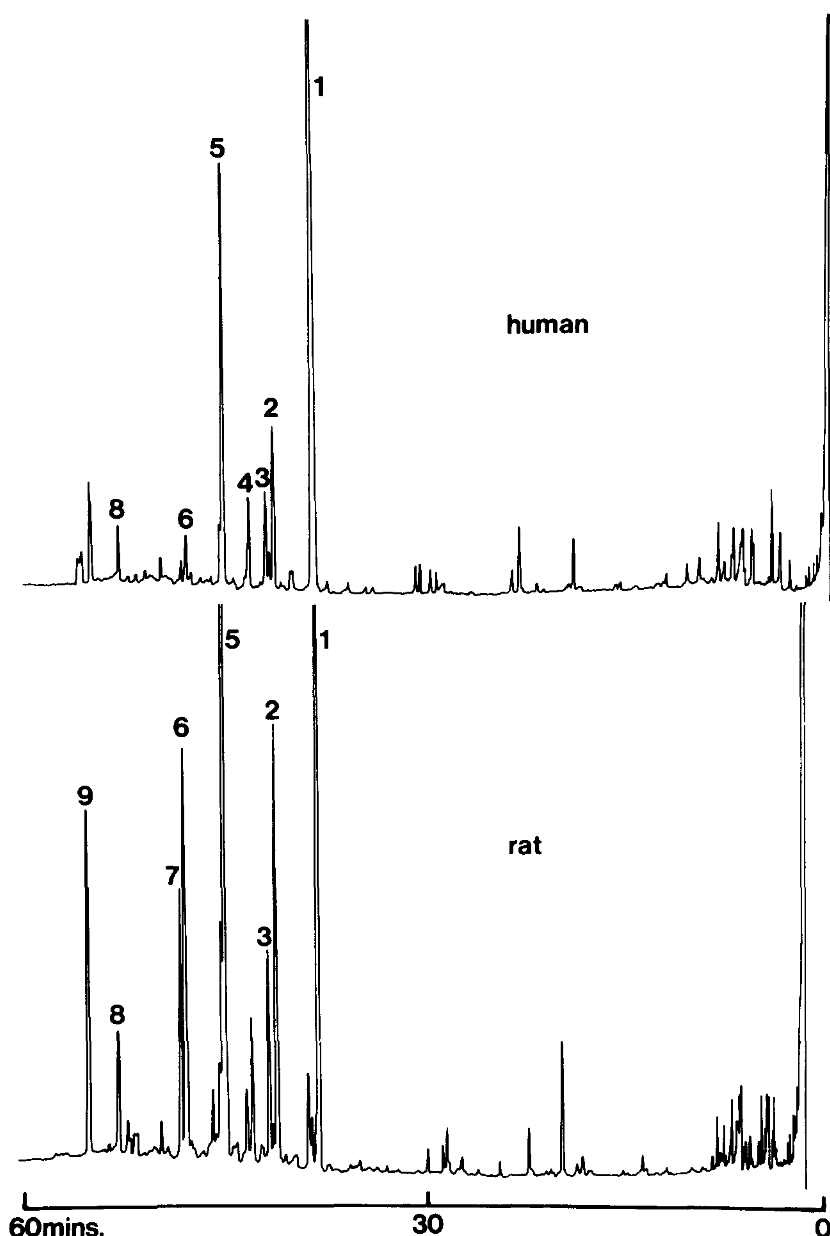

Fig. 3. GLC profiles of TMS ether derivatives of compounds in the neutral fraction from Lipidex-DEAP obtained from analyses of feces of a healthy adult man (upper chromatogram) and an adult female Sprague-Dawley rat (lower chromatogram). The following compounds were identified by GLC–MS analysis: 1, coprostanol; 2, cholesterol; 3, 24-methyl-coprostanol; 4, unsaturated 24-ethyl sterol; 5, 24-ethyl-coprostanol; 6, β-sitosterol; 7, β-sitostanol; 8 and 9, unknown compounds.

fractions can be analyzed by GLC directly following derivatization, however the GLC profile is improved by purifying the derivative on a small column of Lipidex 5000 (13).

Examples of GLC profiles of derivatized, unconjugated bile acids isolated from feces of a healthy adult man and a patient (J) with chronic diarrhea are shown in **Fig. 4.** It is outside the scope of this study to describe in detail the composition of bile acids in feces as determined by the new isolation methods. In agreement with early studies of fecal bile acids in humans (3–5), the healthy subjects excretes predominantly 3α and 3β epimers of 7-dehydroxylated and 12-keto bile acids. In contrast, the patient with diarrhea excretes very few of the recognized principal secondary bile acids and, instead, large quantities of the primary bile acids and their 7-keto derivatives are found.

A further illustration of the applicability of the

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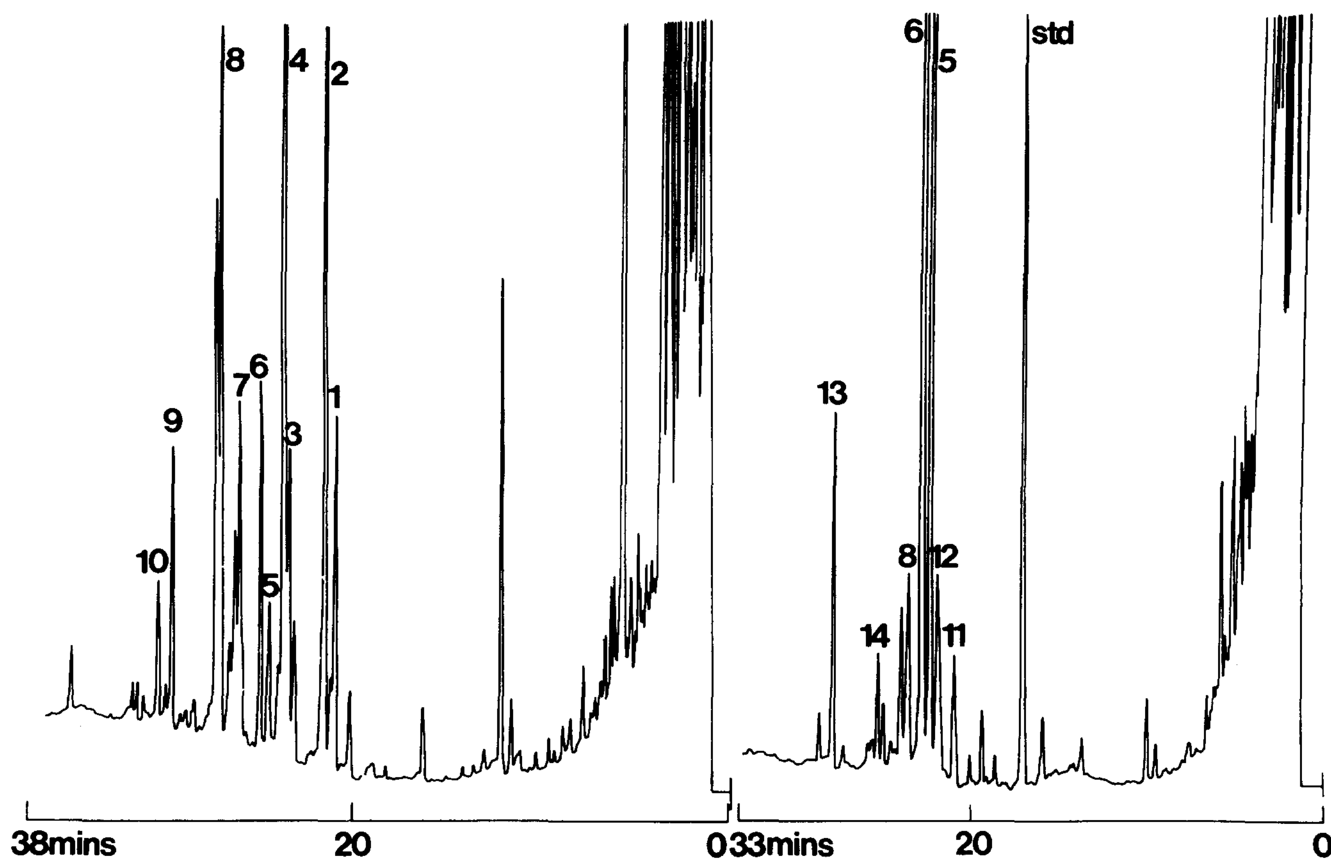

Fig. 4. GLC profiles of the methyl ester TMS ether derivatives of unconjugated bile acids excreted in the feces of a healthy adult man (left) and a male patient (J) with chronic diarrhea (right). Bile acids were identified by GLC–MS and their daily excretions were as follows.

Bile Acid	Healthy	Diarrhea
1. 3β-Hydroxy-5β-cholanoic	37 mg/day	
2. 3α-Hydroxy-5β-cholanoic (lithocholic)	85 mg/day	
3. 3β,12α-Dihydroxy-5β-cholanoic	33 mg/day	
4. 3α,12α-dihydroxy-5β-cholanoic (deoxycholic)	139 mg/day	
5. 3α,7α-Dihydroxy-5β-cholanoic (chenodeoxycholic)	16 mg/day	125 mg/day
6. 3α,7α,12α-Trihydroxy-5β-cholanoic (cholic)	39 mg/day	253 mg/day
7. 3α,7β-Dihydroxy-5β-cholanoic (ursodeoxycholic)	35 mg/day	
8. 3α,7β,12α-Trihydroxy-5β-cholanoic	76 mg/day	18 mg/day
9. 12-Oxo-3β-hydroxy-5β-cholanoic	62 mg/day	
10. 12-Oxo-3α-hydroxy-5β-cholanoic	30 mg/day	
11. 3β,7α-Dihydroxy-5β-cholanoic		10 mg/day
12. 3β,7α,12α-Trihydroxy-5β-cholanoic		18 mg/day
13. 7-Oxo-3α,12α-dihydroxy-5β-cholanoic		60 mg/day
14. 7-Oxo-3α-hydroxy-5β-cholanoic		18 mg/day

method is given in **Fig. 5,** which shows the typical highly complex profile of unconjugated bile acids from feces of adult female rats. This complexity is to be expected from the large number of bile acids present in rat bile (35, 36) as compared to human bile, and from previous studies using packed columns (e.g., 37–40).

The quantitative excretion of the principal bile acids in the samples from the humans and a female rat were within ranges reported in previous studies (3–5). As shown in **Table 5,** the precision of the method for a variety of selected bile acids, as determined by repeated analyses of the three types of fecal samples, was satisfactory.

The fraction from Lipidex-DEAP containing unconjugated bile acids will also contain those acids esterified with fatty acids at C-3. The natural occurrence of such bile acid derivatives was indicated by studies of Norman (19) and Norman and Palmer (20), and recent work by Kelsey et al. (33) has shown that lithocholic acid sulfate can be converted by human intestinal microflora into fatty acyl derivatives of 3β-hydroxy-5β-cholanoic acid. Inclusion of these bile acid derivatives in the analysis

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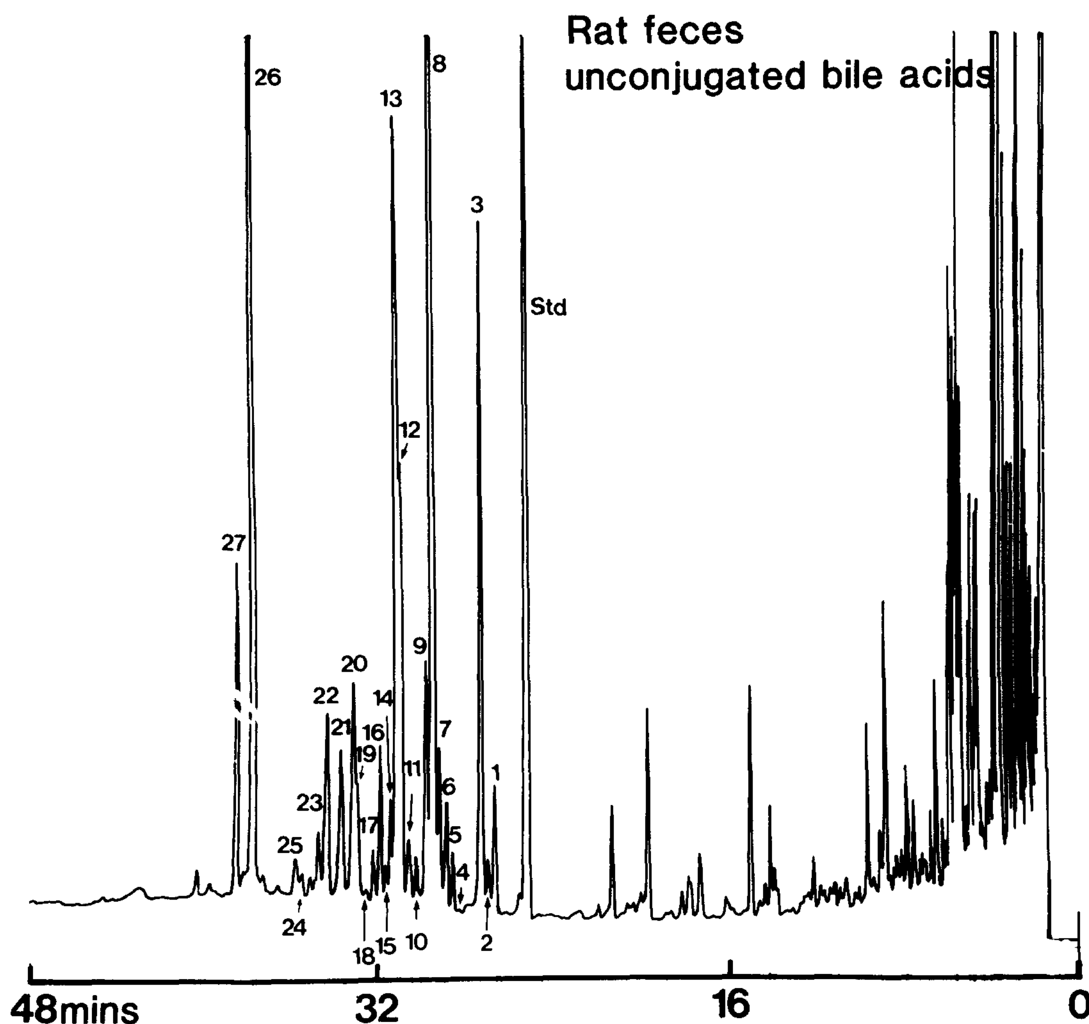

Fig. 5. GLC profile of the methyl ester TMS ether derivatives of unconjugated bile acids excreted in the feces of an adult female Sprague-Dawley rat. The following compounds are indicated. 1, 3β-Hydroxy-5β-cholanoic; 2, 3β-hydroxy-5α-cholanoic; 3, 3α-hydroxy-5β-cholanoic (lithocholic); 4, unknown monohydroxy bile acid; 5, 3α,12α,dihydroxy-5α-cholanoic; 6, 3α,12β-dihydroxy-5β-cholanoic; 7, 3α,12β-dihydroxy-5α-cholanoic; 8, 3α,12α-dihydroxy-5β-cholanoic (deoxycholic); 9, unidentified bile acid; 10, 3α,6β-dihydroxy-5β-cholanoic and 3-oxo-12α-hydroxy-5β-cholanoic; 11, unknown dihydroxy + trihydroxy bile acids; 12, 3α,6β,7α-trihydroxy-5β-cholanoic (α-muricholic); 13, 3α,6α-dihydroxy-5β-cholanoic (hydeoxycholic); 14, 3β,6α-dihydroxy-5β-cholanoic; 15, unknown tetrahydroxy bile acid; 16, 3β,12α-dihydroxy-5α-cholanoic; 17, 12-oxo-3α-hydroxy-5α-cholanoic; 18, unknown trihydroxy bile acid; 19, 3β,12β-dihydroxy-5α-cholanoic; 20, 12-oxo-3α-hydroxy-5β-cholanoic; 21, unknown mono-oxo-monohydroxy bile acid; 22, 3α,6β,7β-trihydroxy-5β-cholanoic (β-muricholic); 23, unknown compound; 24, mono-oxo-dihydroxy and mono-oxo-monohydroxy bile acids; 25, 12-oxo-3β-hydroxy-5α-cholanoic; 26, 3α,6α,7β-trihydroxy-5β-cholanoic (ω-muricholic); 27, 3α,6α,7β-trihydroxy-5α-cholanoic.

will require mild saponification essentially as described for bile acid esters in the neutral fraction. Direct analysis by GLC after derivatization may also be possible in analogy with the analysis of intact cholesteryl esters (41).

Glycine and taurine conjugates. For convenience, enzymatic hydrolysis of this fraction was carried out with cholyglycine hydrolase (42, 43). The efficiency of this enzyme in hydrolyzing glycocholic and taurocholic acids at 37°C overnight was comparable to that at the elevated temperature of 55°C for the shorter period of 4 hr. Although this enzyme will not cleave all bile acid structures, if this fraction is of particular importance, as it may be in conditions affecting intestinal flora, then it is suggested that alkaline hydrolysis be carried out as well. Bile acids were extracted from the hydrolyzate with Lipidex 1000 after adjusting the pH to between 3 and 4. In the fecal samples examined from healthy adult subjects so far, bile acids were barely detectable as glycine- or taurine-conjugates and accounted for <3% of the total bile acids excreted in feces.

Sulfate fraction. At the present time the satisfactory analysis of sulfate conjugates of bile acids can only be performed following a combined solvolytic and hydrolytic step. Considerable controversy exists over the most suitable method to adopt for this deconjugation step, with the result that three principal techniques of sol-

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TABLE 5. Precision of the method determined by replicate analyses of selected unconjugated bile acids in fecal samples from humans and an adult female rat

Bile Acid[a]	Excretion (mg/day) Mean ± SD (n = 4)	CV %	Bile Acid[b]	Excretion (mg/day) Mean ± SD (n = 4)	CV %	Bile Acid[c]	Excretion (μg/day) Mean ± SD (n = 4)	CV %
5αB-3α-ol	1.36 ± 0.19	13.9	Lithocholic	11.5 ± 1.00	8.7	5βB-3β-ol	136 ± 9	7.1
5βB-3β-ol	7.87 ± 0.55	7.0	5βB-3β,7α-ol	2.8 ± 0.1	4.0	5αB-3α-ol	58 ± 4	6.7
Lithocholic	23.49 ± 1.35	5.7	5βB-3β,12α-ol	3.0 ± 0.1	3.3	Lithocholic	657 ± 88	13.4
5βB-3β,12α-ol	1.97 ± 0.32	16.2	Deoxycholic acid	39.1 ± 3.10	8.1	5αB-3α,12α-ol	137 ± 8	5.9
Deoxycholic	43.18 ± 2.70	6.2	Chenodeoxycholic	33.1 ± 2.3	7.1	Deoxycholic	1850 ± 130	6.9
Cholic	7.84 ± 0.74	9.4	Cholic	69.9 ± 3.3	4.7	Hyodeoxycholic	1700 ± 220	12.9
12-oxo-5αB-3α-ol	3.22 ± 0.26	8.1	7-oxo-5βB-3α-ol	7.0 ± 0.8	10.8	5βB-3α,6β-ol	441 ± 19	4.4
12-oxo-5βB-3α-ol	2.59 ± 0.28	10.8	12-oxo-5βB-3α,7α-ol	13.2 ± 0.8	5.9	ω-muricholic	416 ± 56	12.2

[a] Sample from a healthy adult man. B, cholanoic acid, configuration at C-5H and of hydroxyl groups are indicated by Greek letters.
[b] Sample from male patient (A) with chronic diarrhea.
[c] Sample from adult female Sprague-Dawley rat.

volysis are currently practiced. Solvolysis in methanol–acetone–HCl (44) is frequently used but can be time-consuming and will lead to partial formation of acetonides of *cis*-glycol structures, as exist in hyocholic and β-muricholic acids. Dimethoxypropane–concentrated HCl has more recently been employed for the simultaneous solvolysis (45) and methylation (46) of bile acid sulfates. However, this reagent will also yield acetonides, and factors influencing the quantitative solvolysis have yet to be reported. Both of these solvolytic procedures are therefore less desirable for the analysis of fecal samples from rats, in which significant quantities of several 3,6,7-trihydroxy bile acid isomers are present. Solvolysis of this type of sample may be carried out in ethanol–ethyl acetate–H_2SO_4 (9, 12). A drawback of this method is the partial formation of acetates and ethyl esters, and inefficient solvolysis of 7-sulfated bile acids was recently pointed out (47). For the above reasons, two solvolytic procedures were employed and the choice of which to employ will depend upon the type of sample to be analyzed. Solvolysis in tetrahydrofuran–H_2SO_4, which has been found to be most convenient in analyses of sulfates of neutral steroids (48), has yet to be evaluated for bile acid sulfates.

The bile acid sulfates in feces may have a free carboxyl group or be conjugated with glycine or taurine. For this reason and because of the formation of esterified products during solvolysis, preventing the use of enzymatic hydrolysis, an alkaline hydrolysis is required. This may result in formation of artefacts (49–51). In order to minimize transformations of keto bile acids, the hydrolysis was carried out in aqueous solution without addition of alcohols (51). In this way it was also possible to use Lipidex 1000 for the extraction of the deconjugated bile acids. The latter were finally isolated by rechromatography on Lipidex-DEAP before GLC and GLC–MS.

The gas-liquid chromatographic profiles of derivatized bile acids, isolated from the sulfate fractions of samples of feces from a healthy subject and the patient (J) with chronic diarrhea (cf. Fig. 4), are compared in **Fig. 6.** The qualitative differences seen between the unconjugated bile acids (Fig. 4) are also reflected within the sulfate fractions.

Quantitatively, the sulfated bile acids were found to comprise not more than 5% of total bile acids excreted in the feces of a group of seven normal subjects so far studied. These findings are at variance with those in recent reports (52, 53) that stated that bile acid sulfates accounted for up to one-third of the total bile acids excreted in the feces of 14 normal subjects. However, no gas chromatograms were shown and, in view of the methods employed and the complexity of fecal bile acid fractions, it is conceivable that the discrepancy may be accounted for by nonspecific interference in the chromatograms. Islam, Raicht, and Cohen (54) failed to find bile acid sulfates in human feces. However, their method was based on a separation on Sephadex LH-20 which has been shown to exclude taurine-conjugated bile acid sulfates from the sulfate fraction (9). In our study of Japanese adults, using a preliminary version of the present method (55, 56), bile acid sulfates accounted for 1–10% of the total bile acids excreted (57). Although bile acid sulfates are apparently of minor quantitative importance in feces from healthy subjects, changes in patients with intestinal disorders cannot be excluded. There may also be large species differences, as illustrated by the finding of Eyssen, Parmentier, and Mertens (11) that more than 80% of the cholic and chenodeoxycholic acids in feces from mice are present as sulfates.

Capillary column gas–liquid chromatography and mass spectrometry

The complexity of the GLC profiles is evident from the examples shown here, which illustrate the need to use a capillary column to attain separations. This view is strengthened when the chromatograms are compared

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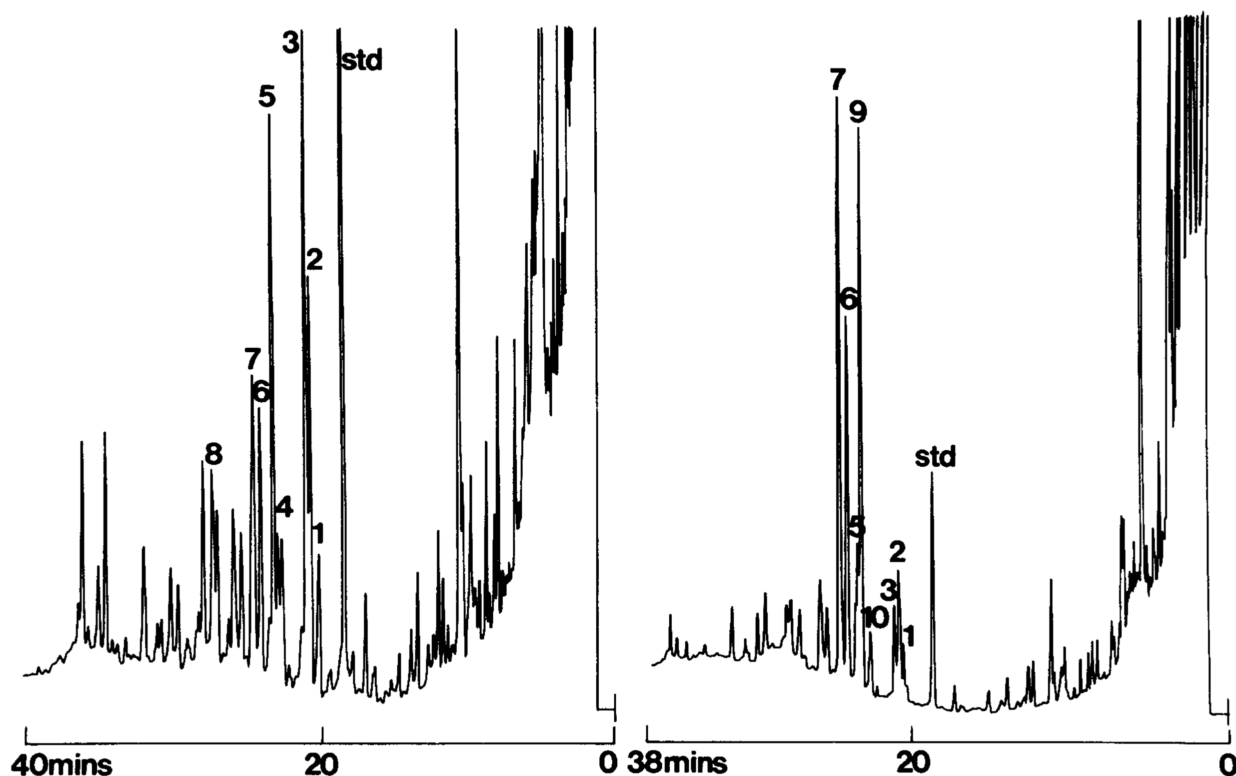

Fig. 6. GLC profiles of methyl ester TMS ether derivatives of the bile acids excreted as sulfates in the feces of a healthy adult man (left) and a male patient (J) with diarrhea (right). The following principal bile acids were identified and the daily excretion (mg/day) was as follows.

Bile Acid	Healthy	Diarrhea
1. 3β-Hydroxy-5β-cholanoic	0.4 mg/day	0.5 mg/day
2. 3α-Hydroxy-5β-cholanoic (lithocholic)	1.4 mg/day	1.1 mg/day
3. Cholesterol	(not determined)	(not determined)
4. 3β,12α-Dihydroxy-5β-cholanoic	0.4 mg/day	
5. 3α,12α-Dihydroxy-5β-cholanoic (deoxycholic)	1.5 mg/day	1.2 mg/day
6. 3α,7α-Dihydroxy-5β-cholanoic (chenodeoxycholic)	0.7 mg/day	3.1 mg/day
7. 3α,7α,12α-Trihydroxy-5β-cholanoic (cholic)	0.8 mg/day	5.0 mg/day
8. 3α,7β,12α-Trihydroxy-5β-cholanoic	0.5 mg/day	
9. 3β-Hydroxy-5-cholenoic		4.8 mg/day
10. 3β,7α-Dihydroxy-5β-cholanoic		0.7 mg/day

with those published previously using packed columns for analysis of bile acids in feces from humans (3–5) and rats (37–40). Even with this increased chromatographic resolution it is evident that many compounds remain unresolved and the solution to this problem cannot be found by changing the type of liquid phase, although using both a nonselective and selective phase may often be of help in identifying unresolved compounds. Selective phases, such as PEG 2000 or Carbowax 20M, which are often used for serum bile acid analysis (58, 59) are less satisfactory for ketonic bile acids and the columns also have shorter life times, necessitating frequent recoating. For these reasons and particularly because of improvements in the commercial manufacture of columns with higher temperature tolerances, a nonselective phase (Silicone OV-1) was employed for the analysis of fecal bile acids.

The identification of a bile acid was based upon the GLC retention data, the complete mass spectrum, and ion current chromatograms constructed of ions characteristic of specific and generalized bile acid structures following the repetitive magnetic scanning of the GLC effluent. These procedures have been discussed fully elsewhere (9).

Quantification was carried out using flame ionization detection and relating the peak height response to that of a known amount of internal standard which was added to the sample prior to derivatization. When temperature programming is used, it is adequate and theoretically sound to use peak height. In theory, peak area should be better, especially if peak width varies with the load in the column. However, in practice it is more difficult to determine the correct base-line, and the uncertain area obtained at the base of the peak causes a

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greater variation than the peak height, which is relatively less influenced by the uncertainty about the baseline. Where authentic bile acid standards were obtained, the values were corrected to account for the differences in responses. In many cases the response factor approximated unity, however bile acids containing an oxo group gave relatively poor peak height responses (40–60%) that required correction (60). This phenomenon is also seen for many neutral steroids that possess a carbonyl group (61).

Applicability of the method

Many methods that have been proposed over the last decade have risen out of a need for simplicity and convenience, e.g., enzymatic and fluorometric measurements, (22, 62–64) with the result that the validity of data obtained by such techniques when applied to feces is questionable. Of the recently described methods using GLC (22, 52, 65–67) none are comprehensive in terms of analytical design, and an extensive characterization of the composition of all classes of bile acids and sterols has not yet been reported.

In the development of the methods described here, efforts have been made to provide a general and relatively simple procedure with sufficient flexibility to allow a number of objectives to be met. Clearly, because of the complexity of the composition of feces, it is impossible to develop a single universal scheme and it is inevitable that there will occur some overlap of certain classes of compounds. In this respect, glucuronide conjugates of bile acids will be distributed between several fractions and, for their accurate determination, alternative procedures should be employed (68). The same is true for conjugated sterols and bile alcohols. For example, cholesteryl sulfate is likely to appear in the taurine-conjugate fraction (cf.: buffer and pH of the solvent eluting steroid sulfates in Setchell et al. (33)) and steroid sulfates may also appear in the bile acid sulfate fraction, however these groups of compounds can be specifically isolated using the same basic scheme with the solvent systems described previously for separation of conjugated neutral steroids (33, 69). An important feature of the method is that groups of bile acids are isolated separately so that necessary chemical transformations such as derivatization, hydrolysis, and solvolysis can be applied selectively only to groups requiring such treatment. This minimizes the amount of artefacts produced.

While the overall technique is complex and time-consuming, particularly if the complete detailed analysis of all groups of compounds is carried out, it should be stressed that the inherent flexibility also renders the method suitable for routine applications. For example, if analyses were restricted to the unconjugated bile acid and sterol fractions, which in most instances will contain the greatest proportion of metabolites, then the analytical time can be reduced to 3 days from the time of collection of the stools.

The type of metabolic profiling of bile acids and related compounds that is described here, utilizing selective group separation and the high resolving power of capillary column GLC, should be useful in investigation of the pathophysiology of the gastrointestinal tract. The isolation methods can also be used in metabolic studies employing isotopically labeled bile acids. Turnover rates and metabolic pathways may differ depending on state of conjugation and initial solvolysis, and hydrolysis will result in loss of information regarding differences in specific labeling of sulfated conjugated and unconjugated bile acids. In this case it is a limitation that the sulfates are not separated with respect to site of sulfation. Alternatively, following group separation, individual metabolites may be separated by high performance liquid chromatographic procedures (28).

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