notes on methodology

Hyocholic acid as internal standard for quantitation of human fecal bile acids

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Summary After hyocholate is added to a fecal homogenate, the bile acids are extracted, purified by thin-layer chromatography, and quantitated by gas–liquid chromatography as methyl ester trifluoroacetates on QF-1 columns.

Supplementary key words gas–liquid chromatography · trifluoroacetates

Quantitative analysis of fecal bile acids is an essential part of studies concerned with sterol balance (1), and a number of methods have been described (2–4). In the method of Grundy, Ahrens, and Miettinen (2), labeled bile acids are added to the fecal homogenates to correct for losses up to but not including GLC. The bile acid methyl esters are then quantitated as their trimethyl silyl ethers, using 5α-cholestane as an internal standard. These authors measured total bile acid peak areas rather than identifying and measuring individual bile acids. Ali, Kuksis, and Beveridge (3) measured individual bile acids as the methyl ester trifluoroacetates on QF-1 columns. They used chenodeoxycholic acid as an internal standard only for the GLC step. Evrard and Janssen (4) were the first to use an internal standard (23-nordeoxycholic acid) that could be added at the initial stage of the fecal bile acid determination and followed through the entire procedure. These authors oxidized the bile acids with chromic acid and then estimated the total bile acids within a particular class (dihydroxy, trihydroxy, etc.).

This report describes the use of hyocholic acid as an internal standard that can be followed through the entire procedure for fecal bile acid determination, including GLC measurement of the individual bile acid methyl esters as their trifluoroacetates.

Reference cholic, chenodeoxycholic, deoxycholic, lithocholic, hyocholic, and hyodeoxycholic acids were obtained commercially and purified by TLC and crystallization (5, 6). [24-^{14}C]Cholic acid (sp act, 30–50 mCi/mmole) was purchased from New England Nuclear, Boston, Mass. It was >97% pure as determined by TLC (radiochromatogram scanner, Packard Instrument Co., Downers Grove, Ill.). Randomly tritiated chenodeoxycholic acid (0.25 mCi/mg) was kindly furnished by Dr. B. A. Kottke. It was prepared by Tracer Lab, Waltham, Mass., using the Wilzbach technique and purified by TLC with free bile acid solvent systems (7, 8). Sodium [24-^{14}C]taurocholate (sp act, 1–5 mCi/mmole) (from Tracer Lab) was furnished to us by Dr. Alan Hofmann. It was >95% pure as determined by radioactivity scanning after TLC with solvent systems used to separate bile acid conjugates (7, 8). Fecal samples were obtained from patients with type II or type IV hyperlipoproteinemia.

Usually, 2-g samples of fecal homogenate were weighed into 100-ml stoppered flasks. To this, 50 μl of labeled free or taurine-conjugated bile acid (0.1–1.0 mg) or 2 mg of hyocholic acid, or both, was added. The samples were then subjected to mild saponification at 110°C for 1 hr (20 ml of 1 N NaOH in 90% ethanol) using the Temp-Blok module heater (Lab-Line Instruments, Melrose Park, Ill.) in which only a quarter of the tube was inserted. The neutral sterols were extracted with petroleum ether as described by Miettinen, Ahrens, and Grundy (9). To the remaining aqueous phase, usually 25–27 ml, 2 ml of 10 N NaOH and 5 ml of ethanol were added, and the mixture was heated for 5 hr at 110°C in a Temp-Blok module heater. Heating for 5 hr was carried out in a 100-ml tube with a quarter of the tube inside the heater. Due to the condensing effect of the long tube, the total volume did not decrease by more than 5–8 ml during the boiling. After cooling, the mixture was brought back to its original volume with ethanol, if needed, and the pH was brought to 2–3 with concentrated HCl. After additional cooling, each tube was extracted once with 50 ml of chloroform–methanol 2:1 (v/v) and twice with 50 ml of chloroform. The organic phases were pooled, evaporated to dryness, and made up to 20 ml with chloroform–methanol 2:1, and 0.2-ml aliquots were taken for counting. About 2–4 ml of the chloroform–methanol 2:1 extract was then dried and redissolved in 0.5 ml of ethyl ether–methanol 1:1, methylated with diazomethane (6), and then subjected to TLC.

The thin-layer plates were developed first in benzene to remove fatty acids and then in isooctane–isopropanol–acetic acid 60:20:0.5 (v/v/v) (2). The area containing the bile acids down to the origin was scraped into vials. The bile acid methyl esters were eluted from the gel with two 25-ml portions of methanol and then with 25 ml of methanol–acetone 1:9 (v/v). The bile acids were analyzed by GLC on 1% QF-1 (100–120 mesh) as their trifluoroacetates and were identified by their retention times as described in detail previously (7, 8). An F & M model 402 high-efficiency gas chromatograph was used; operating conditions were: column, 215°C; flash heater, 240°C; detector, 250°C; carrier gas, helium, 50 ml/min.

The efficiency of the extraction and of the subsequent steps preceding GLC was initially checked by adding a

Abbreviations: GLC, gas–liquid chromatography; TLC, thin-layer chromatography.

TABLE 1. Reproducibility of method and recovery of added bile acids

Experiment	Bile Acid Added	Recovery, Mean ± SD
	Saponification and extraction[a]	%
1	[^{14}C]Cholic acid[b]	91.1 ± 5.1 (9)
2	[^{14}C]Taurocholic acid[c]	88.0 ± 1.3 (5)
3	[^{14}C]Chenodeoxycholic acid[d]	90.3 ± 2.8 (5)
4	Hyocholic acid[e]	87.7 ± 2.2 (5)
	Thin-layer chromatography	
1	[^{14}C]Cholic methyl ester[f]	96.7 ± 3.9 (5)
2	[^{14}C]Chenodeoxycholic methyl ester[g]	96.5 ± 2.3 (4)
3	Hyocholic methyl ester[h]	95.2 ± 1.5 (5)

[a] About 2 g of fecal homogenate containing labeled bile acids or hyocholic acid was saponified and extracted as described in the text. Aliquots of the extracts were taken for counting.

[b] Mass of cholic acid, 0.24–2.4 μmoles; radioactivity added (dpm), 46,879–50,052.

[c] Mass of taurocholic acid, 0.38–1.1 μmoles; radioactivity added (dpm), 13,554–16,642.

[d] Mass of chenodeoxycholic acid, 0.24–2.4 μmoles; radioactivity added (dpm), 75,554.

[e] Measured by GLC with hyodeoxycholic acid as internal standard; mass of hyocholate, 1.2–4.8 μmoles.

[f] Mass of cholic acid methyl ester, 0.24–2.4 μmoles; radioactivity added (dpm), 11,980–18,755.

[g] Mass of chenodeoxycholic acid methyl ester, 0.24–2.4 μmoles; radioactivity added (dpm), 12,334–15,515.

[h] Measured by GLC with hyodeoxycholic acid as internal standard; mass of hyocholic acid, 1.2–2.4 μmoles.

known amount of [^{14}C]cholic acid and calculating its recovery. The mean recovery of added cholic acid was 91.1 ± 5.1% through the extraction and saponification procedures (Table 1). The mass of cholic acid in the radioactive standard was 0.24–2.4 μmoles; this difference in mass did not affect the recovery of the labeled cholic acids. With [^{14}C]taurocholate (0.38–1.1 μmoles) the mean recovery was 88.0 ± 1.3%. The difference, 3.1%, in the mean recoveries of free and conjugated bile acids during saponification and extraction procedures is of particular interest to show that no specific loss of conjugated bile acid occurred during hydrolysis procedures. These recoveries also validate the efficiency of the extraction and saponification procedures. The recovery of tritiated chenodeoxycholic acid and hyocholic acid paralleled that of cholic acid (Table 1).

During TLC of standard bile acid methyl esters and bile acid methyl esters obtained from feces (0.24–2.4 μmoles) and subsequent elution, the recoveries were nearly quantitative (Table 1). Under these conditions the elution of hyocholate (up to 2.4 μmoles) exceeded 93%. It can be seen from Table 1 that the elution of hyocholic acid methyl ester was comparable to that of the methyl esters of cholic and chenodeoxycholic acids. For successful elution of fecal bile acids exceeding 2.4 μmoles, it is desirable to use up to 50 ml of methanol and, if necessary, a final elution with 25 ml of methanol–acetone 1:9 (v/v). During the hyocholate recovery studies it was found necessary to scrape the bile acid area very close to the origin during TLC to obtain maximal yields. The recoveries during extraction and TLC of hyocholate and labeled bile acids

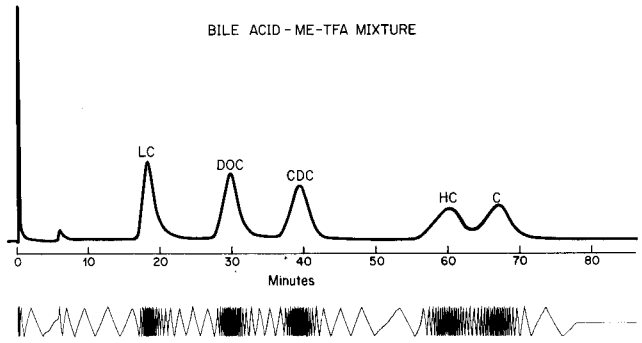

Fig. 1. GLC of artificial mixture of bile acid methyl ester trifluoroacetates on 1% QF-1 column. Column conditions as described in the text. Peak identification: LC, lithocholic; DOC, deoxycholic; CDC, chenodeoxycholic; HC, hyocholic; and C, cholic. Molar composition of the mixture (%): LC, 20.4; DOC, 21.8; CDC, 20.8; HC, 18.5; and C, 18.5. Total weight of the mixture was 52.2 mg; weight of the mixture injected was 20 μg.

added to the feces by the present procedure were comparable with those of Grundy et al. (2).

The GLC estimation of the bile acid methyl esters as trifluoroacetates was validated (Fig. 1) by making mixtures of known weights of bile acids and checking their relative responses as described previously (7, 8). In the range of 0.2–5 μg, a relatively linear response was obtained for all bile acids. The responses of various dihydroxy bile acids were similar. The detector response and retention times of other bile acids in relation to hyocholic acid are summarized in Table 2. It can be seen that the molar response of hyocholic acid is comparable to that of other bile acids. It is noteworthy that hyocholic acid is well separated from all other fecal bile acids and does not overlap with any known keto acids (Fig. 2). The above results show that hyocholic acid and other bile acids are recovered to comparable extents during extraction, TLC, and GLC. The overall recovery of hyocholate (1.2–4.8 μmoles) during the whole procedure, monitored by GLC with a known amount of hyodeoxycholate, was 80.6–90.1%.

The use of hyocholic acid as an internal standard for fecal bile acid determination is justified because: (*1*) it can be followed through the entire procedure along with other bile acids; (*2*) it is not present in normal human feces (10); and (*3*) it can be completely resolved from cholic acid and other bile acids that occur in normal human feces. The separation shown for the bile acid trifluoroacetates agrees well with previous reports (3, 7). In our analysis of fecal samples from eight subjects, the concentration of cholic acid was less than 0.5% of the total bile acids.

This method also can be used when the feces contain significant amounts of cholic acid, which might occur in patients treated with antibiotics. With fecal samples containing up to 5 μg of cholic acid per injection (0.5 mg/g feces), there was practically no overlap of its peak with hyocholate. With up to 10 μg per injection, there was an

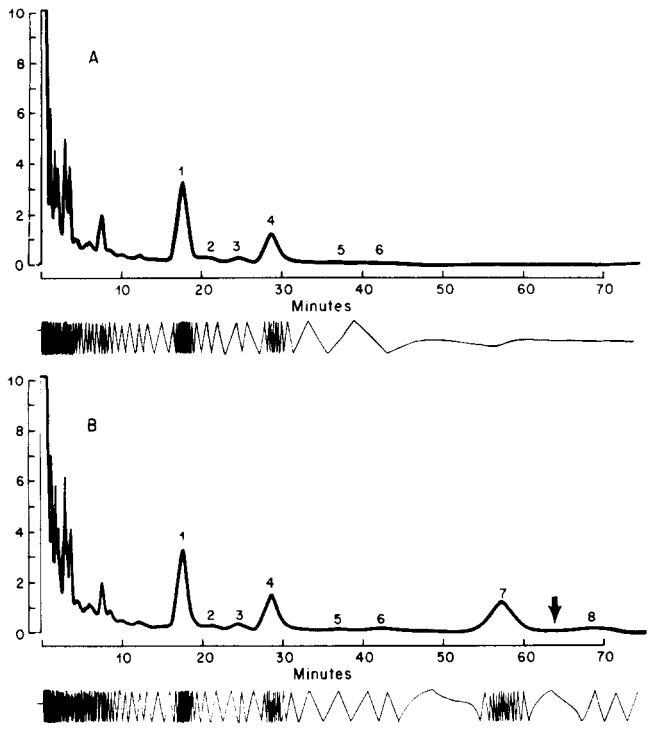

Fig. 2. GLC separation of human fecal bile acids as their methyl ester trifluoroacetates on QF-1 columns in absence (*A*) and presence (*B*) of internal standard, hyocholate. Peak identification: 1, lithocholate; 2, unknown; 3, 3β,12α-dihydroxycholanoate; 4, deoxycholate; 5, 3α,12β-dihydroxycholanoate; 6, chenodeoxycholate; 7, hyocholate; and 8, 7-ketolithocholate. Column conditions as described in text. Arrow indicates the position of cholic acid, if present (retention time relative to hyocholate, 1.12).

overlap of less than 5%. When extraordinary concentrations of cholic acid are found, fecal samples with (*A*) and without (*B*) hyocholate should be processed. The ratio of cholic acid to another bile acid (for example, deoxycholic acid) in sample *B* (with no hyocholate) should be determined. By using this ratio, the cholic acid concentration in sample *A* can be calculated accurately and added to the other bile acid amounts.

In this study, the following fecal bile acid compositions (%) were obtained: lithocholic acid, 57.3; deoxycholic acid, 35.0; 3β,12α-dihydroxycholanoic acid, 3.3; unknown acids, 1.2; chenodeoxycholic acid, <0.2; cholic acid, <0.5; and 3-keto-7α-hydroxycholanoic and 7-ketolithocholic acids, 2.0. In six samples (from the same day's fecal collection) processed simultaneously, the mean ($\pm$ SD) concentration of total bile acids was 2.69 $\pm$ 0.25 mg/g of feces.

Hyocholate also can be used as an internal standard for fecal bile acid determination in both normal and steatorrheic stools. In steatorrhea, stools might contain large amounts of fatty acids after saponification. The fatty acids can be purified by chromatography on 0.5-mm-thick layers of silica gel G. Alternatively, the fatty acids can be purified by column chromatography as described by Ali et al. (3) or Grundy et al. (2). However, in our experience with fecal samples from a number of normal and hypolipoproteinemic subjects, purification by column chromatography was not found necessary.

TABLE 2. Retention times and detector response of various bile acids in relation to hyocholic acid [a]

Bile Acid	Retention Times Relative to Hyocholic Acid	Percentage Molar Response Relative to Hyocholic Acid [b]
Lithocholic	0.31	105.1
Deoxycholic	0.50	103.3
Chenodeoxycholic	0.66	103.1
Hyodeoxycholic	0.75	103.1
Cholic	1.12	99.6
7-Ketolithocholic	1.33	99.3

[a] As methyl ester trifluoroacetates on 1% QF-1 (100–120 mesh on Chromosorb W). Other conditions as described in the text.
[b] Linear range of response up to 5 μg.

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