A method for the accurate measurement of isotope ratios of chenodeoxycholic and cholic acids in serum

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Summary A method for the extraction of bile acids from serum is described that enables the stable isotopic content of chenodeoxycholic acid and cholic acid to be determined accurately to levels as low as the natural ^{13}C abundance. The method uses Sep-Pak C_{18} reverse phase cartridges both for extraction and purification procedures. Free bile acids, bile acid conjugates, and 3-monosulfated bile acid conjugates are recovered in high yield from the Sep-Pak in methanol–water 75:25 after first removing impurities with hexane and methanol–water 40:60 washes. Other important features of the method include the use of enzymatic rather than alkaline hydrolysis of bile acid conjugates, the use of ammonia as the reagent gas for chemical ionization mass spectrometric measurement of isotopic ratios, and the exclusion of all extraneous components in the final sample from the ion source. This method should be applicable to kinetic studies of bile acids using bile acids labeled with stable isotopes and serum measurements, and provides an alternative sampling point in the enterohepatic circulation to conventional duodenal bile samples requiring intubation.—DeMark, B. R., G. T. Everson, P. D. Klein, R. B. Showalter, and F. Kern, Jr. A method for the accurate measurement of isotope ratios of chenodeoxycholic and cholic acids in serum. *J. Lipid Res.* 1982. **23:** 204–210.

The quantity of a primary bile acid in the enterohepatic circulation and the rate of its loss and replacement by synthesis are customarily estimated by isotope dilution techniques introduced by Lindstedt (1–3). The interpretation of the washout of a tracer dose of bile acid from duodenal bile requires the assumption that the size of the pool is constant during the period of measurement. Under such circumstances, the concentration of label in the bile acid follows a single exponential decay function that can be represented by a model in which the tracer is miscible in a single physiological pool. To date, further deconvolution of bile acid kinetics in human subjects has been limited by two factors. The first is that duodenal bile samples preclude resolution of any short-term events in the kinetic behavior of labeled bile acids, because the gall bladder acts as an integrating chamber for most of the circulating bile acid between meals. Second, the possibility of sampling at other points in the enterohepatic circulation, specifically in peripheral plasma or serum, has not been possible using radioactive tracers. The reason for this limitation is that the concentrations of bile acids in the peripheral circulation normally are so low that accurate measurement of their specific activities would require administration of unacceptably large quantities of radioactivity.

The only practical method for conducting bile acid kinetic measurements in serum depends upon the use of bile acids labeled with stable isotopes. This method is feasible only because the isotopic concentration in the individual bile acids is measured by gas–liquid chromatography–mass spectrometry techniques. These techniques permit the proportion of labeled to unlabeled spe-

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cies of bile acid molecules to be determined in the same sample at the same time in a single measurement. Since these measurements can be made on acceptably small samples (1–4 ml of whole blood), involve no radioactive exposure, eliminate the need for daily duodenal intubation, and offer a different vantage point from which to observe the kinetic behavior of bile acids, we have addressed the central methodological requirement to conduct such measurements.

This requirement is the ability to isolate bile acids from the serum matrix in such purity that the isotopic measurements are independent of the many other organic components present in serum. This ability is demonstrable when the individual bile acids isolated from control serum display the same isotopic composition as pure standards of the same acids. The ability to obtain the correct value for the natural abundance of stable isotopes present in each bile acid corresponds to the attainment of natural background counting statistics in radioactive studies. There is, however, one important difference: the content of ^{13}C relative to ^{12}C is 10^8 higher than the proportion of ^{14}C to ^{12}C in nature. Thus with a natural abundance of approximately 1.11% of all carbon in nature being ^{13}C, a molecule with 24 carbon atoms in its structure, such as a bile acid, has a probability of 24 × 0.0111 or 0.2644 of containing at least one ^{13}C atom. Alternatively, the natural abundance of ^{13}C in such a molecule can be expressed as the ratio of ^{13}C-containing molecules to ^{12}C molecules, as 0.2664 or 26.64%. The ability to detect the presence of (specifically labeled) [24-^{13}C]bile acid would depend upon the accurate measurement of a significant difference above the value of 26.64%.[4] If one is limited to an increase of 10% in this value, as opposed to 1% or 0.1% as a significant increase, the ability to detect the decline in labeled bile acid following oral administration of the tracer would be diminished greatly. Hence the highest possible accuracy and precision are desirable in this measurement.

Procedures for the isolation of bile acids from bile for stable isotope ratio measurements used in the past have been aided by the relatively high concentrations (mmol/ml) present in bile. By contrast, the concentration in serum usually is a few nanomoles per milliliter and samples must be purified extensively to obtain accurate quantitation of isotopic abundance ratios. Since the end measurement is determination of the ratio between two molecular species, the absolute recovery of the individual

bile acids is of less importance than the purity of the samples. Nonetheless, with very small samples, the ion statistics ultimately will depend upon the amount of material recovered.

To date, only one report has appeared on the use of bile acids labeled with stable isotopes for serum bile acid kinetics. Using a clean-up procedure based on Amberlite XAD-2 extraction of serum bile acids, Tateyama et al. (4) reported a correlation between serum and biliary chenodeoxycholate kinetic parameters after administration of [11,12-^{2}H]chenodeoxycholic acid to four subjects. Cholic acid kinetics were not studied and no data were provided on the accuracy of their serum chenodeoxycholate isotope ratio measurements.

We report here a procedure for the purification and analysis of the primary bile acids from normal serum that yields accurate and reproducible isotope ratios at the level of natural isotopic abundance.

EXPERIMENTAL

Materials

Unlabeled bile acid standards were obtained from Supelco, Inc. (Bellefonte, PA) and were more than 98% pure by GLC and TLC. Glycine- and taurine-conjugated bile acids and 3-monosulfated bile acid conjugates, prepared by the methods of Tserng et al. (5–7), were supplied by the Bioanalytical Center at Argonne National Laboratory. ^{14}C-Labeled bile acids were obtained from New England Nuclear Corp. (Boston), Amersham (Chicago), and Mallinckrodt (St. Louis) and were pure by TLC and scintillation counting. Organic solvents were either reagent or certified grade (Fisher Scientific Co., Fairlawn, NJ). Diethyl ether and hexanes were redistilled. 2,2-Dimethoxypropane was used as purchased from Aldrich Chemical Co. (Milwaukee, WI). 2-Mercaptoethanol was obtained from Matheson, Coleman and Bell (Los Angeles, CA); cholylglycine hydrolase (*Clostridium perfringens welchii* extract) was from Sigma Chemical Co. (St. Louis, MO); and Sep-Pak C_{18} cartridges were from Waters Associates (Milford, MA).

Serum bile acid purification

A Sep-Pak C_{18} reversed phase cartridge was attached to a 12-ml plastic syringe and was washed with 10 ml of methanol followed by 10 ml of distilled water (DW), as recommended by the manufacturer. A 5-ml serum sample was diluted with 5 ml of 0.1 N NaOH and introduced onto the Sep-Pak cartridge via the syringe and a Harvard infusion pump at a rate of 1 ml/min.

The Sep-Pak then was washed at 3–5 ml/min in the following sequence: 5 ml of 0.1 NaOH, 10 ml of DW,

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[4] This calculation omits, for simplicity's sake, the contributions of ^2H, ^{15}N, and ^{17}O made to the abundance of the isotopic satellite occurring at one mass unit above the main molecular species, and also neglects the additional carbon atoms introduced by the derivatization methods used to prepare the bile acids for gas–liquid chromatography–mass spectrometry. Routine programs that compute the M + 1/M as well as M + 2/M ratios are available for any molecular structure of known composition.

10 ml of hexane, 10 ml of DW, and 10 ml of methanol–DW 40:60. The hexane wash is essential to prevent a substantial loss of bile acid material in the methanol–DW 40:60 wash. Free or conjugated bile acids were eluted with 20 ml of methanol–DW 75:25 at 1 ml/min. The eluate was collected in a round-bottom flask and the solvent was removed by flash evaporation at 37°C. The residue was dissolved (with sonication) in 1.0 ml of DW and solvolysis was accomplished by the method of Javitt, Lavy, and Kock (8) by adding 7.0 ml of 2,2-dimethoxypropane and 2 drops of concentrated HCl. After a 2-hr reaction time at room temperature, the reaction mixture was transferred to a test tube and taken to dryness under N_2. Enzymatic hydrolysis was performed according to the method of Karlaganis and Paumgartner (9). The residue remaining after solvolysis was dissolved in 4 ml of DW and a few drops of 0.2 M acetic acid were added to adjust the pH to 5.6–6.0. This was followed by 0.4 ml of 0.025 M Na acetate buffer (pH = 5.6), 0.2 ml of 0.75% mercaptoethanol, 0.2 ml of 1.86% EDTA, and 5 units of cholylglycine hydrolase (12 U/mg). A unit of enzyme activity is defined as the amount of enzyme necessary for hydrolysis of 1 μmole of cholylglycine in 5 min at pH 5.6, 37°C. After a 2-hr reaction time at 37°C, the hydrolysis mixture was cooled to room temperature, the pH was adjusted to 1.0 with concentrated HCl, and the free bile acids were extracted three times with 7 ml of diethyl ether. The combined ether extracts were dried under N_2 at 37°C. The free acids were converted to their methyl esters by reaction with 2 ml of methanol, 2 ml of 2,2-dimethoxypropane, and 1 drop of concentrated HCl, at room temperature for 30 min (10). Solvents were removed under N_2 at 37°C and the acetate derivatives of the bile acid methyl esters were formed by the procedure of Roovers, Evrard, and Vanderhaeghe (11). To the dried methyl esters was added 0.5 ml of a solution of 10 ml of acetic anhydride, 14 ml of acetic acid, and 0.1 ml of 70% perchloric acid at 0°C for 15 min. The reaction was quenched by the addition of 4 ml of DW and the resulting suspension was vortexed and then injected onto a clean Sep-Pak C_{18} cartridge previously washed with methanol and DW as before. The adsorbed bile acid derivatives were washed with 10 ml of DW and the non-polar impurities, which included the major proportion of cholesterol acetate and acetylated methyl lithocholate, were removed with a 3.0-ml heptane wash. After an additional 10-ml DW wash, the diacetylated and triacetylated bile acid methyl esters were eluted with 20 ml of methanol–DW 75:25 at a fluid rate of 1 ml/min. The eluate was taken to dryness by flash evaporation and the residue was transferred to Reacti-Vials (Pierce, Rockford, IL) with acetone. The solvent was removed under N_2 at 22°C and the vials were sealed with a Teflon-lined cap and stored at 4°C until ready for analysis.

Recovery of bile acids, bile acid conjugates, bile acid sulfates, and bile acid methyl ester acetates

In order to establish the appropriate washing and elution conditions for bile acid conjugates, pooled serum spiked with glycine or taurine conjugates of lithocholic acid (LCA), deoxycholic acid (DCA), chenodeoxycholic acid (CDCA), and cholic acid (CA) was carried through the Sep-Pak procedure. Qualitative TLC (12) was employed to test for the presence of the eight conjugates in the hexane and aqueous methanol extracts and in a subsequent 100% methanol wash. Similarly, the elution characteristics of sulfated bile acid conjugates were monitored by TLC (6, 7) to identify the fraction containing the 3-monosulfated glycolithocholate, taurolithocholate, glycocholate, and taurocholate after these sulfated bile acid conjugates were subjected to the identical extraction procedure as the nonsulfated conjugates.

Recovery in the initial Sep-Pak extraction was measured as follows: ^{14}C-labeled DCA, CDCA, and CA, as well as ^{14}C-labeled glycine and taurine conjugates of DCA and CA were added to aliquots of pooled serum. This serum was carried through the initial Sep-Pak extraction procedure described above and the percent recovery in the methanol–DW 75:25 eluate was determined.

The recovery of the three major bile acid methyl ester acetates in the final Sep-Pak purification step was determined as follows. ^{14}C-Labeled DCA, CDCA, and CA were methylated and acetylated as described above and the specific activity of each was determined prior to adsorption onto the Sep-Pak. This specific activity then was compared to the specific activity in the methanol–DW 75:25 eluate.

Since ^{14}C-labeled glycine conjugates of LCA, DCA, and CDCA, and the taurine conjugate of CDCA were not available for direct recovery measurements, we compared the distribution of standard mixtures of unlabeled glycine and taurine conjugates before and after the first Sep-Pak extraction and elution. Relative recovery was estimated by GLC analysis (13) of the bile acid methyl ester acetate derivatives. Similarly, we compared the relative recovery of the bile acid methyl ester acetate derivatives before and after the second Sep-Pak extraction and elution.

Isotope ratio measurements

Methyl ester acetate derivatives of normal serum bile acids were analyzed by GLC–MS stable isotope ratiometry. The gas chromatograph was a Varian series 1400 (Varian Associates, Palo Alto, CA) with a glass column (1 mm × 183 cm) packed with 1% Poly S-179 (13) on 100/120 mesh Gas Chrom Q (Applied Science Labs, Inc., State College, PA). Column temperature was maintained isothermally at 265°C; the injector and GLC out-

let-mass spectrometer inlet temperatures were 285°C. Helium was the carrier gas at a flow rate of 9.0 ml/min.

Under these conditions the chenodeoxycholate derivative had a retention time of 11.0 min and the cholic acid derivative emerged at 14.5 min. To minimize source contamination, the GLC effluent was directed to a waste flow connection for the first 7.5 min of each analysis. After the cholic acid derivative eluted from the column, the GLC effluent again was switched to a waste flow connection and the column oven temperature was raised to 305°C for 5 min. Mass spectrometry was carried out in a Biospect quadrupole mass spectrometer (Scientific Research Instruments, Baltimore, MD) operating in the chemical ionization mode with ammonia reagent gas. The ion source temperature was maintained at 160°C and the source pressure was 1.0 torr. Under ammonia chemical ionization conditions, the most abundant ions produced for unenriched methyl ester acetates of chenodeoxycholate and cholate are the ammonium adduct ions (M + 18) at m/z 508 and m/z 566, respectively (14). A stable isotope ratiometer-multiple ion detector (SIRMID), designed and constructed at Argonne National Laboratory, Argonne, IL (15), was employed to quantitate the ratio of the ion intensities 509/508 for the chenodeoxycholic acid derivative at unenriched, or natural isotopic abundance. Similarly, the ratio of the ion intensities 567/566 was quantitated for the derivative of unlabeled cholic acid. These M + 1/M ratio measurements provide the background abundance values necessary for kinetic studies using [24-¹³C]-labeled bile acids.

RESULTS

Recovery experiments from serum using Sep-Pak cartridges

Table 1 shows the efficiency of the Sep-Pak extraction and elution procedure for several radiolabeled free bile acids and bile acid conjugates. Greater than 90% recovery of the serum bile acids and bile acid conjugates was achieved in the methanol–DW 75:25 eluate. Qualitative TLC (12) for the presence or absence of bile acids and their conjugates in the successive washes with hexane, methanol–DW 40:60, methanol–DW 75:25, and a final 100% methanol wash gave results consistent with the radiochemical results when a series of spiked serum samples were analyzed. In addition, these TLC results indicated that free LCA and its conjugates were recovered to a similar degree in the methanol–DW 75:25 eluate as CDCA and DCA conjugates. Qualitative confirmation of sulfated bile acid recovery also was obtained in separate TLC analyses (6, 7) of the Sep-Pak washes after extraction from serum spiked with 3-monosulfated gly-

colithocholate, taurolithocholate, glycocholate, and taurocholate.

The achievement of sample purity suitable for isotopic abundance measurements was not without selective losses in the bile acid composition. This is illustrated by the results of further recovery experiments with ¹⁴C-labeled bile acid methyl ester acetates after a second Sep-Pak procedure. This purification step resulted in the complete recovery of the cholic acid derivative, but approximately 15% of diacetylated bile acid methyl esters were lost in the 3.0 ml heptane wash (Table 2). Subsequent GLC (13) analysis of a standard mixture of acetylated bile acid methyl esters indicated that methyl lithocholate monoacetate is removed almost completely by the heptane wash. The chromatograms of Fig. 1 show that cholesterol monoacetate as well as lithocholate derivative are removed with heptane when bile acid derivatives from a bile sample are analyzed before and after Sep-Pak purification.

Isotope ratio measurements

The natural abundance of stable isotopes (isotope ratio) in methyl chenodeoxycholate diacetate and methyl cholate triacetate was measured by GLC–MS–SIRMID in twelve unspiked serum samples from ten different normal volunteers. The results are summarized in Table 3, together with the results obtained for pure standards of the bile acid derivatives. It can be seen that the ratios measured in the serum samples are indistinguishable from those measured in pure standards for both primary bile acid derivatives.

DISCUSSION

The use of bile acids labeled with stable isotopes e.g., 24-¹³C forms, eliminates the radiation hazard associated with ³H and ¹⁴C and permits studies in children (16) and in pregnant women (17). The extension of these techniques to serum samples requires the ability to measure the isotope ratio of isolated bile acids with very high precision during the period of time that the tracer dose is diluted by newly synthesized bile acid. The concentration of ¹³C in the isolated bile acids does not fall to zero, but rather to that level characterized by the natural abundance (isotope ratio) of ¹³C in these molecules. This ratio is known for the pure compounds and, when ammonia chemical ionization mass spectrometry is used to analyze the methyl esters of bile acid acetates, the proportion of molecules with one ¹³C to those with no ¹³C is 33.24% for CDCA and 35.25% for CA. This ability has been demonstrated in the measurement of CDCA and Ca isolated from control sera by the method described in this communication.

Five factors proved to be critical in the achievement

TABLE 1. Recovery of ^{14}C-labeled bile acids, bile acid conjugates, and bile acid methyl ester acetates from Sep-Pak cartridge

		Absolute Percent Recovery[a]		
	N	DCA[b]	CDCA	CA
		$\bar{X} \pm SD$		
Free acids[c]	3	102.0 ± 2.0	95.4 ± 2.3	98.4 ± 1.9
Conjugates[c]				
Glycine	3			95.3 ± 5.1
Taurine	6	91.0 ± 2.6		96.6 ± 5.3
Methyl ester acetates[d]	3	84.3 ± 7.0	86.8 ± 1.0	95.7 ± 5.7

[a] All recoveries were from the methanol–DW 75:25 elution fraction, total volume 20 ml, flow 1 ml/min. The elution system is described under Methods.
[b] Abbreviations: DCA, deoxycholic acid; CDCA, chenodeoxycholic acid; CA, cholic acid.
[c] Recovery using initial Sep-Pak procedure.
[d] Recovery using final Sep-Pak procedure.

of pure bile acid samples for isotopic analysis: the use of the C_{18} Sep-Pak for extraction and purification from serum, the use of enzymatic hydrolysis, the use of a second Sep-Pak clean-up procedure on the derivatized bile acids, the use of ammonia gas for chemical ionization, and, finally, the diversion of the gas chromatographic effluent from the ion source, except during the elution of the peaks of interest.

The use of C_{18} Sep-Pak reverse phase cartridges to extract bile acids and bile acid conjugates from serum is probably the most important aspect of the sample preparation procedure. We found that XAD-2 (18) or XAD-7 (19) resins were unsatisfactory for accurate isotope ratio determinations, especially for cholic acid derivatives. The Sep-Pak cartridge offers many advantages over other methods for the extraction and purification of serum bile acids. Whitney and Thaler (20) have shown that a single cartridge is able to concentrate up to 50 mg of taurocholate or taurocholate-3-sulfate and that recovery of these compounds was complete over a range of concentrations from 1.25 to 12.5 μM and at solvent flow rates of 2–20 ml/min. In this work we have demonstrated further that the cartridge efficiently extracts the four major free bile acids and their glycine and taurine conjugates from serum. The 3-monosulfated glycine and taurine conjugates of LCA and CA also were shown to be extracted, thus extraction of all bile acid conjugates and sulfates from serum can be achieved using the Sep-Pak cartridge.

Once adsorbed onto the Sep-Pak cartridge, bile acids and their conjugates and sulfates can be removed conveniently with 5.0 ml of methanol (20). However, we

TABLE 2. Composition of standard mixtures of bile acid conjugates or methyl ester acetates following Sep-Pak purification

		Relative Percent Distribution			
	N	LCA[a]	DCA	CDCA	CA
		$\bar{X} \pm SD$			
Glycine conjugates	4				
Before Sep-Pak		14.2 ± 1.5	35.3 ± 1.5	22.1 ± 2.7	28.1 ± 2.9
After Sep-Pak I		15.6 ± 2.3	36.9 ± 1.0	21.5 ± 1.5	26.0 ± 0.4
P value		NS[b]	NS	NS	NS
Taurine conjugates	4				
Before Sep-Pak			30.5 ± 0.4	43.6 ± 1.8	25.8 ± 2.1
After Sep-Pak I			37.2 ± 3.4	38.1 ± 2.1	24.7 ± 3.4
P value			<0.05	<0.05	NS
Methyl ester acetates	4				
Before Sep-Pak II		14.2 ± 1.5	35.3 ± 1.5	22.1 ± 2.7	28.1 ± 2.9
After Sep-Pak II		<1.0	35.4 ± 3.6	25.0 ± 1.9	45.3 ± 4.7
P value		≪0.001	NS	NS	<0.01

[a] Abbreviations: LCA, lithocholic acid; DCA, deoxycholic acid; CDCA, chenodeoxycholic acid; CA, cholic acid.
[b] NS, not significant.
Relative percent distribution was determined by GLC.
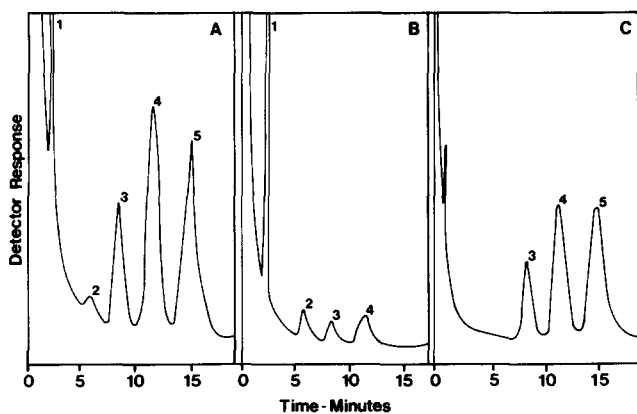
Fig. 1. GLC chromatograms of human biliary bile acid sample after methylation and acetylation. Bile was used for illustration of the separation procedures to enable visualization of the composition of both the retained and discarded fractions. 1A, Before final Sep-Pak purification procedure; 1B, heptane eluant after adsorption to Sep-Pak; 1C, subsequent methanol–DW 75:25 eluate. Peak 1, cholesterol acetate; peak 2, methyl LCA monoacetate; peak 3, methyl DCA diacetate; peak 4, methyl CDCA diacetate; and peak 5, methyl CA triacetate. Chromatographic conditions: 1 mm × 183 cm glass column packed with 1% Poly S-179 (13) on Gas Chrom Q. Oven temperature was 255°C and N_2 was the carrier gas at 12 ml/min.

found that considerable purification can be achieved by selective solvent washes prior to elution of the adsorbed material. Hexane removes non-polar material from the adsorbed bile acid fraction, and the cartridge then can be washed successively with water and 40% methanol in water before eluting the bile acid fraction with methanol–DW 75:25. In our experience, the use of 100% methanol was not necessary to attain complete recovery of the bile acid fraction.

Enzymatic hydrolysis of the bile acid conjugates was adopted in this procedure for two reasons: the enzymatic process produced fewer artifacts than alkaline hydrolysis (21), thereby resulting in fewer interfering impurities in the ratio measurements; and, in confirmation of previously reported work (22), produced complete hydrolysis of the conjugates.

A second Sep-Pak procedure was used for the final purification of the acetylated bile acid methyl esters to

obtain the values of isotopic abundance equal to theory. This procedure is essential for isotope ratio measurements but is neither required for, nor applicable to, measurements of bile acid composition in serum. The reason for this distinction is that the procedure sacrifices approximately 15% of the diacetylated bile acid methyl esters and almost all of the methyl lithocholate monoacetate in order to remove the monoacetylated sterols and cholesterol acetate. Both of the latter components contribute to the interfering impurities in the isotope ratio measurements.

The use of ammonia gas for chemical ionization has been shown to enhance the sensitivity of bile acid detection by a factor of 2–3 over isobutane ionization (14). Ammonia also is known to be a more selective ionization reagent than other reagent gases commonly used (23) and yields base peaks for acetylated methyl esters of CDCA and CA that are 137 mass units higher than under isobutane CI conditions. Isotope ratio measurements at these higher m/z values are less susceptible to interference from lower molecular weight impurities.

Finally, diversion of the GLC effluent to the waste pump at all times, except when the bile acid peaks of interest were emerging, proved to reduce contamination of the ion source significantly. This procedure was completed after each chromatographic injection by raising the column oven temperature by 40°C for 5 min to remove any retained materials that might interfere with subsequent analyses.

During the development of this procedure 5 ml of serum was used. However, with capillary columns and improved instrumental sensitivity, it should be possible to reduce this sample size to 0.5–1.0 ml of serum. This would render the technique applicable to young children, but probably it could not be used in neonatal studies until further reductions in sample size were introduced. This procedure does open the possibility of conducting bile acid kinetic measurements in serum samples through the use of bile acids labeled with stable isotopes. While this may eventually replace bile collection by duodenal intubation, its most important function may be to provide a new vantage point from which to study bile acid kinetics in human subjects.▮▮

TABLE 3. Primary bile acid isotope ratio measurements[a]

	Methyl Chenodeoxycholate Diacetate 509/508 × 100		Methyl Cholate Triacetate 567/566 × 100	
	X̄ ± SD	CV	X̄ ± SD	CV
Standards n = 5	33.24 ± 0.15	0.45%	35.25 ± 0.14	0.40%
Serum n = 12	33.18 ± 0.12	0.36%	35.22 ± 0.20	0.57%

[a] The ratios of ion intensities shown were determined by GLC–MS–stable isotope ratiometry using ammonia as ionizing reagent gas. For further details, see text.

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