

Highly simplified method for gas-liquid chromatographic quantitation of bile acids and sterols in human stool

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Abstract A simple method for the gas-liquid chromatographic quantitation of human fecal bile acids and sterols is described where bile acids are subjected to n-butyl ester derivatization, without prior isolation from the stool, followed by trimethylsilylation of the sterols and bile acids. Under these conditions, bile acid derivatives are well resolved from each other and from the trimethylsilyl ether derivatives of fecal sterols and no overlap occurs. The method was shown to be highly reproducible and recoveries were similar to those obtained with other methods used for fecal bile acid analysis. Application of the method for bile acid and sterol analysis in human stool is described.—Batta, A. K., G. Salen, K. R. Rapole, M. Batta, P. Batta, D. Alberts, and D. Earnest. **Highly simplified method for gas-liquid chromatographic quantitation of bile acids and sterols in human stool.** *J. Lipid Res.* 1999. 40: 1148–1154.

Supplementary key words bile acids • bile acid n-butyl ester-trimethylsilyl ethers • capillary gas-liquid chromatography • human fecal bile acids • fecal sterols • fatty acids

Bile acids are the major catabolic products of cholesterol and facilitate the excretion of bile lipids including cholesterol, and the absorption of dietary lipids including fat-soluble vitamins, via their detergent action. During their enterohepatic circulation, approximately 5% of bile acids escape reabsorption and seep into the colon where they are subjected to modification by intestinal bacteria to form secondary bile acids. There is significant circumstantial evidence to suggest that secondary bile acids, in particular the 7 α -dehydroxylated bile acid, deoxycholic acid, act as co-carcinogens in colon cancer. Thus, increased amounts of deoxycholic acid have been reported in patients with colon cancer as compared with controls by some authors, but not all (1–3). However, fecal bile acid pattern is highly complex, due to extensive bacterial metabolism of bile acids during intestinal transit and various mono-, di-, and/or trioxo compounds and iso- (3 β -hydroxy), urso- (7 β -hydroxy), and ligo- (12 β -hydroxy) bile acids have been reported (4–6). Thus, although increased amounts of deoxycholic acid may be associated

with colon polyp formation, it is possible that some of the other secondary bile acids may also be co-carcinogenic.

We have recently shown that ursodeoxycholic acid results in reduction of colon polyp in experimental rats (7). We have now extended this study to humans, and as part of the study, we needed to screen a large number of hospital out-patients for fecal bile acid measurements. Although, several gas-chromatographic (GLC) methods have been used for the purpose (4, 5, 8, 9), they are all cumbersome and time-consuming and are not applicable for routine screening purposes. We describe a simple method for simultaneous quantitation of fecal bile acids and sterols, which was found to be highly reproducible and gives results comparable with other well established methods.

EXPERIMENTAL

Reagents and chemicals

Ursodeoxycholic and ursocholic acids were gifts from Tokyo Tanabe, Japan. Nor-cholic acid was synthesized in our laboratory according to literature (10). Other bile acids were purchased from Steraloids (Wilton, NH). Cholesterol, campesterol, sito-

Abbreviations: The following abbreviations and trivial names have been used: GLC, gas-liquid chromatography; TMS, trimethylsilyl; Sil-prep, hexamethyldisilazane; trimethylchlorosilane; pyridine, 3: 1: 9; lithocholic acid, 3 α -hydroxy-5 β -cholanoic acid; deoxycholic acid, 3 α , 12 α -dihydroxy-5 β -cholanoic acid; iso-deoxycholic acid, 3 β , 12 α -dihydroxy-5 β -cholanoic acid; chenodeoxycholic acid, 3 α , 7 α -dihydroxy-5 β -cholanoic acid; cholic acid, 3 α , 7 α , 12 α -trihydroxy-5 β -cholanoic acid; ursodeoxycholic acid, 3 α , 7 β -dihydroxy-5 β -cholanoic acid; ursocholic acid, 3 α , 7 β , 12 α -trihydroxy-5 β -cholanoic acid; nor-deoxycholic acid, 3 α , 12 α -dihydroxy-24-nor-5 β -cholan-23-oic acid; nor-cholic acid, 3 α , 7 α , 12 α -trihydroxy-24-nor-5 β -cholan-23-oic acid; 12-oxo-lithocholic acid, 12-oxo, 3 α -hydroxy-5 β -cholanoic acid; coprostanol, 5 β -cholestan-3 β -ol; cholesterol, 5-cholesten-3 β -ol; 24-methylcoprostanol, 24-methyl-5 β -cholestan-3 β -ol; campesterol, 24-methyl-5-cholesten-3 β -ol; 24-ethylcoprostanol, 24-ethyl-5 β -cholestan-3 β -ol; stigmaterol, 24-ethyl-5,22-cholestadien-3 β -ol; sitosterol, 24-ethyl-5-cholesten-3 β -ol, sitostanol, 24-ethyl-5 α -cholestan-3 β -ol, fucosterol, stigmasta-5,24(28)-dien-3 β -ol; and lanosterol, 4,4,14-trimethyl-cholesta-8,24-dien-3 β -ol.

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sterol, coprostanol, fucosterol, stigmasterol, lanosterol, 4-cholesten-3-one, and cholestanone were from Steraloids, Inc. 24-Methyl- and 24-ethylcoprostanol were isolated from the sterol fraction of human stool by a combination of thin-layer chromatography and high-performance liquid chromatography and their structures were confirmed by mass spectra. All fatty acids used in the study were purchased from Aldrich Chemical Co. (Milwaukee, WI). Methyl esters of the bile acids and fatty acids were prepared by addition of 0.1 ml of 3% anhydrous methanolic hydrochloric acid (Aldrich Chemical Co.) to 5–20 μg of the respective bile acid or fatty acid and were kept at room temperature for 2 h followed by evaporation of solvent at 55°C under N_2 . All compounds were >98% pure as judged by GLC of the trimethylsilyl (TMS) ether derivatives and exhibited mass spectral fragmentation patterns compatible with their structures. Sil-prep (hexamethyldisilazane: trimethylchlorosilane: pyridine, 3: 1: 9) used for preparation of TMS ether derivatives of the bile acid esters was purchased from Alltech Associates (Deerfield, IL).

Gas chromatography

A Hewlett-Packard model 6890 gas chromatograph equipped with a flame ionization detector and an injector with a split/splitless device for capillary columns was used for all separations. The chromatographic column consisted of a chemically bonded fused silica CP-Sil-5 CB (stationary phase, 100% dimethylsiloxane) capillary column (25 m \times 0.22 mm I.D.) (Chrompack, Raritan, NJ) and helium was used as the carrier gas. The GLC operating conditions were as follows: injector and detector temperatures were 260°C and 290°C, respectively. After injection, oven temperature was kept at 100°C for 2 min, then programmed at a rate of 35°C/min to a final temperature of 278°C (11).

Gas chromatography–mass spectrometry

Mass spectrometry of sterols and bile acids, when needed, was carried out on a Hewlett-Packard model 5988 gas chromatograph–mass spectrometer using a 25-m fused silica CP-Sil-5 CB capillary column.

Butyl ester formation

Bile acids or fatty acids (10–20 μg each) were taken in *n*-butanol (200 μl) and 50 μl concentrated hydrochloric acid was added. The contents were heated at 60°C for 4 h and solvents were removed at 60°C.

Trimethylsilylation

The esterified bile acid or the sterol (5–10 μg) was reacted with 100 μl of Sil-prep for 30 min at 55°C. Solvents were evaporated at 55°C under N_2 and the TMS ether derivative formed was taken in 100 μl of hexane and 1 μl was injected into the GLC column. The retention times of the various bile acids were calculated relative to that of nor-cholic acid.

Quantitation of fecal bile acids and sterols

To 10–15 mg freeze-dried stool (weighed exactly) was added internal standard (nor-cholic acid, 20 μg) in 200 μl of *n*-butanol followed by 50 μl concentrated hydrochloric acid and the contents were subjected to butyl ester formation as described above. The esterified product was directly subjected to trimethylsilylation. The TMS ether derivatives formed were taken in 200 μl of hexane, centrifuged to separate the stool debris, and 1–2 μl of the clear supernatant was injected into the GLC column.

Quantitation of fecal bile acids and sterols by sodium hydroxide/solvent extraction method

Freeze-dried stool (10–15 mg), to which 20 μg of nor-cholic acid and 20 μg 5 α -cholestane were added, was digested with 1 ml

of 1 N sodium hydroxide for 1 h at 90°C in a screw-cap tube (12). After cooling, the product was diluted with water (5 ml) and repeatedly extracted with *n*-hexane (4 \times 3 ml). The *n*-hexane was evaporated to dryness and the residue was subjected to trimethylsilylation. An aliquot was used for GLC to quantitate the neutral sterols. The aqueous layer after extraction of neutral sterols was acidified to pH 1 with 5 N hydrochloric acid followed by extraction with ethyl acetate (4 \times 3 ml). Ethyl acetate layer was washed with water to neutrality, evaporated to dryness, and the residue was subjected to butyl ester formation and trimethylsilylation. An aliquot was then used for GLC to quantitate bile acids.

Quantitation of fecal bile acids and sterols by Soxhlet extraction method

Freeze-dried stool (10–15 mg) was transferred into a small paper thimble, together with 20 μg nor-cholic acid and 20 μg of 5 α -cholestane, and was subjected to continuous extraction with 1% ammoniacal ethanol for 16 h in a Soxhlet extractor (8). Ethanol was evaporated to dryness and the residue was taken up in 5 ml of 0.5 N sodium hydroxide. The neutral sterols were then extracted with *n*-hexane (4 \times 5 ml). The *n*-hexane was evaporated to dryness and the residue was subjected to trimethylsilylation. An aliquot was used for GLC to quantitate the neutral sterols. The aqueous solution after removal of neutral sterols was acidified to pH 1 with 5 N hydrochloric acid and bile acids were isolated and derivatized as described above. An aliquot was then used for GLC.

Quantitation of fecal bile acids and sterols after extraction with chloroform–methanol

Freeze-dried stool (10–15 mg), to which nor-cholic acid (20 μg) and 5 α -cholestane (20 μg) were added, was heated with 5 ml chloroform–methanol 2:1 at 65°C for 0.5 h in a screw-cap tube. After centrifugation, the supernatant was collected and the residual fecal material was again extracted with chloroform–methanol as described above. The combined solution from four such extractions was evaporated to dryness, the residue was taken up in 5 ml of 0.5 N sodium hydroxide, and the neutral sterols and bile acids were extracted as described above under the Soxhlet extraction method. Aliquots were used for quantitation of neutral sterols and bile acids by GLC.

RESULTS

In an earlier publication, Child, Aloe, and Mee (13) showed that the acetate derivatives of the *n*-butyl esters of several bile acids and fatty acids and of a number of sterols were resolved from each other on GLC and the various compounds could be quantitated in presence of each other. We recently showed that the trimethylsilyl ether derivatives of the *n*-butyl esters of common bile acids were eluted later than all plasma sterols, including late eluting sitosterol, thus obviating the need for complete removal of sterols during plasma bile acid analysis (14). In an extension of our method, we have found that when a mixture of several fecal fatty acids, sterols, and bile acids was subjected to the conditions of *n*-butyl ester followed by trimethylsilyl ether formation and injected into the gas chromatograph, each class of compounds was resolved from the others. **Table 1** shows the retention times of the *n*-butyl ester–trimethylsilyl ether derivatives of a number of fatty acids, bile acids, and sterols that are usually reported in

TABLE 1. GLC retention times of trimethylsilyl ether-n-butyl esters of bile acids, trimethylsilyl ethers of sterols, and n-butyl esters of fatty acids on CP-Sil-5-CB capillary column

TMS Ether of	RRT ^a	TMS Ether of	RRT ^a
Nor-CA-n-butyl ester	1.000	24-methylcoprostanol	0.744
LCA-n-butyl ester	1.034	24-ethylcoprostanol	0.845
3-Oxo-5 β -cholanoic acid-n-butyl ester	1.086	campesterol	0.833
DCA-n-butyl ester	1.119	campestanol	0.836
CDCA-n-butyl ester	1.159	sitosterol	0.935
CA-n-butyl ester	1.174	sitostanol	0.950
UDCA-n-butyl ester	1.191	fucosterol	0.943
UCA-n-butyl ester	1.248	lanosterol	0.926
Coprostanol	0.660	palmitic acid	0.356
Cholesterol	0.737	heptadecanoic acid	0.371
Cholestanol	0.738	stearic acid	0.389
Cholestanone	0.729	palmitoleic acid	0.352
4-Cholesten-3-one	0.797	oleic acid	0.385
Stigmasterol	0.883	linoleic acid	0.389
		arachidonic acid	0.433

The n-butyl ester of fatty acid, n-butyl ester-TMS ether of the bile acid, or the TMS ether of the sterol (0.1 μ g in 1 μ l hexane) was injected onto a fused-silica CP-Sil-5 CB capillary column. The injector and detector temperatures were kept at 260°C and 290°C, respectively. After injection, oven temperature was kept at 100°C for 2 min, then programmed at a rate of 35°C/min to a final temperature of 278°C. Nor-CA, nor-cholic acid; LCA, lithocholic acid; DCA, deoxycholic acid; CDCA, chenodeoxycholic acid; CA, cholic acid; UDCA, ursodeoxycholic acid; UCA, ursocholic acid.

^a Retention times are expressed relative to that of the n-butyl ester-trimethylsilyl ether of nor-cholic acid (retention time, 22.360 min).

human stool. Clearly, n-butyl esters of fatty acids are eluted from the GLC column as a group well separated from the trimethylsilyl ethers of sterols, which are in turn eluted before the n-butyl ester-trimethylsilyl ethers of bile acids, and there is no overlap between the various classes of compounds. With appropriate temperature programming, better resolutions between individual fatty acids may be obtained and a more complex mixture of fatty acids may also be resolvable. We have already shown that the retention times of the n-butyl ester-trimethylsilyl ether derivatives of the bile acids are highly reproducible and, for amounts of bile acids ranging from 0.02 to 0.2 μ g injected onto the column, the detector response, as shown by the integrator, is linear (14). Even though the n-butyl ester-trimethylsilyl ethers of chenodeoxycholic acid and cholic acid did not show baseline resolution, when known amounts of these bile acid derivatives were injected together, the detector response as shown by the integrator corresponded to the amounts injected in a range of 0.02–0.2 μ g of the bile acid (14). Under the derivatization conditions, dehydration of bile acids was found to be minimal (13).

In an effort to apply our method for quantitation of bile acids in fecal samples, we weighed approximately 10 mg of freeze-dried human stool and subjected it to the derivatization procedure; an aliquot of the hexane extract was then subjected to GLC. The secondary bile acids, lithocholic acid and deoxycholic acid, were the predominant bile acids in the stool, with smaller amounts of isodeoxycholic acid and 12-ketolithocholic acid (Fig. 1). Sterols were completely resolved from the bile acids and did not interfere with bile acid quantitation as shown in Fig 1.

Thus, the major fecal sterols were eluted from the column between 14 and 21 min. Sitosterol (retention time, 20.907 min), the major late eluting fecal sterol, was eluted well before nor-cholic acid, the bile acid with the shortest retention time (22.360 min) and was used as the internal standard. There was no overlap between the two groups of compounds. In addition to the bile acids, cholesterol and its bacterial metabolite, coprostanol, and plant sterols, campesterol and sitosterol, and their bacterial metabolites, 24-methyl-5 β -cholestan-3 β -ol (24-methylcoprostanol), 24-ethyl-5 β -cholestan-3 β -ol (24-ethylcoprostanol), stigmasterol, and sitostanol were all present and well separated from each other and the bile acids (Fig. 1). All major peaks seen in the chromatogram were identified as due to bile acid derivatives or the trimethylsilyl ether derivatives of sterols, as confirmed by their mass spectral fragmentation pattern.

Our method was found to be reproducible for fecal sterol and bile acid analysis and the recoveries of bile acids and sterols on repeat analysis of fecal samples were found to be similar to those obtained by methods where rigorous solvent extractions are used (Table 2). Thus, the bile acids and sterols were quantitated within 5% range in three different analyses from a given fecal sample. Also, the amounts of individual sterols and bile acids obtained were highly comparable with those obtained when bile acids were first extracted with ethanol in a Soxhlet apparatus or with chloroform-methanol, while ethyl acetate extraction of the acidified stool yielded lower values for lithocholic acid, apparently due to incomplete extraction (Table 2). A comparison of fecal bile acids and sterols in five healthy individuals as obtained by our direct derivatization method and three solvent extraction methods is given in Table 3 and Table 4. Lithocholic acid and deoxycholic acid were the predominant fecal bile acids in all subjects and were found to be present in almost equal amounts. Cholesterol and coprostanol were the major fecal sterols and significant amounts of the plant sterols, campesterol and sitosterol, were also present, but their corresponding 5 β -H derivatives were not as abundant. The amounts of both sterols and bile acids obtained by our direct derivatization method were comparable to those obtained by solvent extraction methods except that direct extraction of acidified stool showed somewhat reduced amounts of lithocholic acid (Table 3).

DISCUSSION

Because of efficient bacterial deconjugation, bile acids in the stool are present almost completely in the unconjugated form and therefore the bile acid hydrolysis step can usually be avoided during fecal bile acid quantitation. However, a major problem in fecal bile acid analysis lies in their quantitative extraction from the stool and highly cumbersome methods have been used to extract fecal bile acids. Furthermore, stool also contains neutral sterols, including cholesterol and plant sterols and their bacterial metabolites, and also fatty acids, which may interfere in

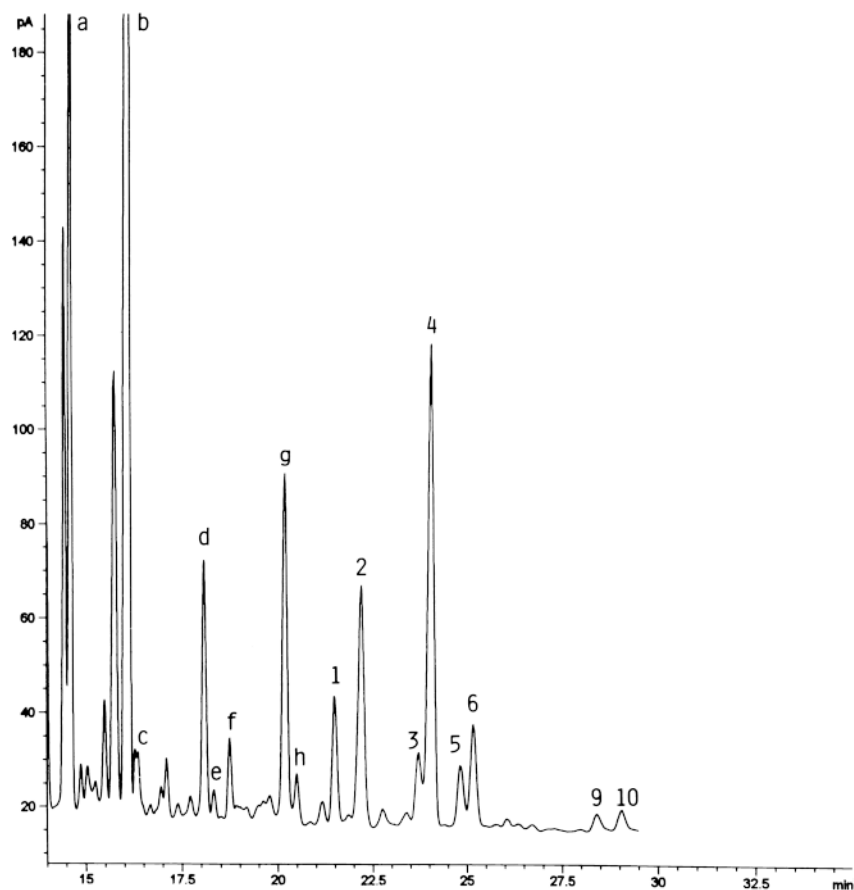


Fig. 1. GC chromatogram of sterols and bile acids present in stool from a healthy control. Ten mgs of freeze-dried stool containing 20 μ g nor-cholic acid was subjected to derivatization as described in the Experimental section. After dissolving in 200 μ l hexane, 1 μ l was injected into the GC column. Chromatographic conditions were as described in the Experimental section. Peak identification: 1, nor-cholic acid; 2, lithocholic acid; 3, iso-deoxycholic acid; 4, deoxycholic acid; 5, chenodeoxycholic acid; 6, cholic acid; 9, 3-oxo,12 α -hydroxy-5 β -cholanoic acid; 10, 12-oxo-lithocholic acid; a, coprostanol; b, cholesterol; c, 24-methylcoprostanol; d, campesterol; e, 24-ethylcoprostanol; f, stigmasterol; g, sitosterol, and h, sitostanol.

GLC analysis. Eneroth, Hellstrom, and Sjoval (8) used continuous extraction of aliquots of homogenized stool for 16 h with chloroform-methanol, hydrolyzed the extract with strong alkali, and removed neutral compounds by solvent extraction followed by acidification and isolation of bile acids by continuous extraction with ethyl ether. The bile acids were further purified by preparative TLC as the methyl esters and prepared for GLC. In another elaborate method developed by Grundy, Ahrens, and Miettinen (9), stool was homogenized with an equal volume of water and an internal standard of [14 C]deoxycholic acid was added followed by mild alkaline hydrolysis. The neutral sterols were extracted out with petroleum ether and the aqueous suspension was subjected to rigorous alkaline hydrolysis. After acidification, bile acids were extracted with chloroform-methanol 2:1, subjected to chromatography over Florisil, and the purified bile acid fraction was subjected to methyl ester formation followed by preparative TLC to remove the fatty acids. Bile acid fraction was then isolated and aliquots were subjected to radioactivity measurement, to correct for recovery, and to GLC. In alternate methods, stool has been extracted with

ammoniacal alcohol, methanol-hydrochloric acid, acetic acid-toluene, and bile acids were extracted after removal of neutral sterols (15-18).

As fatty acids are less strongly retained on the capillary GLC columns, bile acids can usually be quantitated in the presence of fatty acids. However, sterols still need to be separated, as they can interfere with bile acids during GLC analysis. Thus, the GLC retention time of the TMS ether of cholesterol is close to that of the methyl ester of lithocholic acid on a CP-Sil-5 CB capillary column, while the plant sterols, campesterol and sitosterol, which are usually present in the stool, are eluted in the general bile acid region (14). Because the retention times of bile acids increase when their esters with higher homologs are used instead of the methyl ester-trimethylsilyl ethers (19), Tsaconas et al. (20) prepared the isobutyl ester-TMS ethers of bile acids and found that most common bile acids were well resolved from sterols on capillary GLC. However, we found that although the isobutyl ester-TMS ethers of bile acids were eluted later than the TMS ether of cholesterol on a CP-Sil-5 CB capillary column, the plant sterol, sitosterol, a major fecal sterol, was eluted later than lithocholic

TABLE 2. Reproducibility of quantitation of fecal sterols and bile acids

Compound	Current Method ^a	Soxhlet Extraction ^b	Folsch Extraction ^c	NaOH/Extraction ^d
	<i>μg/mg dry stool</i>			
LCA	1.8 ± 0.1	1.7 ± 0.2	1.6 ± 0.2	1.3 ± 0.3
Iso-DCA	0.5 ± 0.05	0.5 ± 0.1	0.4 ± 0.1	0.4 ± 0.1
DCA	3.5 ± 0.2	3.3 ± 0.4	3.3 ± 0.3	3.1 ± 0.5
Coprostanol	5.9 ± 0.3	5.4 ± 0.5	5.8 ± 0.4	5.04 ± 0.6
Cholesterol	12.2 ± 0.5	12.4 ± 0.6	11.9 ± 0.6	11.4 ± 0.6
24-Methylcoprostanol	0.7 ± 0.1	0.8 ± 0.2	0.9 ± 0.2	0.7 ± 0.3
Campesterol	2.2 ± 0.1	2.4 ± 0.1	2.3 ± 0.3	2.1 ± 0.3
24-Ethylcoprostanol	0.3 ± 0.05	0.5 ± 0.1	0.7 ± 0.2	0.6 ± 0.2
Sitosterol	3.7 ± 0.2	3.7 ± 0.4	3.6 ± 0.4	3.3 ± 0.4

Mean ± SD of analysis performed on 3 separate samples of the freeze-dried human fecal sample used in Fig. 1. Gas-chromatographic conditions are described in Table 1.

^a Stool (10–15 mg), to which 20 μg of each of nor-cholic acid and 5α-cholestane was added, was directly subjected to n-butyl ester formation followed by TMS ether derivatization. After addition of hexane (200 μl), 2 μl was used for GC. Sterols were quantitated against 5α-cholestane and bile acids were quantitated against nor-cholic acid as the internal standard.

^b Stool (10–15 mg), to which 20 μg of each of nor-cholic acid and 5α-cholestane was added, was continuously extracted for 18 h with ammoniacal ethanol in a Soxhlet extractor. Sterols were extracted with n-hexane and bile acids were then extracted with ethyl acetate. Sterol fraction was subjected to silylation and the bile acid fraction was subjected to n-butyl ester formation followed by TMS ether derivatization. After addition of hexane (200 μl) to each fraction, 2 μl was used for GC as described above.

^c Stool (10–15 mg), to which 20 μg of each of nor-cholic acid and 5α-cholestane was added, was extracted with chloroform-methanol 2:1 (4 × 5 ml). Sterols were extracted with n-hexane after which bile acids were extracted with ethyl acetate. Sterols and bile acids were quantitated by GC exactly as described above under the Soxhlet extraction method.

^d Stool (10–15 mg), to which 20 μg of each of nor-cholic acid and 5α-cholestane was added, was digested with 1 N sodium hydroxide at 90°C for 1 h, diluted with water (5 ml), and sterols were extracted with n-hexane. Bile acids were then extracted with ethyl acetate. Sterols and bile acids were quantitated by GC exactly as described above under the Soxhlet extraction method.

acid. Furthermore, nor-deoxycholic acid and nor-cholic acid, routinely used as internal recovery standards, were also eluted in the same region as the sterols.

In another method, Child et al. (13) prepared the n-butyl ester-acetate derivatives of bile acids and showed that these bile acid derivatives were resolved from sterols commonly present in stool. They used the method for fecal bile acid analysis after solvent extraction of stool but without prior separation of fecal sterols. Faced with several drawbacks of acetate-n-butyl ester derivatives of bile acids, including reduced sensitivity of acetate derivatives (11) and increased retention times, we recently prepared the n-butyl ester-TMS ether derivatives of bile acids and found that the method could be conveniently used to quantitate plasma bile acids even with incomplete removal of plasma sterols (14). It was also found that the n-butyl ester-TMS ether derivative of nor-cholic acid was eluted later than the major fecal sterols and this bile acid could safely be used as an internal recovery standard.

In our attempt to apply this derivatization method for fecal bile acid analysis, and in the process to further simplify the method for routine screening of fecal samples,

TABLE 3. Bile acid analysis in human stool by gas-chromatography: comparison of different methods

Bile Acid	Current Method ^a	Soxhlet Extraction ^b	Folsch Extraction ^c	NaOH/Extraction ^d
	<i>μg/mg dry stool</i>			
LCA ^e	3.1 ± 1.2	2.5 ± 0.9	2.8 ± 1.1	2.2 ± 0.6
IsoDCA	1.0 ± 0.5	0.9 ± 0.5	1.0 ± 0.5	0.9 ± 0.5
DCA	3.1 ± 1.4	3.2 ± 1.5	3.1 ± 1.7	3.0 ± 1.2
Other ^f	0.5 ± 0.5	0.3 ± 0.3	0.4 ± 0.3	0.4 ± 0.3
Total	7.7 ± 2.8	6.9 ± 2.7	7.3 ± 2.8	6.5 ± 2.2

Mean ± SD from five healthy subjects. Gas-chromatographic conditions are described in Table 1.

^a Freeze-dried stool (10–15 mg), to which 20 μg of each of nor-cholic acid and 5α-cholestane was added, was directly subjected to n-butyl ester formation followed by TMS ether derivatization. After addition of hexane (200 μl), 2 μl was injected into the gas-chromatograph. Bile acids were quantitated against nor-cholic acid as internal standard.

^b Freeze-dried stool (10–15 mg), to which 20 μg of each of nor-cholic acid and 5α-cholestane was added, was continuously extracted for 18 h with ammoniacal ethanol in a Soxhlet extractor. Sterols were extracted with n-hexane and then bile acids were extracted with ethyl acetate and subjected to n-butyl ester formation followed by TMS ether derivatization. After addition of hexane (200 μl), 2 μl was used for GC as described above.

^c Freeze-dried stool (10–15 mg) was extracted with chloroform-methanol 2:1 (4 × 5 ml). After evaporation of solvents, the residue was taken in 0.5 N sodium hydroxide and sterols were extracted with n-hexane. Bile acids were then extracted with ethyl acetate and subjected to n-butyl ester formation followed by TMS ether derivatization. After addition of hexane (200 μl), 2 μl was used for GC as described above.

^d Freeze-dried stool (10–15 mg) was digested with 1 N sodium hydroxide at 90°C for 1 h, diluted with water (5 ml), and sterols were extracted with n-hexane. Bile acids were then extracted with ethyl acetate and subjected to n-butyl ester formation followed by TMS ether derivatization. After addition of hexane (200 μl), 2 μl was used for GC as described above.

^e LCA, 3α-hydroxy-5β-cholanoic acid; DCA, 3α,12α-dihydroxy-5β-cholanoic acid; Iso-DCA, 3β,12α-dihydroxy-5β-cholanoic acid.

^f Includes chenodeoxycholic acid, cholic acid, ursodeoxycholic acid, ursocholic acid, 3-oxo,12α-hydroxy-5β-cholanoic acid, and 12-ketolithocholic acid.

we digested freeze-dried stool (together with nor-cholic acid as internal standard) with sodium hydroxide and extracted out neutral sterols followed by extraction of bile acids which were directly derivatized for GLC. A comparison of this method with earlier methods that used more rigorous solvent extraction for isolation of fecal bile acids (8, 9) showed lower levels of lithocholic acid by sodium hydroxide/solvent extraction of the stool (Table 2) thus suggesting that this bile acid needed more rigorous solvent extraction. All methods were, however, generally comparable for quantitation of fecal sterols (Table 2). Thus, the non-polar fecal sterols are quantitatively isolated by repeated extraction with hexane, whereas the more polar bile acids need rigorous extraction procedures. It is also likely that the bile acids are tightly bound to the bacterial debris in the stool and are, therefore, difficult to extract quantitatively (21).

Of the several other methods reported for extraction of fecal bile acids, we were attracted by the method of van den Ende et al. (17) where they used methanol-concentrated hydrochloric acid to extract bile acids from fecal samples. As the use of methanol-hydrochloric acid would result in at least partial methyl ester formation of bile

TABLE 4. Sterol analysis in human stool by gas-chromatography: comparison of different methods

Sterol	Current Method ^a	Soxhlet Extraction ^b	Folsch Extraction ^c	NaOH/ Extraction ^d
	<i>μg/mg dry stool</i>			
Coprostanol	9.3 ± 5.6	9.1 ± 5.9	9.2 ± 5.3	9.0 ± 5.1
Cholesterol	11.6 ± 7.1	11.9 ± 8.1	11.3 ± 7.7	11.4 ± 7.3
24-Methylcoprostanol	1.0 ± 0.2	0.9 ± 0.2	0.9 ± 0.2	1.1 ± 0.3
Campesterol	2.5 ± 1.1	2.4 ± 0.9	2.3 ± 1.0	2.2 ± 1.2
24-Ethylcoprostanol	0.6 ± 0.2	0.5 ± 0.2	0.7 ± 0.3	0.6 ± 0.3
Stigmasterol	1.3 ± 0.4	1.4 ± 0.2	1.4 ± 0.5	1.4 ± 0.3
Sitosterol	3.5 ± 1.2	3.9 ± 1.4	3.7 ± 1.1	3.3 ± 1.5
Total	29.8 ± 12.7	30.1 ± 13.2	29.5 ± 12.9	29.0 ± 13.3

Mean ± SD from five healthy subjects. Gas-chromatographic conditions are described in Table 1.

^a Freeze-dried stool (10–15 mg), to which 20 μg of each of nor-cholic acid and 5α-cholestane was added, was directly subjected to n-butyl ester formation followed by TMS ether derivatization. After addition of hexane (200 μl), 2 μl was used for GLC. Sterols were quantitated against 5α-cholestane as internal standard.

^b Freeze-dried stool (10–15 mg) was continuously extracted for 18 h with ammoniacal ethanol, in a Soxhlet extractor; sterols were extracted with n-hexane and subjected to TMS ether derivatization. After addition of hexane (200 μl), 2 μl was used for GLC as described above.

^c Freeze-dried stool (10–15 mg) was extracted with chloroform–methanol 2:1 (4 × 5 ml). After evaporation of solvents, the residue was taken in 0.5 N sodium hydroxide and sterols were extracted with n-hexane and subjected to TMS ether derivatization. After addition of hexane (200 μl), 2 μl was used for GLC.


^d Freeze-dried stool (10–15 mg) was digested with 1 N sodium hydroxide at 90°C for 1 h, diluted with water (5 ml), and sterols were extracted with n-hexane and subjected to TMS ether derivatization. After addition of hexane (200 μl), 2 μl was used for GLC as described above.

acids in the presence of small amounts of water (17), we considered that this would enhance extraction of bile acids, particularly lithocholic acid, from the feces, bile acid methyl esters being more soluble in organic solvents. Thus, hydrochloric acid may free the bile acids bound to the bacteria with simultaneous esterification. With this in mind, we considered treating the freeze-dried stool directly with n-butanol/hydrochloric acid with the aim to extract fecal bile acids and convert them into their n-butyl esters. Treatment of the stool contents with Sil-prep would now form the TMS ethers of the bile acid esters and of the neutral sterols, which could be solubilized in n-hexane and used for GLC in the usual way. As we have already shown that the n-butyl ester–TMS ethers of bile acids are well resolved from the TMS ethers of sterols, including sitosterol, on capillary GLC (14), and as fatty acids are known to elute significantly earlier from the column than sterols, fecal sterols and bile acids could be easily analyzed in a single chromatographic run (Fig. 1).

When compared with two methods that used rigorous solvent extraction for isolation of bile acids, our method was found to be highly compatible for both fecal sterol and bile acid analysis (Tables 3 and 4) and lithocholic acid, the highly insoluble and most difficult bile acid to isolate quantitatively from the stool, was at least as well extracted by the present method as by the solvent extraction methods. On the other hand, our direct derivatization procedure is very simple and all extractions and pre-purification of bile acid fractions are eliminated. In fact, the whole procedure amounts to the direct derivatization of stool for GLC analysis as if bile acids were not tightly bound to the bacterial debris. The method is thus extremely convenient for screening purposes when large number of samples need to be analyzed. The weakness of the method is that it cannot be used for fecal bile acid

analysis under certain pathological conditions, where bile acids in the stool may remain conjugated with glycine and/or taurine. An added advantage of n-butyl ester derivatization with hydrochloric acid at elevated temperatures is that any sterol esters are also hydrolyzed. Thus, in an experiment when cholesterol stearate and cholesterol heptadecanoate were subjected to the above derivatization procedure, both compounds were completely hydrolyzed to produce the TMS ether of cholesterol and the n-butyl ester of the corresponding fatty acid. Ironically, treatment with methanol–hydrochloric acid at room temperature for 4 h failed to hydrolyze these fatty acid esters of cholesterol while complete hydrolysis was observed at 60°C. It is likely that polymerized or esterified deoxycholic acid, suspected to be present in feces (22, 23), is also converted into deoxycholic acid under the acidic conditions used for derivatization.

In summary, we have developed a highly simplified method for fecal sterol and bile acid analysis where the bile acids are directly converted into their n-butyl ester–TMS ether derivatives and sterols are converted to the TMS ether derivatives, without prior isolation from the stool. In this method, the elaborate extraction of bile acids from stool, removal of neutral sterols, and chromatographic purification of bile acids prior to derivatization for GLC are avoided. This not only renders the method easy to perform but the usual errors that occur during multiple extractions are also eliminated. Under most circumstances, the method is applicable to stool analysis, where more than 98% bile acids are present in unconjugated form and their n-butyl ester–TMS ethers can be directly formed. However, this method for fecal bile acid analysis is not applicable in certain pathological conditions or in germ-free animals, where conjugated bile acids are expected in the stool. We have used this method for quantitation of bile acids in human stool and obtained amounts of bile acids com-

parable to those obtained by established methods that used rigorous solvent extraction of bile acids and removal of neutral sterols before derivatization and quantitation by GLC. 

This work was supported by U.S. Public Health Service grant HL-17818, a grant from the Veterans Affairs Research Service, Washington, DC, and National Cancer Institute grant PO1-CA-41108. The contents of this publication are solely the responsibility of the authors and do not necessarily represent the official views of the National Cancer Institute.

Manuscript received 21 September 1998 and in revised form 5 February 1999.

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