RAPID ANALYSIS OF HUMAN FECAL BILE ACIDS

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ABSTRACT

A rapid, accurate, precise method for determining human fecal bile acids is reported. Feces are homogenized and then briefly extracted with boiling absolute ethanol. A portion of the extract is evaporated to dryness and the residue heated with mild alkali to hydrolyze bile acid 3α-hydroxyl esters. Aliquots of hydrolyzed crude extract are treated with resazurin reagent which effects a series of enzyme catalyzed reactions in which bile acid free 3α-hydroxyls are first oxidized to 3-oxo-groups in a reaction catalyzed by 3α-hydroxysteroid dehydrogenase. Resulting protons are transferred to β-nicotinamide adenine dinucleotide, yielding reduced β-nicotinamide adenine dinucleotide (β-NADH). β-NADH then reduces nonfluorescent resazurin to fluorescent resorufin in a reaction catalyzed by diaphorase. Developed fluorescence, which is proportional to the extract aliquots bile acid content, is excited at 565 nm and read at 580 nm, wavelengths which lie in a spectral region in which there is minimal fecal pigment absorption. 3-Oxo-bile acids and bile acid 3α-sulfates are extracted in the procedure but reduction and/or solvolysis is necessary before quantification.

INTRODUCTION

Several problems attend current human fecal bile acid determinations resulting in time-consuming procedures which, in some cases, are of questionable accuracy. First, fecal pigments which interfere with colorimetric, fluorometric and gas-liquid chromatographic procedures, must be removed before quantification. Purification is time-consuming, results in bile acid losses and is, in most cases, incomplete. A second problem is due to the wide variety of bile acids present in fecal extracts (1). Such extracts can be assayed for total free and conjugated and individual bile acids by gas-liquid chromatography (2), but time-consuming purification and derivative preparation procedures are necessary. Enzymatic methods can also be used (3-6). These utilize hydroxysteroid oxidoreductase (HSO) catalyzed oxidation of bile acid 3α-hydroxyls coupled with

proton transfer to β-nicotinamide adenine dinucleotide (β-NAD). Resulting β-NADH is determined colorimetrically or fluorometrically; however, fecal pigments absorbing at 340 nm interfere.

Preliminary studies (7,8) suggest that a fluorometric method involving indirect reduction of nonfluorescent resazurin to fluorescent resorufin by bile acids may overcome these difficulties. In this method, bile acids in crude extracts are determined using a series of reactions (9) in which HSO first catalyzes oxidation of bile acid 3α-hydroxyl groups coupled with proton transfer to β-NAD yielding β-NADH. β-NADH then reduces nonfluorescent resazurin yielding fluorescent resorufin in a reaction catalyzed by diaphorase (10). Fluorescence, which is proportional to the extracts bile acid content, is excited at 565 nm and read at 580 nm, wavelengths which lie in a region of minimal fecal pigment absorption. Studies of the precision, accuracy and sensitivity of the assay are presented which show that it is a simple, rapid and accurate method for determining human fecal bile acids.

MATERIALS (chemicals)

All chemicals were reagent grade and all solvents were distilled before use. Bile acids were purchased from Research Plus Steroid Laboratories, Denville, NJ, USA; Calbiochem, San Diego, CA, USA, and Steraloids, Wilton, NH, USA. Their purity was checked by thin-layer chromatography. Those found impure were purified using preparative thin-layer chromatography. Hydroxysteroid oxidoreductase Grade II (a mixture of EC 1.1.1.50 and EC 1.1.1.51), 3α-hydroxysteroid oxidoreductase (EC 1.1.1.50), diaphorase Grade II-L (EC 1.6.4.3.), β-NAD Grade AA-1 and tris base were purchased from Sigma Chemical Co., St. Louis, MO, USA; silica gel HR from E. Merck, Darmstadt, FGR, and Darco G-60 from Baker Chemical Co., Phillipsburg, NJ, USA. Resazurin was purchased from Aldrich Chemical Co., Inc. Milwaukee, WI, USA and was used without further purification. Deoxycholate-24-^{14}C was obtained from New England Nuclear, Boston, MA, USA. The radiochemical purity of this material exceeded 99%.

Resazurin reagent

Two mg resazurin, 100 mg β-NAD, 6.4 units of HSO and 37 units of diaphorase† were dissolved in 100 ml of 0.05 M pH 7.4 phosphate buffer containing 19.1 mg sucrose, 0.1 μg dithioerythritol (Sigma); 7.5 mg

†Diaphorase concentration can be increased to speed fluorescence development, if desired.

ethylene diamine tetra-acetic acid, and 50 mg bovine serum albumin (Sigma). One unit of HSO was defined to oxidize 1 μmole of substrate per minute in the presence of β-NAD at pH 8.9 and 25°C. One unit of diaphorase was defined to oxidize 1 μmole of NADH per minute at pH 7.5 and 25°C using 2,6 dichlorophenolindophenol as an electron acceptor. This reagent is stable for two days at 3°C. It is stable for at least one month when stored at -40°C.

Resazurin blank reagent

Same composition as resazurin reagent except that HSO is omitted. Same or better stability.

Tris buffer 0.1 M pH 9

Twelve and one-tenth grams of tris base is dissolved in 900 ml water. The pH is adjusted to 9 with 1 M hydrochloric acid and the solution diluted to 1 liter.

Phosphate buffer 0.05 M pH 7.4

Thirty and nine-tenths ml of 0.5 M potassium dihydrogen phosphate and 90 ml of 0.5 M disodium hydrogen phosphate are mixed and diluted to three liters.

Instrumentation

Farrand Model No. 104232 recording spectrofluorometer
Beckman Model No. 25 recording spectrophotometer
Barber-Colman Series 5000 gas-liquid chromatograph, equipped with hydrogen flame detector
Packard Tri-Carb liquid scintillation spectrometer, Model No. 526

METHODS

Human Fecal Bile Acid Determination

The mass of a feces sample (frozen until analyzed) is determined and the material transferred to a blender. Twice the sample's mass of water is added and the mixture thoroughly homogenized. About 20 ml of homogenate is transferred to a weighed 250 ml round bottom boiling flask and its mass determined. Seventy ml of absolute ethanol is added and the flask's content refluxed for 30 minutes. The liquid phase is separated by filtration. The residue is washed three times with 20 ml portions of hot alcohol and the combined liquid phase adjusted to 100 ml. A 4 ml aliquot is evaporated to dryness in a calibrated vial. The residue is dissolved in 2 ml of 3 M NaOH and heated at 100°C for two hours. After cooling, the pH is adjusted to ≈ 9 and the mixture's volume adjusted to 10 ml with 0.1 M pH 9 tris buffer. The sample is now ready for fluorescence development. The scheme outlined in Table I is used when mixing samples and reagents.

Tubes T, H and SS are prepared in duplicate for each sample. Tubes T_{BLK}, H_{BLK} and std are prepared in duplicate for each series of samples using the same lot of resazurin and resazurin blank reagents. In practice, tris buffer, sample aliquot and standard solution are added to 10 ml test tubes first; then, resazurin and resazurin blank reagents are added. The tube's contents are thoroughly mixed and incubated at room temperature for 30 minutes. The samples are then transferred to fluorometer cuvettes and fluorescence determined (excitation at 565 nm, reading at 580 nm). If fluorescence of 'T' mixtures shows that bile acid concentration in the 10 µl sample exceeds 1 µg, a smaller aliquot must be used to avoid exceeding resazurin reagent's linear range.

Calculations:

$$\text{mM bile acid} = \frac{T-H-\left(T_{BLK}-H_{BLK}\right) \times 0.0625^{*}}{SS-T} \times \frac{\text{Total homogenate wt}}{\text{homogenate aliquot wt}}$$

*This factor is the product of the m moles of deoxycholic acid standard (2.5×10^{-6}) and the sample aliquot's dilution factor with respect to the homogenate aliquot (2.5×10^{4}) and must be adjusted if other than a 10 µl sample is used.

TABLE I

ANALYTICAL REAGENT MIXING SCHEME

Tube	Resazurin reagent†	Resazurin blank reagent†	Tris buffer 0.1 M pH 9†	Sample	Standard deoxycholate solution*
	ml	ml	ml	µl	µl
T	0.5	-	2.4	10	-
H	-	0.5	2.4	10	-
SS	0.5	-	2.4	10	10
H_{BLK}	-	0.5	2.4	-	-
T_{BLK}	0.5	-	2.4	-	-
Std‡	0.5	-	2.4	-	10

*2.5×10^{-6} m moles deoxycholic acid/10 µl tris (1 µg deoxycholic acid /10 µl tris buffer)

† See Materials

‡ Std does not enter calculations but is included to check standard recovery (SS-T)

Fecal extract purification

a) Solvent extraction:

A 4 ml aliquot of untreated crude fecal extract (total volume 100 ml) is chosen; approximately 0.1 μCi deoxycholate-24-^{14}C is added, mixed and a 1-2 μl aliquot removed for scintillation counting. The balance of the material is quantitatively transferred to a nickel crucible and evaporated to dryness. The residue is dissolved in 2 ml of 3 M NaOH and the sample autoclaved at 150°F for three hours to hydrolyze any conjugated bile acids. Following hydrolysis, the sample is transferred to a teflon screw-capped test tube and extracted three times with 3 ml of petroleum ether (B.P. 36-54°C) to remove neutral steroids, hydrocarbons and neutral pigments. The petroleum ether phase is discarded and the aqueous phase acidified (pH 1) and extracted three times with 3 ml petroleum ether (B.P. 36-54°C) to remove fatty acids and relatively nonpolar, weakly acidic material. After discarding the petroleum ether phase, the acidified aqueous phase is further extracted three times with 3 ml diethyl ether to recover the "acidic fraction" containing bile acids. The aqueous phase is discarded and the ether phase washed twice with ether-saturated water. The ether fraction is then evaporated to dryness and the residue dissolved in 3 ml of benzene:methanol 1:1 (v/v). This solution is mixed for about a minute at room temperature with 0.5 g Darco G-60, and the residue washed three times with 3 ml aliquots of benzene:methanol 1:1 (v/v). The combined filtrate and washings are evaporated to dryness and the residue dissolved in 10 ml of 0.1 M pH 9 tris buffer. One or two μl of solution is placed in a scintillation counting vial and total deoxycholate-24-^{14}C counts per minute (CPM) determined. Comparison of total CPM in the extract aliquot at the beginning and end of purification makes it possible to determine bile acid loss and make corrections in assays. This procedure can be interrupted at any stage and aqueous phases and extracts analyzed for ^{14}C-activity and bile acid content.

b) Thin-layer chromatography:

Six to 12 μl aliquots of crude fecal extract are applied to 14 channel 20 x 20 silica gel HR coated (250 μ), activated (100°) for one hour, thin-layer chromatography plates. After drying, the plates are developed with petroleum ether:diethyl:ether:glacial acetic acid 80:20:1 (v/v). The developed plates are dried at room temperature and 1 cm areas centered on the origins and containing free and conjugated bile acids, are marked and subsequently transferred to centrifuge tubes. Bile acids are eluted from the silica gel by adding two and four-tenths ml of 0.5 M pH 9 tris buffer to the contents of each tube. The tubes are mixed to suspend the silica gel and then heated for 15 minutes at 100°C.

This procedure effectively separates cholesterol, cholesterol esters, triglycerides, free fatty acids, hydrocarbons and some pigments from free and conjugated bile acids.

Thin-layer chromatographic separation of fecal bile acids

Six to 12 µl aliquots of concentrated (10 times) crude fecal extract are applied to 14 channel 20 x 20 cm silica gel HR coated (250 µ) activated (100°C for one hour) thin-layer chromatography plates. Aliquots of a mixture of standard bile acids are applied to adjacent channels. After drying the plates are developed using ethyl acetate:2,2,4-trimethylpentane:glacial acetic acid 10:10:2 (v/v). Each channel on the developed plate is marked with a sharp stylus at 0.5 cm intervals starting 1 cm below the origin. Five-tenths cm silica gel sections are transferred to individual centrifuge tubes by scraping with a sharpened spatula. Two and four-tenths ml of 0.1 M pH 9 tris buffer is added to each tube. The content of each tube is thoroughly mixed and the tubes places in a boiling water bath for 15 minutes after which they are mixed again. After cooling, bile acids are determined: five-tenths ml of resazurin reagent is added to each centrifuge tube. After standing at room temperature for 25 minutes the tubes are centrifuged (800 g) briefly, and their content transferred to spectrofluorometer cuvettes. Fluorescence is excited at 565 nm and read at 580 nm. Scrapings from unused channels are used as blanks. The bile acid content of each silica gel section is then calculated from the reading.

Tests showed that silica gel HR did not interfere with fluorescence development and that bile acids were quantitatively recovered from plates using the extraction procedure outlined above.

Fluorescence development time curves

Two and four-tenths ml of 0.1 M pH 9 tris buffer is placed in a 3 ml spectrofluorometer cuvette. One to 10 µl of an aqueous or methanolic solution of extract containing bile acid is added and the cuvette's contents mixed. The cuvette is placed in a recording spectrofluorometer; 0.5 ml of resazurin or resazurin blank reagent introduced and the recorder started.

RESULTS AND DISCUSSION

Although a method using resazurin has been successfully employed for direct quantification of nonsulfated serum bile acids (9), the complex mixture of pigments, steroids and other lipids found in crude fecal extracts necessitates studies to prove that bile acid titers determined by the resazurin method (see Methods for procedure) in such extracts are accurate.

A) Fecal pigments

Successful application of the resazurin method for bile acid analysis in crude human fecal extracts requires minimal absorbance in the 500 to

600 nm region. The curve in Fig. 1 is the absorption spectrum of a typical crude human fecal bile acid ethanol extract. Absorption is low, from 500 to 690 nm, but increases rapidly at wavelengths shorter than 450 nm. It therefore appears that fecal pigments should have minimal effects on fluorescence measurements in the resazurin method.

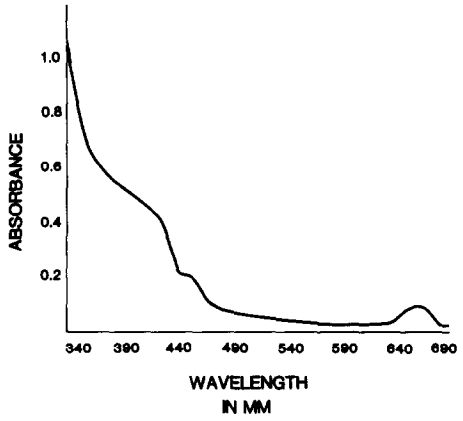

Fig 1 Absorption spectra of an average crude human fecal bile acid ethanol extract.

Fluorescence development - standard bile acids

Since fecal bile acid extracts contain a wide variety of bile acids (1), the development time and stability of fluorescence for a number of these acids treated with resazurin reagent was determined. Fluorescence development time was determined (see Methods) using equimolar quantities of bile acids. The results are shown in Table II.

TABLE II

FLUORESCENCE DEVELOPMENT TIME FOR DIFFERENT BILE ACIDS* TREATED WITH RESAZURIN REAGENT

5β-cholan-24-oic acid	Development time**
	Min.
3α-hydroxy- (lithocholic acid)	8
3α,6α-dihydroxy- (hyodeoxycholic acid)	8
3α,7α-dihydroxy- (chenodeoxycholic acid)	6
3α,12α-dihydroxy- (deoxycholic acid)	5
3α,6α,7α-trihydroxy- (hyocholic acid)	8
3α,7α,12α-trihydroxy- (cholic acid)	9
3α,-hydroxy-7-oxo-	14
3α-hydroxy-12-oxo-	5
3α-hydroxy-7,12-dioxo-	16
3α,12α-dihydroxy-7-oxo-	16
-N-(2-sulfoethyl)-amide of (taurine conjugate of)	
3α-hydroxy-	7
3α,7α-dihydroxy-	4
3α,12α-dihydroxy-	5
3α,7α,12α-trihydroxy-	9
-N-(carboxymethyl)-amide of (glycine conjugate of)	
3α-hydroxy-	8
3α,12α-dihydroxy-	4
3α,7α,12α-trihydroxy-	9

* Equimolar quantities (10 µl of 0.2 mM)
**Average of two determinations

Development time is relatively short for deoxycholic and lithocholic acids, the main bile acids found in most human bile acid extracts (Fig. 3). 7-Oxo-bile acids which are usually present in very small quantities have considerably longer development times. Further tests showed that once fluorescence maxima was attained, readings were stable for at least 90 minutes. The data show that the 30 minute development time used in the method is more than sufficient to effect maximum

fluorescence development when fecal extracts are used. It must be kept in mind, however, that the absolute development time is dependent on bile acid concentration as well as structure. Therefore, extract dilution and aliquot selection are important considerations.

Linearity and sensitivity of the resazurin method - standard bile acids

The linear range and sensitivity of the method was determined using a number of different standard bile acids (cholic, deoxycholic, lithocholic, and 3α-hydroxy-5β-cholan-12-oxo-24-oic acids). For these tests, different amounts of the bile acids were treated with resazurin and resazurin blank reagents. After 30 minutes fluorescence was determined. The data showed that fluorescence readings were the same and directly proportional to concentrations from 0 to 0.02 μmoles (0 to 8 μg for cholic acid) for all bile acids studied.

Human fecal bile acid extract fluorescence development curves

Fluorescence development curves were studied using a number of different crude human bile acid extracts. Fig. 2 presents graphs which illustrate fluorescence development vs time (see Methods) using a typical crude human fecal bile acid extract treated with resazurin reagents prepared with three different grades of hydroxysteroid oxidoreductase (HSO). It is evident that impurities in resazurin reagent prepared with partially purified HSO react with non-bile acid impurities in crude fecal bile extracts yielding slowly increasing amounts of fluorescence with time. On the other hand, extracts treated with resazurin reagent containing column purified 3α-HSO yield satisfactory curves.

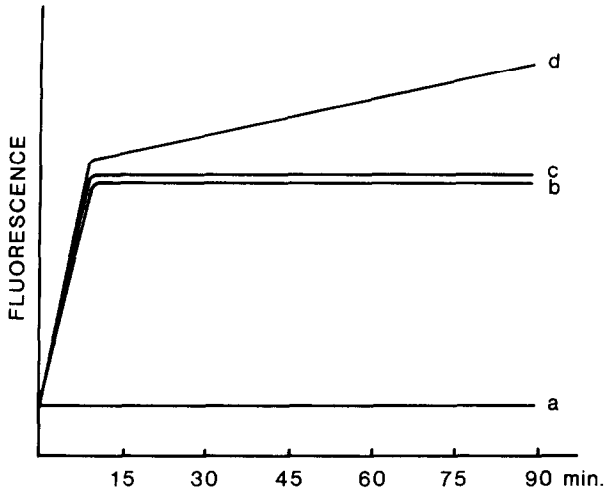

Fig. 2 Fluorescence development curves for crude human fecal bile acid extracts treated with a) resazurin blank reagent, b) resazurin reagent containing Sigma, column purified 3α-hydroxysteroid oxidoreductase, c) resazurin reagent containing Nyegaard column purified 3α-hydroxysteroid oxidoreductase, d) resazurin reagent containing Sigma Grade II partially purified hydroxysteroid oxidoreductase.

The results of these studies show that if crude human fecal bile acid extracts are to be assayed for total bile acids without purification, resazurin reagent prepared with column purified 3α-HSO must be used. If partially purified or crude HSO preparations are used in preparing the reagent, results will be high; in some cases - severely so.

If column purified 3α-HSO is unavailable for resazurin reagent preparation, crude fecal bile acid extracts can be purified by thin-layer chromatography (TLC) before treatment with resazurin reagent. Extracts are applied to TLC plates which are developed with a solvent system (petroleum ether:diethyl ether:glacial acetic acid 80:20:1 (v/v)) which mobilizes impurities but leaves bile acids at the origin (see Methods). Bile acids eluted from these plates yield satisfactory fluorescence development curves when treated with resazurin reagent containing impure HSO.

Bile standard recoveries

Having established that molar fluorescent yield of different bile acids are identical and satisfactory fluorescence development curves are attained when crude fecal bile acid extracts are treated with resazurin reagent, we studied the recovery of a bile acid standard added to different aliquots of crude extract. Duplicate tubes containing 0 to 20 µl of crude human fecal bile acid extract were used. One µg of deoxycholic acid was added to half the tubes. Resazurin reagent was added and fluorescence determined after 30 minutes. The data in Table III show that recoveries were excellent through 15 µl in all cases. In some cases, recoveries decreased when 20 µl of crude fecal extract was used. Since recoveries sometimes decrease with large extract aliquots, standard additions are a necessary part of the procedure.

TABLE III

RECOVERIES OF BILE ACID STANDARD ADDED TO CRUDE HUMAN BILE ACID EXTRACTS

Type of human feces sample	Standard Recovery µg*					
	Extract aliquot (µl)					
	0	2	5	10	15	20
	µg					
Normal	1.01	0.98	1.02	0.98	0.94	0.90
Normal	1.00	1.00	0.98	1.02	0.94	0.86
Low serum HDL	0.98	0.99	1.02	1.00	0.92	0.86
Diarrhea (undefined)	1.02	1.00	1.00	0.96	0.97	0.98

* 1 µg of standard deoxycholic acid added to each aliquot

Identity of fluorescent yield with bile acid titer of human crude fecal bile acid extracts

Although recoveries were good when bile acid standards were added to various aliquots of crude fecal bile acid extract, this does not prove that fluorescence developed when the extracts are treated with resazurin reagent is exclusively due to bile acids. It is possible

that foreign substances, imitating bile acid properties, are present in extracts, giving rise to fluorescence. To investigate this possibility, aliquots of corresponding crude and charcoal purified (see Methods) fecal extracts and a mixture of standard bile acids were chromatographed on adjacent channels of a thin-layer chromatography plate and the distribution of bile acids was determined in each channel (see Methods). The results are shown in Fig. 3. Almost all fluorescence developed in channels containing crude and charcoal purified extracts is found in regions corresponding to those in which deoxycholic, 3α-hydroxy-12-oxo-5β-cholan-24-oic and lithocholic acids are found. This data provides strong evidence that fluorescence developed when crude fecal extracts are treated with resazurin reagent is due to bile acids.

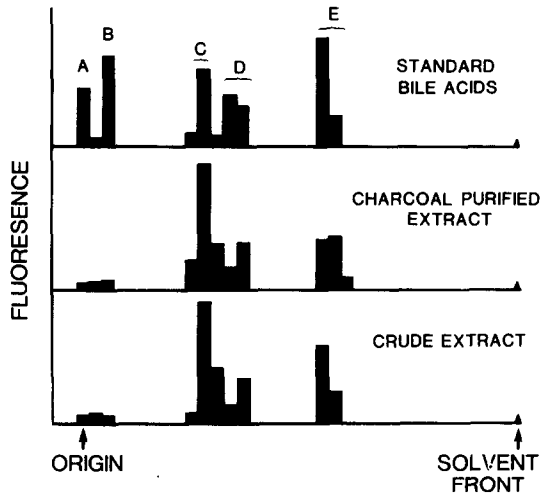

Fig. 3 Thin-layer chromatographs of a mixture of standard bile acids and crude and charcoal purified human fecal bile acid ethanol extracts. Developing solvent: ethyl acetate:2,2,4-trimethylpentane:glacial acetic acid 10:10:2 (v/v). Fluorescence developed with resazurin reagent. Activated silica gel HR 250 μ plates used. A) taurocholic, B) cholic, C) deoxycholic, D) 3α-hydroxy-12-oxo-5β-cholan-24-oic, E) lithocholic acids.

Necessity for mild alkaline hydrolysis of crude human fecal extracts

The bile acid content of fecal bile acid extracts was assayed (see Methods) following varying degrees of purification (see Methods).

Bile acid content was the same for all extracts at purification stages beyond high temperature alkaline hydrolysis. The content of crude unhydrolyzed extracts, however, was 10-50% lower. Studies showed that mild alkaline hydrolysis of crude extracts yielded hydrolysates with the same bile acid content as those derived from high temperature hydrolysis. Treatment of crude extracts with 1 M NaOH at 100°C for two to three hours was optimum. Thin-layer chromatographic studies proved that apparent increased bile acid content of extracts following hydrolysis, was exclusively due to increased bile acid content.

Standard recovery and overall precision of the method

A number of 72 hour human feces samples were collected and repeatedly analyzed. Results are shown in Table IV. Overall precision averaged about 7% and standard recoveries were excellent in all cases. Table IV also contains data which can be used to compare results obtained using the resazurin method and gas-liquid chromatography according to Grundy et al (2). The results agree reasonably well in all cases.

TABLE IV

PRECISION, ACCURACY AND OVERALL RECOVERY OF FECAL BILE ACID DETERMINATION

Human feces sample	Bile acid excretion		Standard recovery (resazurin method)	
	Resazurin method	Gas-liquid chromatography	Added**	Recovered
	mg/24 hr*		mg	
Normal	239 ± 15‡(8)†	252***	25.0	24.5 ± 1.00‡(8)†
Normal	263 ± 11 (8)	-	25.0	24.9 ± 1.52 (8)
Normal	194 ± 10 (8)	181	25.0	25.1 ± 1.32 (8)
Low serum HDL	86 ± 6 (8)	-	25.0	23.8 ± 1.00 (8)
Low serum HDL	126 ± 8 (8)	-	25.0	24.5 ± 1.64 (8)
Diarrhea (unspecified)	630 ± 15 (8)	655	25.0	25.1 ± 1.46 (8)
Diarrhea (unspecified)	302 ± 17 (8)	283	25.0	25.0 ± 1.80 (8)

* In terms of deoxycholic acid
** Deoxycholic acid added to 20 ml of fecal homogenate
† Number of times analyzed
‡ Standard deviation
***Average of two determinations

The goals of the present investigation were to develop an accurate, precise method for determining human fecal bile acids which would be simple and rapid. These goals have, for the most part, been achieved. However, a few problems remain.

First, 3-oxo-bile acids are not determined by the present method since a bile acid 3α-hydroxyl is necesssary to effect resazurin reduction. We have developed a method for determining relatively pure 3-oxo-bile acids (11-13). The oxo-acids are reduced with sodium borohydride yielding 3α- and 3β-hydroxy bile acids. These acids are quantified according to Iwata and Yamasaki (14) using a mixture of 3α- and 3β-hydroxysteroid oxidoreductases to catalyze oxidation. Preliminary studies suggest that quantitative reduction of 3-oxo-bile acids in crude fecal extracts with borohydride is possible. Resazurin reagent containing a mixture of 3α- and 3β-hydroxysteroid oxidoreductase can then be used to quantify the reduced acids. These experiments suggest that 3-oxo-bile acids constitute a very small percentage of human fecal bile acids in almost all cases.

Second, sulfated bile acids while extracted in our procedure are not determined, since even vigorous, high temperature alkali treatment fails to hydrolyze these compounds. Of course, it is possible to include a solvolysis step in the procedure. Unfortunately, solvolysis, as practiced, is very time-consuming (15-16). Further work will be necessary before a shortened procedure possibly can be developed.

Third, free fatty acids, if present in moderate to large quantities in feces, cause analytical difficulties since they inhibit fluorescence development in the resazurin method. If internal standard fluorescence development is inhibited more than 60% in a determination, extract purification is necessary. The method outlined under "Fecal extract purification, thin-layer chromatography" in the Methods Section, is excellent. Sixty-percent fluorescent inhibition, however, is unusual since in analysis of over 100 feces specimens, we have never encountered more than 15% inhibition, and this is unusual.

One very important point to note is that the amount of bile acid in a sample aliquot used for fluorescence development should not exceed

1 μg. If this amount is exceeded, fluorescence in standard addition samples may not develop fully in 30 minutes. This is not as much of a problem as it may seem, however, since we have seldom encountered a sample which exceeded this limit when the dilution schedule outline was followed.

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