Steady-state kinetics of serum bile acids in healthy human subjects: single and dual isotope techniques using stable isotopes and mass spectrometry

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Abstract Techniques have been developed for the measurement of the complete steady-state kinetics of both chenodeoxycholic (CDCA) and cholic (CA) acid and the pool size of deoxycholic acid (DCA) from the serum of healthy subjects using stable isotopes and capillary gas-liquid chromatography-mass spectrometry (GLC-MS). Serum bile acids were purified by a method employing a C18 chromatographic cartridge, acid solvolysis, enzymic hydrolysis, methylation, a C8 chromatographic cartridge, and TMS-ether derivatization. Fifty mg each of [24-^{13}C]CDCA and [24-^{13}C]CA was given to five healthy subjects and kinetics were measured from serum and bile. In each case, the measurements from serum (S) equalled those from bile (B) (CDCA (S vs. B): fractional turnover rate (FTR) (d^{-1}) 0.17 ± 0.03 vs. 0.18 ± 0.04; pool (g) 0.64 ± 0.1 vs. 0.68 ± 0.14, synthesis (g d^{-1}) 0.12 ± 0.03 vs. 0.1 ± 0.03; CA (S vs. B): FTR (d^{-1}) 0.28 ± 0.05 vs. 0.29 ± 0.07, pool (g) 0.84 ± 0.29 vs. 0.82 ± 0.29, synthesis (g d^{-1}) 0.24 ± 0.10 vs. 0.25 ± 0.12). In addition, a dual isotope technique for measuring the steady-state kinetics of CDCA was developed using [11,12-^{2}H]CDCA, [24-^{13}C]CDCA, and a single sample of serum. In ten subjects, the FTR, pool, and synthesis of CDCA measured from serum was similar to that measured from bile. Finally, a technique for estimating the deoxycholic acid (DCA) pool from serum using the ratio of the 370 ion of DCA to that of CDCA was developed. In summary, these data demonstrate that the steady-state kinetics of CDCA and CA and the pool size of DCA can be measured from the serum of healthy subjects.—**Everson, G. T.** Steady-state kinetics of serum bile acids in healthy human subjects: single and dual isotope techniques using stable isotopes and mass spectrometry. *J. Lipid Res.* 1987. **28:** 238-252.

Supplementary key words bile acid kinetics • mass spectrometry • stable isotopes

The steady-state kinetics of bile acids were first measured in intact man by Lindstedt in 1957 using radiolabeled cholic acid and bile sampling (1). ^{14}C-Labeled cholic acid was administered orally and the specific activity of [^{14}C]cholic acid in duodenal bile was measured over the next 10 to 15 days. The specific activity decreased monoexponentially with time, i.e., the turnover of cholic acid obeyed first order kinetics. Subsequently, it has been shown by others that the turnover of all the bile acids of man obey first order kinetics (2). In these studies, duodenal bile samples were required for analysis and, thus, repeated nasoduodenal intubation was necessary. However, few individuals tolerate repeated nasoduodenal intubation and, therefore, serial studies of the same individual are difficult to achieve. Methods have been developed to reduce the number of samples required for analysis but still intubation is required (3, 4). For these reasons, we and others have developed techniques for measuring steady-state bile acid kinetics in intact healthy humans using serum, stable isotopes, and gas-liquid chromatography-mass spectrometry (GLC-MS) (5-10).

This report describes our experience with the analysis of serum bile acid kinetics in intact healthy human subjects. The main objectives of these studies were: *1)* to develop methods for the accurate measurement of the isotope ratios of serum bile acids, and *2)* to validate the measurement of kinetics from serum by comparing kinetics measured from serum with those from bile. In addition, a dual isotope technique for measuring steady-state kinetics was developed so that the number of samples required for GLC-MS analysis could be reduced. The latter technique ultimately reduces the cost of performing kinetic studies using serum samples by reducing the time (labor) required for sample processing and GLC-MS data acquisition.

Abbreviations: CDCA, chenodeoxycholic acid; CA, cholic acid; DCA, deoxycholic acid; GLC-MS, gas-liquid chromatography-mass spectrometry; IR, isotope ratio; MR, molar ratio; TMS, trimethylsilyl; HMDS, hexamethyldisilazine; FTR, fractional turnover rate.

MATERIALS AND METHODS

Materials

[24-^{13}C]CDCA, [11,12-^{2}H]CDCA, [24-^{13}C]CA and unlabeled standards of CDCA and CA were obtained from Argonne National Laboratories, Argonne, IL or MSD Isotopes, Montreal, Canada. These compounds were greater than 99% pure as judged by thin-layer chromatography, gas–liquid chromatography, and mass spectrometry. Sep-Pak C18 chromatographic cartridges were purchased from Waters Associates, Inc., Milford, MA. C8 chromatographic cartridges (Baker 10) were obtained from J. T. Baker Chemical Company, Phillipsburg, NJ. Cholylglycine hydrolase was purchased from Sigma Chemical Company, Inc., St. Louis, MO. The synthetic octapeptide of cholecystokinin (CCK-8, Kinevac®) was purchased from Squibb Pharmaceuticals, Princeton, NJ. All other solvents and materials used in the sample preparation were of standard reagent grade.

Methods

These studies were approved by the University of Colorado Human Subjects Committee. Subjects were paid volunteers and gave written informed consent. All were healthy and had normal serum levels of bilirubin, ALT, AST, alkaline phosphatase, and γ-glutamyl transferase.

Protocols[1]

Single isotope technique. Five subjects were studied. A 2-hr postprandial serum sample was obtained prior to administration of isotopes for determination of the natural abundance of the isotope ratios of CDCA and CA. Subsequently, subjects ingested 50 mg each of [24-^{13}C]CDCA and [24-^{13}C]CA. Serum and bile were then sampled from 1 to 4 days after the administration of isotopes. A nasoduodenal tube was placed each morning after an overnight fast and gallbladder contraction was stimulated with an intravenous injection of CCK-8, 0.02 μg/kg. Thirty to 40 ml of dark bile (gallbladder bile) was readily obtained by gravity drainage. This bile was mixed and only 1–2 ml was saved for subsequent determination of isotope ratios. The rest of the bile was returned to the subject via the nasoduodenal tube. Ten ml of blood was obtained approximately 1 hr after all the aspirated bile had been returned to the patient (usually 1.5-2 hr after the administration of CCK-8). On some days, we could not obtain a bile sample because of poor nasoduodenal tube position or subject intolerance to the tube. On those days, serum was sampled 2 hr after ingestion of a regular meal. Serum and bile samples were stored at −20°C until they were analyzed.

We also determined the time of the peak level of serum bile acid concentration in seven subjects ingesting regular meals. Gallbladder emptying and refilling was monitored using realtime ultrasonography (11) to determine whether periods of gallbladder emptying preceded peaks in bile acid concentrations. Three of the subjects underwent serum sampling and measurement of gallbladder volume hourly for 30 hr. In addition, in these three subjects, plus four others, serum was obtained and gallbladder volume was measured fasting and 2 hr after each regular meal for 4 days. Total serum bile acid concentration was determined in each of these serum samples by an enzymic spectrophotofluorometric method (12).

Dual isotope technique. Ten subjects were studied. A 2-hr postprandial serum sample was obtained for determination of the natural abundance of the isotope ratios of CDCA. Subsequently, subjects ingested 35 mg of [11,12-^{2}H]CDCA. Exactly 24 hr later, each subject ingested 35 mg of [24-^{13}C]CDCA. At least 24 hr after administration of the last isotope, each subject underwent sampling of serum and bile. After an overnight fast, a nasoduodenal tube was placed and bile and serum were sampled as described above. In this study, bile and serum were sampled fasting as well as after stimulation of gallbladder contraction. In some subjects, bile and serum were sampled on 2 days. In the subject shown in Fig. 6, serum sampling was continued for 6 days.

Sample preparative method. Serum samples were adjusted to pH 9, sonicated, and heated to 60°C for 30 min to inhibit protein binding of bile acids (13). Bile acids were extracted using a C18 chromatographic cartridge, washed twice with 5 ml of distilled water and 5 ml of methanol–water 1:3, and eluted from the cartridge with 5 ml of methanol–water 3:1. The eluate was brought to dryness and the residue was solvolyzed using 0.5 ml of distilled water, 3.5 ml of dimethoxypropane, and 1 drop of concentrated HCl for 16 to 24 hr at room temperature (14). Samples were then taken to dryness under nitrogen, and incubated overnight at 37°C with 25 units of cholylglycine hydrolase, 0.4 ml of 2.5 mM sodium acetate at pH 5.6, 0.2 ml of 1.86% (w/w) EDTA in water, 0.2 ml of 0.75% (v/v) mercaptoethanol in water, and 1 ml of water (15). The glycine and taurine conjugates of all bile acids are greater than 95% hydrolyzed under these conditions. After hydrolysis, the pH was adjusted to 1 with concentrated HCl and bile acids were extracted twice with 5 ml of diethyl ether. The pooled ether extracts were dried under nitrogen and methylated

[1]In addition to the studies reported in this paper, we had previously studied nine other subjects (9, 10) using a published sample preparation and analytical method (5). However, we were not able to consistently measure the kinetics of both CDCA and CA from serum in these nine subjects. In 50% of these studies, the recovery of bile acid from serum was complete, the enrichment by the isotope was sufficient, and the kinetics from serum were similar to those obtained from bile. The poor results in the other 50% were due to several factors: variable recoveries of bile acids from samples, presence of uncharacterized contaminants, and variation in the mass spectrometer measurements of isotope ratios. For these reasons, a new sample preparation method was developed. Our results with this method are the subject of this paper.

by reaction with 1 ml of methanol, 1 ml of 2,2-dimethoxypropane, and 1 drop of HCl for 1 hr at room temperature (16). After drying under nitrogen, samples were dissolved in 0.4 ml of ethyl acetate. Once dissolved, 4 ml of hexane was added and the sample was applied to a C8 chromatographic cartridge. After washing the sample on the cartridge with 5 ml of heptane, 5 ml of hexane, and 5 ml of ethyl acetate–hexane 1:19, bile acid methyl esters were eluted with ethyl acetate–hexane 1:1 (**Fig. 1**). After elution, samples were dried and the methyl esters were then converted to their corresponding trimethylsilyl (TMS) ether derivatives by the addition of 0.2 ml of pyridine, 8 drops of hexamethyldisilazine (HMDS), and 4 drops of trimethylchlorosilane (17). Samples were allowed to react for 2 hr at 45°C and then dried under nitrogen. TMS derivatives were extracted twice with 2 ml of hexane and dried. Samples were stored in 0.1 ml of hexane–pyridine–HMDS 98:1:1 and 1 to 2 μl of this solution was injected for GLC–MS analysis.

Capillary gas–liquid chromatography mass spectrometry of bile acid methyl ester trimethylsilylether derivatives. The capillary GLC system used in these studies was a Hewlett-Packard model #5790. The column was a 30 m × 0.25 mm fused silica column, DB-1 (J & W Scientific, Rancho Cordova, CA) coated with a 0.25 μm film thickness of SE-30. The initial column temperature was held at 230°C for 4 min. It was then increased to 295°C at a rate of 20°C/min. Final temperature was held at 295°C until completion of the run. The injector and interface between the chromatograph and the mass spectrometer were maintained at 295°C throughout the run. All bile acids eluted from the column within 15 min (**Fig. 2**).

For mass spectrometric analyses, the effluent of the capillary column was directed into the ion source of a Hewlett-Packard benchtop quadrupole mass selective detector, model #5970, operating in the electron impact ionization mode. During the course of these studies we also used a magnetic sector scanner (VG Micromass 16, VG Instruments, Inc., Stanford, CT) but found that measurements were less precise than with the HP #5970. Ions at m/z 370, 371, 372 for CDCA and m/z 458, 459, for CA were measured by selected ion monitoring. The isotope ratios (371/370, 372/370 for CDCA, and 459/458 for CA) from samples after administration of isotopes were compared to those of unenriched samples.

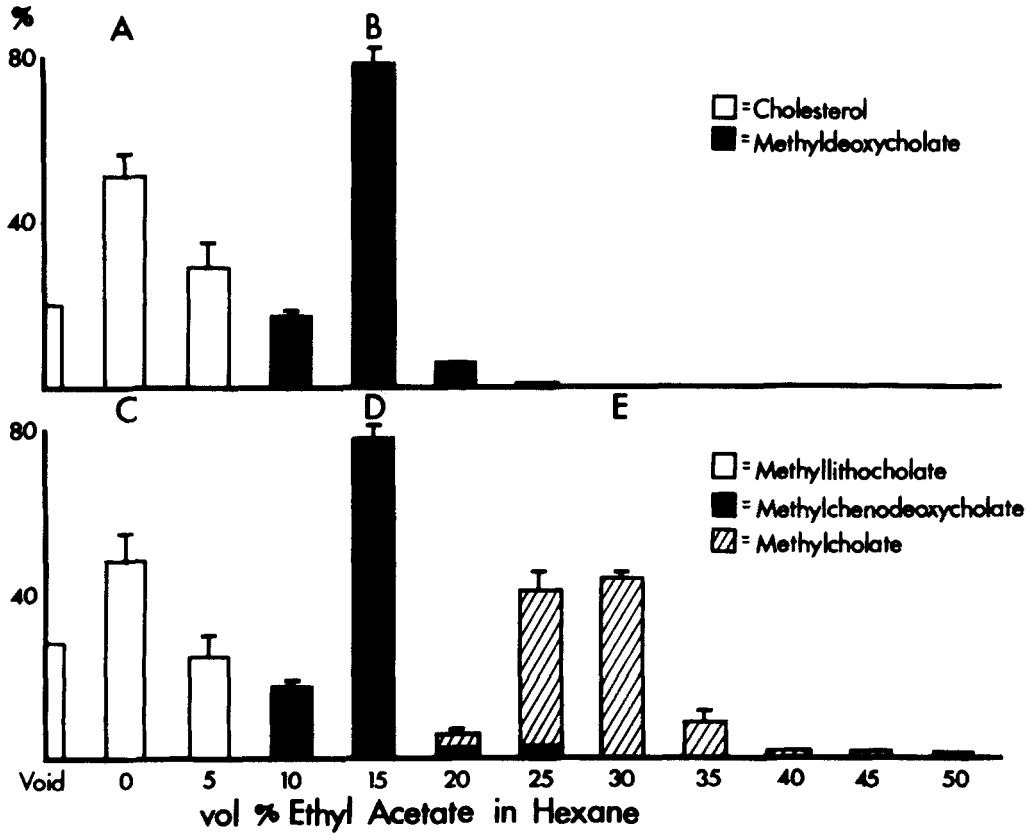

Fig. 1. The elution of cholesterol (A) and the methyl esters of deoxycholate (B), lithocholate (C), chenodeoxycholate (D) and cholate (E) from the C8 chromatographic cartridge is shown. All bile acid methyl esters elute prior to the 40% ethyl acetate fraction. Recoveries were determined by capillary GLC using known amounts of standards of the above compounds (n = 3 for each compound).

Measurement of isotopic enrichment of serum and bile. To measure steady-state kinetics using serum, one assumes that the enrichment of serum bile acid by labeled bile acid is identical to the enrichment of the entire pool of bile acid by labeled bile acid. We tested this assumption directly by comparing the isotope ratios measured from serum with those from bile. Even though less than 1% of the pool of a given bile acid resides in serum at any given time, the isotope ratios from serum were identical to those from bile (see Results below and Appendix B).

Calculation of kinetics by single isotope technique. The enrichment in isotope ratios (ΔIR) of CDCA and CA after administration of isotope was calculated by substracting the isotope ratios of unenriched from enriched samples. Thus,

$$\Delta IR = IR_t - IR_s \qquad Eq.\ 1)$$

where IR_t and IR_s are the isotope ratios of enriched and unenriched bile acid, respectively. Over the range of our data, ΔIR was linearly related to the molar ratio of labeled to unlabeled bile acid. Thus, the molar ratio (MR) of the labeled to unlabeled bile acid in each sample of serum and bile was calculated from ΔIR (18) using appropriate standard curves (**Fig. 3**). Steady-state kinetics were determined from linear regression analysis of the plot of Ln(MR) versus time. As determined by the method of least squares, this analysis yielded a single straight line that exhibited monoexponential decay. The slope of this line was the turnover rate (FTR). Pool size (PS) was calculated from the y intercept ($LnMR_o$) using the following equation,

$$PS = \frac{Dose}{e^{Ln(MR_o)}} \times \frac{MW_U}{MW_L} \qquad Eq.\ 2)$$

where dose is the amount (mg) of label given, MR_o is the molar ratio at time, t = 0, MW_U is the molecular weight of the unlabeled bile acid, and MW_L the molecular weight of the labeled bile acid. Steady-state synthesis (Syn) is then calculated from

$$Syn = PS \times FTR \qquad Eq.\ 3)$$

The steady-state kinetics of bile acids obtained from the serum and bile of each individual were compared.

Calculation of kinetics by dual isotope technique.[2] A modification of the method of Vantrappen, Rutgeerts, and Ghoos (4) was used. Fractional turnover rate (FTR) was calculated from the following equation (see appendix A for derivation)

$$FTR = Ln\left(\frac{MR_{t_1}}{mr_{t_2}}\right) \qquad Eq.\ 4)$$

where MR_{t_1} = the molar ratio of [11,12-^{2}H]CDCA to unlabeled CDCA in the sample and mr_{t_2} = the molar ratio of [24-^{13}C]CDCA to unlabeled CDCA in the sample. Pool size and synthesis rates were then calculated by equations 2 and 3, above.

Deoxycholic acid pool size. The deoxycholic acid pool ($Pool_{DCA}$) was not measured by isotope dilution but was estimated by comparison of the serum concentration of DCA to that of CDCA. The assumptions in this estimate of the DCA pool were as follows. *1)* The ratio of serum concentrations of DCA to CDCA were equal to the ratio of their pools; and *2)* the recoveries of CDCA and DCA from the preparative method were similar. $Pool_{DCA}$ was calculated from

$$Pool_{DCA} = \frac{I_{370DCA}}{I_{370CDCA}} \times K \times Pool_{CDCA} \qquad Eq.\ 5$$

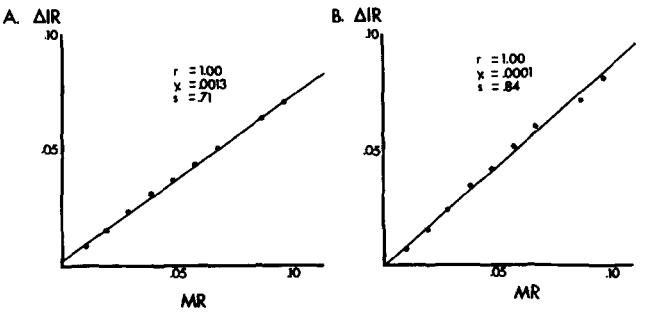

Fig. 3. The calibration curves for [24-^{13}C]CDCA (panel A) and [24-^{13}C]CA (panel B) are shown. The enrichment in isotope ratio (ΔIR) is plotted against the molar ratio (MR) of labeled to unlabeled bile acid. The standard deviations of the delta isotope ratio measurements ranged from 0 to 0.0021 for each of the points in the standard curve.

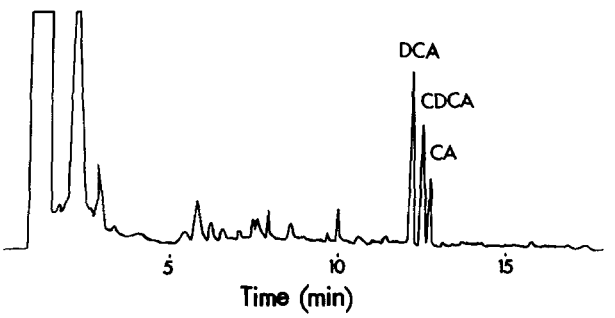

Fig. 2. Capillary gas chromatogram of a sample of normal human serum. Conditions: DB-1 fused silica column 0.25 mm × 30 m, SE-30 at 0.25 μm film thickness, He carrier gas; injector and detector T, 295°C; initial column T°, 230°C; ramp 20°C/min; final column T°, 295°C. Abbreviations: DCA, deoxycholic acid; CDCA, chenodeoxycholic acid; CA, cholic acid.

[2]All analytical methods and equations used in the calculation of steady-state kinetics by the dual isotope technique were first validated in vitro using a system of beakers and serial dilutions of labeled CDCA by unlabeled CDCA. The results of these experiments (n = 9) were: given FTR = 0.223 d^{-1}, measured FTR = 0.232 ± 0.010 d^{-1}, given pool = 0.501 g, measured pool = 0.495 ± 0.021 gm, given synthesis = 0.112 g d^{-1}, measured synthesis = 0.115 ± 0.013 g d^{-1}.

where I_{370DCA} was the intensity of the 370 ion of DCA, $I_{370CDCA}$ was the intensity of the 370 ion of CDCA, and K was a factor relating the differences in fragmentation of DCA and CDCA. K was determined by

$$K = R_{STD} - (I_{370DCA}/I_{370CDCA})_{STD} \quad Eq.\ 6)$$

where R_{STD} was the DCA:CDCA molar ratio of a standard calibration mixture of DCA and CDCA. I_{370DCA} and $I_{370CDCA}$ are the respective intensities of the 370 ions of the DCA and CDCA standards. The value of K was 3.81 ± 0.18 (mean ± SD) over the course of these studies.

After an overnight fast, most of the DCA and CDCA pool is stored within the gallbladder. Thus, the ratio of the concentrations of DCA and CDCA in gallbladder bile should approximate the ratio of the pool of DCA to that of CDCA. We compared the DCA:CDCA ratios from samples of serum with those from duodenal bile obtained after stimulation of gallbladder contraction as a means of validating our estimate of the DCA pool. To assess the effects of fasting, we compared DCA:CDCA ratios in fasting serum with those from serum obtained after gallbladder contraction in ten pairs of samples from six separate subjects.

Statistical analysis

The basic assumption for measuring kinetics from serum, that serum bile acids were in isotopic equilibrium with the entire bile acid pool, was tested by comparison of isotope ratios measured from serum with those from bile using the paired *t*-test. Variations in the measurements of isotope ratios by various mass spectrometers (see Table 1) were expressed as coefficients of variation (CV%). CV% was calculated from

$$CV(\%) = \frac{\sqrt{\frac{\Sigma(R_i - \bar{R})^2}{n}}}{\bar{R}} \times 100\%$$

where $\bar{R}$ is the mean of isotope ratio measurements, R_i is the isotope ratio of a given measurement, and n is the number of measurements. Kinetics were calculated from Ln/linear regression of molar ratio versus time. Kinetics measured from serum were compared to those from bile by paired *t*-test. Further kinetic analysis of the disappearance curve of labeled bile acid failed to disclose more than a single pool model to explain the disappearance of labeled compound. The data were entered into the following equation and fit to one, two, or three exponentials by the method of nonlinear least squares (19),

$$y = ae^{-k1t} + be^{-k2t} + ce^{-k3t}$$

where y is the molar ratio of labeled to unlabeled bile acid in a given sample; t is the sampling time; a, b, and c are constants defining the pool sizes for three compartments; k_1, k_2, and k_3 are constants defining the rates of disappearance of labeled compound. In all cases the data could only be fit to a single- and not a two- or three-compartmental model.

RESULTS

Evaluation of experimental protocols

All subjects preferred sampling by venipuncture to sampling by nasoduodenal intubation. In addition, serum samples were rapidly obtained whereas 15 min to 3 hr was required to obtain a single bile sample by nasoduodenal intubation. In some instances bile could not be obtained (see plots in Fig. 5). The 50-mg dose used in the single isotope studies enriched the CDCA pool from 6.1 to 10.6% and the CA pool from 4.5 to 10.2%. The 35-mg dose used in the dual isotope study enriched the CDCA pool from 2.3 to 12.0%. Since isotope ratio measurements are more accurate at higher enrichments, larger doses (50 mg) are necessary when sampling for longer intervals (> 3 days). Lower doses (35 mg) can be used in the dual isotope technique because single samples are obtained just 1 to 2 days after administration of isotopes.

Evaluation of preparative and analytical methods

The coefficients of variation of the isotope ratio measurements from a variety of mass spectrometers are shown in **Table 1.** Measurements from serum had slightly larger errors than those from bile. The least errors in measurements from both serum and bile were achieved with the HP #5970 benchtop mass spectrometer. The specific features of the preparative method and GLC-MS analysis that contributed to the improved precision are evaluated below.

Recoveries of free and conjugated bile acids from the C18 chromatographic cartridge are greater than 95% in the methanol-distilled water 3:1 fraction. Trihydroxylated bile acids elute at lower concentrations of methanol than dihydroxylated bile acids. Although most of the cholesterol in the samples is retained on the cartridge, a small, variable amount may elute with bile acids in the methanol-water 3:1 fraction.

An overnight incubation and a large amount of the enzyme cholylglycine hydroxylase was used to achieve complete hydrolysis of conjugated bile acids in serum. This was necessary since hydrolysis was incomplete after shorter incubations (mean ± SD of six experiments: % hydrolysis

TABLE 1. Coefficients of variation of isotope ratio measurements from various mass spectrometers

GLC-MS Apparatus	Coefficients of Variation			
	CDCA		CA	
	S	B	S	B
BS	0.3-2.6	0.1-0.7	0.3-1.5	0.1-0.7
F	0.1-1.2	0.1-0.6	0.1-2.1	0.1-0.6
HP	0.1-0.4	0.1-0.5	0.1-0.5	0.1-0.5
VG	0.3-3.8	0.2-3.1	0.2-8.0	0.2-4.2

Abbreviations: CDCA, chenodeoxycholic acid; CA, cholic acid; S, serum; B, bile; BS, Biospect quadrupole; F, Finnigan MAT 212 quadrupole; HP, Hewlett-Packard #5970 quadrupole; VG, VG Micromass 16.

of [^{14}C]cholyltaurine added to serum at 2 hr = 69 ± 15, 4 hr = 77 ± 20 and at 24 hr = 94 ± 4).

The sample purity demonstrated by the chromatogram in Fig. 2 is mainly due to the C8 chromatographic cartridge. We found that acidification and hexane extraction of the sample after elution from the C18 cartridge did not adequately remove nonpolar contaminants. Thus, we used the C8 cartridge to obtain a purer bile acid fraction. Cholesterol and the methyl esters of bile acids eluted from the C8 cartridge as shown in Fig. 1. Nonpolar contaminants from serum, cholesterol, and methyl lithocholate were removed in the early nonpolar washes. The methyl esters of the dihydroxylated bile acids could be eluted with ethyl acetate–hexane 1:4 (v/v) and all bile acid methyl esters are removed by ethyl acetate–hexane 1:1 (v/v).

Isotope ratio measurements

The unenriched (natural abundance) isotope ratios from the serum were within 0.0025 of those of pure standards for all studies. The standard deviations of duplicate or triplicate measurements of isotope ratios for a given sample of serum were usually less than 0.0015 and similar to the standard deviations for measurements from bile (Appendix B). The average absolute difference ± standard deviation between measurements made from serum and bile were: 0.0022 ± 0.0022 (P = NS) for the 371/370 ratio of CDCA, 0.0015 ± 0.0014 (P = NS) for the 372/370 ratio of CDCA, and 0.0036 ± 0.0040 (P = NS) for the 459/458 ratio of CA.

Gallbladder emptying and serum bile acid levels

The pattern of gallbladder emptying and concentration of serum bile acids over 30 hr is shown in **Fig. 4** for three subjects. The gallbladder emptied within 2 hr of a regular breakfast and remained contracted throughout the day, not affected by ingestion of regular meals. This pattern of gallbladder contraction is similar to that which we have previously observed in healthy subjects ingesting regular meals (20, 21). Although there were only minor fluctuations in gallbladder volume, serum concentrations of bile acids fluctuated considerably. As expected, bile acid concentrations were lowest in the fasting state and rose promptly within 2 hr after gallbladder contraction. However, peak levels of serum bile acid were not observed after the first meal of the day but rather after the second or third. In all seven subjects that were studied for 4 consecutive days, peak serum bile acid concentrations consistently occurred after the noon or evening meal. These relatively late rises in serum bile acid concentration were not related to gallbladder emptying since little, if any, refilling and emptying of the gallbladder had occurred during the day.

Bile acid kinetics

Single isotope technique. The plots of Ln (MR × 100) versus t from both serum and bile for both CDCA and CA

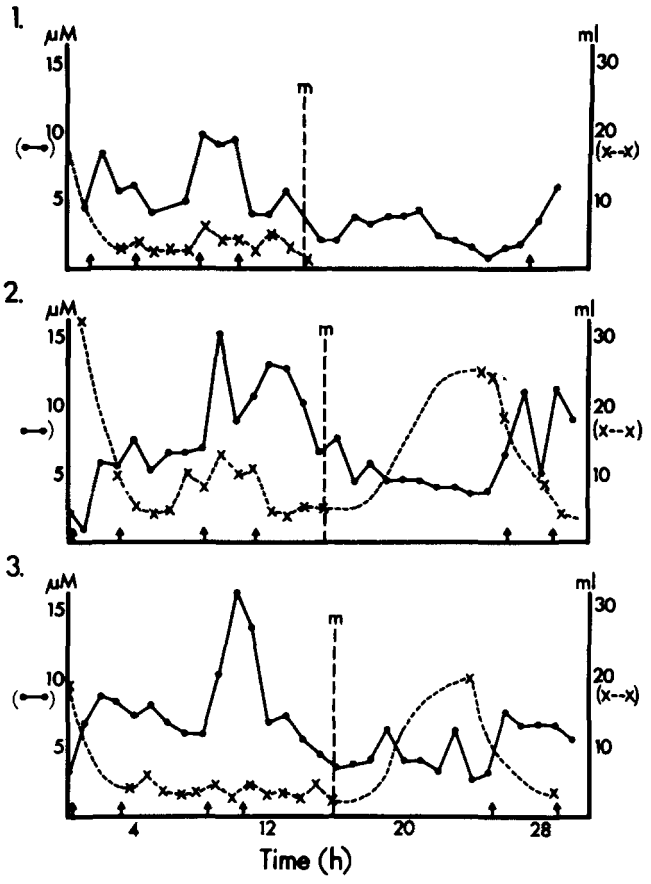

Fig. 4. Hourly serum bile acid concentration (●—●) and gallbladder volumes (x--x) for 30 hr. Meals are indicated by arrows (↑); m, midnight. Bile acid concentration was highest after the evening meal. Gallbladder volume remained small throughout the day while subjects were awake and ingested regular meals.

for each subject are shown in **Fig. 5.** The raw data of isotope ratios from which the molar ratios were calculated are given in appendix B. Natural abundance isotope ratios for both CDCA and CA were similar for all subjects. The 50-mg dose of isotope was 6.1 to 10.6% of the total CDCA pool and 4.6 to 10.2% of the total CA pool. The disappearance of isotope from both bile and serum obeyed monoexponential decay. In most cases the data for Ln (MR × 100) and the regression lines for serum and bile were identical.

The steady-state kinetics of CDCA and CA for all subjects are given in **Table 2.** In all cases, the kinetics from serum and bile were similar for both CDCA and CA. These data indicate that there is isotopic equilibrium between serum and bile in normal healthy subjects.

Dual isotope technique. An example of the data from a study of a single individual is shown in **Fig. 6.** In addition to the standard 2 days of bile and serum sampling, this subject underwent sampling of serum for an additional 3 days. Both isotopes of CDCA exhibit the same monoexponential decay (from linear regression analysis of Ln (MR × 100) vs. t, [^{13}C]CDCA slope = 0.48, [^{2}]CDCA

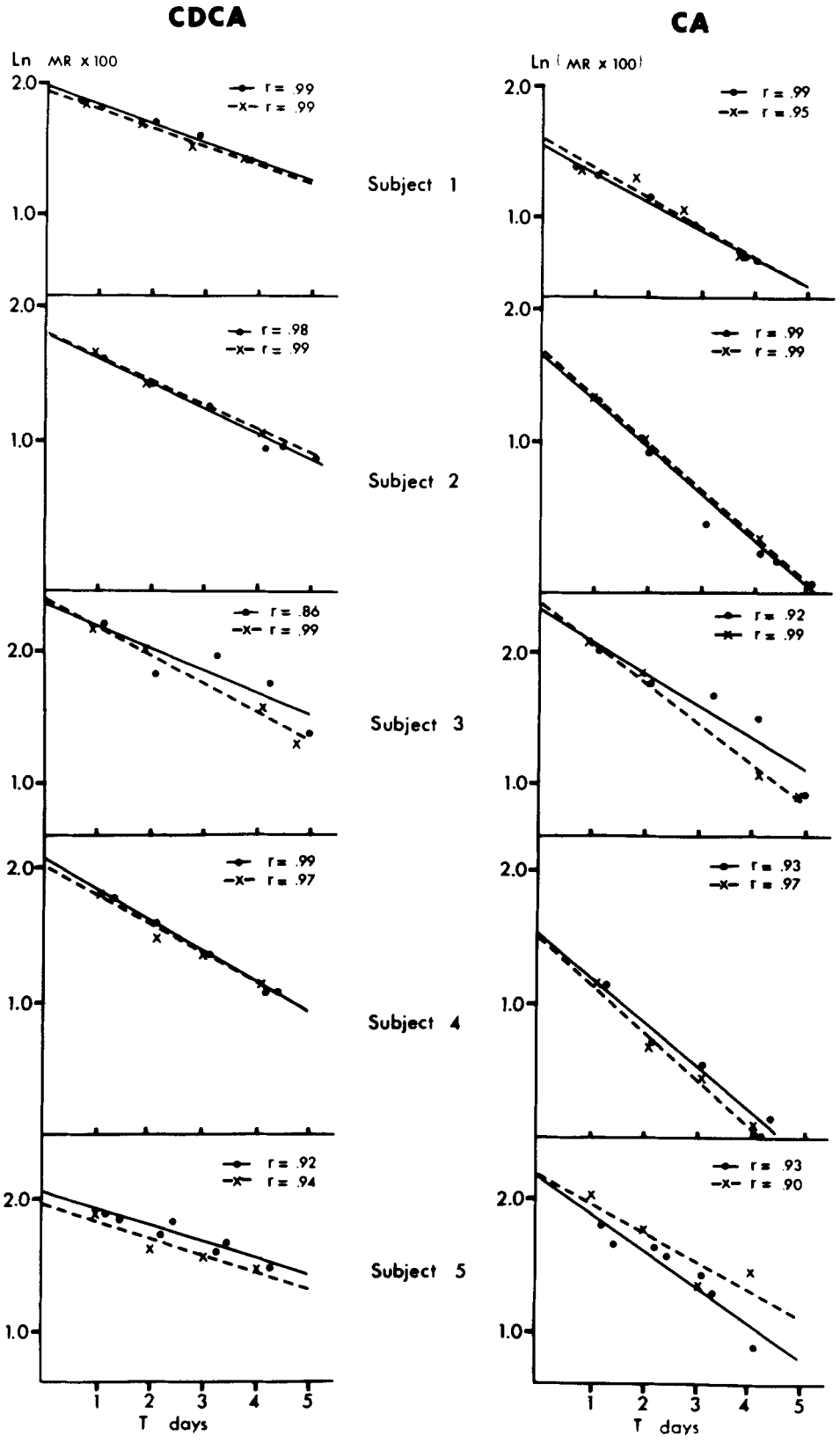

Fig. 5. The plots of Ln(MR × 100) versus t for each of the subjects participating in the single isotope study. The molar ratio from serum (●) was similar to that from bile (x) at comparable time points for each subject. In addition, the regression lines defining monoexponential decay from serum (—) were similar to those from bile (--). Abbreviations: CDCA, chenodeoxycholic acid; CA, cholic acid; Ln(MR × 100), natural logarithm of 100 times the molar ratio of labeled to unlabeled bile acid.

TABLE 2. Comparison of serum and biliary bile acid kinetics: single isotope technique

Subject	Bile Acid Kinetics					
	FTR(d^{-1})		Pool (g)		Syn (g d^{-1})	
	S	B	S	B	S	B
			CDCA Kinetics			
1	0.14	0.14	0.70	0.74	0.10	0.10
2	0.19	0.19	0.82	0.82	0.16	0.15
3	0.17	0.22	0.47	0.46	0.08	0.10
4	0.23	0.21	0.62	0.68	0.14	0.15
5	0.12	0.14	0.66	0.72	0.08	0.09
Mean ± SD	0.18 ± 0.03	0.18 ± 0.04	0.64 ± 0.13	0.68 ± 0.14	0.12 ± 0.03	0.12 ± 0.03
			CA Kinetics			
1	0.21	0.23	1.07	1.01	0.23	0.23
2	0.34	0.35	0.96	0.95	0.33	0.33
3	0.25	0.31	0.49	0.47	0.12	0.15
4	0.33	0.36	1.09	1.12	0.36	0.40
5	0.28	0.22	0.57	0.56	0.16	0.12
Mean ± SD	0.28 ± 0.05	0.29 ± 0.07	0.84 ± 0.29	0.82 ± 0.29	0.24 ± 0.10	0.25 ± 0.12

Abbreviations: FTR, fractional turnover rate; Syn, synthesis rate; CDCA, chenodeoxycholic acid; CA, cholic acid; SD, standard deviation. Steady-state kinetics were determined from multiple samples of serum (S) or bile (B) after administration of [24-^{13}C]CDCA and [24-^{13}C]CA.

slope = 0.50. For all studies, isotope ratios from serum equalled those from bile at comparable time points (refer to appendix B for isotope ratio data). It is important to note that small errors in the measurement of isotope ratios could result in larger errors in the measurement of kinetics by this technique. When significant error is introduced into the measurements, such as occurs at low serum concentrations of bile acid and at low isotope enrichment, then kinetics will be inaccurate. Thus, kinetics can be accurately measured only from samples obtained 24–48 hr after the last isotope is administered. Isotopic enrichment is too low in subsequent days to give accurate results with the dual isotope technique.

CDCA kinetics were calculated from single samples that were obtained within 24–30 hr of the last administered isotope. The raw data from these samples are displayed in appendix B. The measurements of the natural abundance of the isotope ratios are similar in all studies. The isotope ratios between serum and bile were nearly identical (by paired *t*-test there was no significant difference in the isotope ratios measured from serum compared to bile). The isotope ratios between fasting and stimulated serum were similar, although in most cases isotope ratios from stimulated serum were slightly lower than fasting serum. These data indicate isotopic equilibrium between serum and bile and between fasting and stimulated samples of serum. As shown in **Fig. 7A–C**, kinetics from serum and bile were similar in all subjects. Although synthesis and FTR were the same between serum and bile, CDCA pool was 8.2% greater when measured from serum: (serum vs. bile) pool (g) 0.78 ± 0.43 vs. 0.72 ± 0.38, t = 2.78, df = 9, $P < 0.025$; synthesis (g/d) 0.20 ± 0.10 vs. 0.21 ± 0.13, t = 0.12, df = 9, P = NS; FTR (d^{-1}) 0.31 ± 0.18 vs. 0.32 ± 0.18, t = 1.69, df = 9, P = NS.

DCA pool. The proportion of I_{370DCA} to $I_{370CDCA}$ was increased in fasting, compared to stimulated serum (0.24 ± 0.19 vs. 0.13 ± 0.07, $P < 0.03$). In nine out of ten subjects, the DCA:CDCA ratios from stimulated samples of serum were similar to those from stimulated samples of bile (serum vs. bile, 0.16 ± 0.14 vs. 0.14 ± 0.11). In one subject who had the highest proportion of DCA, the DCA:CDCA ratio was much greater from serum (0.79) than from bile (0.48). As a result of the increase in DCA:CDCA ratio and

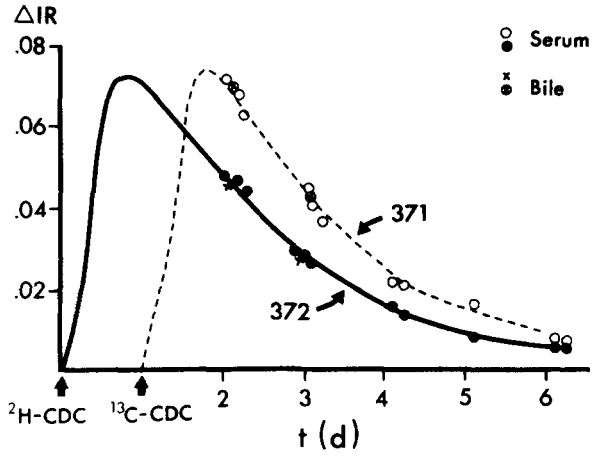

Fig. 6. Example of a kinetic study by dual isotope technique in one subject. Both isotopes (372 = [11,12-^{2}H]CDCA and 371 = [24-^{13}C]CDCA) exhibit similar monoexponential decay. Measurements from serum (○ or ●) and bile (x or ⊗) are identical at similar time points.

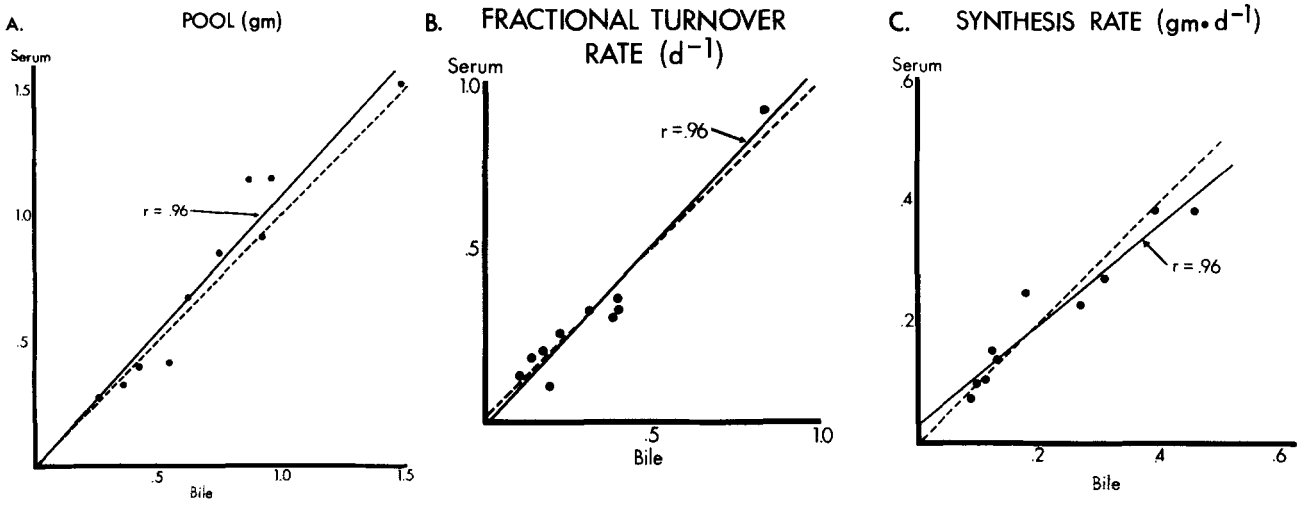

Fig. 7. Kinetics from serum and bile, measured by dual isotope technique, are shown. In all cases measurements from serum and bile were similar and not significantly different from the line of identity. The dashed line is the line of identity.

the slightly larger CDCA pool, the DCA pool size measured from stimulated serum was larger than that measured from stimulated bile (serum vs. bile, 0.56 ± 0.50 vs. 0.39 ± 0.28, $P < 0.05$).

DISCUSSION

Sample preparative methods

Many of the techniques used in our studies have been recently reviewed (22, 23). The C18 chromatographic cartridge is accepted as an important initial step in the processing of serum bile acids. However, accurate measurement of the isotope ratios of serum bile acids requires that the sample be essentially free of nonpolar contaminants and neutral sterols, especially cholesterol. In addition, the yield of bile acid through the preparative method must be sufficient to insure an adequate amount of bile acid for analysis by GLC-MS. Accordingly, we had developed a method where the recovery of bile acid from serum was satisfactory for measurement of isotope ratios (5). However, samples were insufficiently pure and thus, we were only partially successful in our initial studies of the measurement of serum bile acid kinetics (9, 10). For these reasons, our original method was considerably modified. Three changes were necessary to achieve the sample purity that is depicted by the chromatogram in Fig. 2. First, the washes and eluting solvents used in the initial C18 chromatographic cartridge step were modified to produce consistent and quantitative recoveries of all unconjugated and conjugated bile acids. Second, purification of bile acid methyl esters by the C8 chromatographic cartridge eliminated nearly all nonpolar contaminants from the sample. Finally, capillary gas-liquid chromatography was substituted for packed column gas-liquid chromatography and resulted in complete separation of all bile acids from each other and from the few remaining contaminants.

In our original method (15), the acetate derivatives of the methyl esters of bile acids were purified by a C18 chromatographic cartridge. This purification included water washes. Although acetate derivatives are not hydrolyzed by these washes, TMS derivatives are. Thus, the use of TMS derivatives required that a new procedure be developed. Initially, we attempted to purify samples by hexane extractions after samples had been eluted from the first C18 chromatographic cartridge as had been done by others (6, 23). Although some purification was achieved, significant contamination remained. For these reasons, a C8 chromatographic cartridge was used to purify the methyl esters of bile acids. This step completely separated the methyl esters of the di- and trihydroxylated bile acids from neutral lipid and other nonpolar contaminants.

Karlaganis and Paumgartner (24) validated the use of capillary GLC-MS for measuring serum bile acids. The original system measured TMS-ether derivatives on a 20 m × 13 mm glass capillary column with a crystal layer of barium carbonate coated with polyethyleneglycol. They reported run times of less than 15 min with complete resolution of bile acids from each other. Recently, the same group has used a 25 m × 0.33 mm fused silica column with an OV-1701 coating and reported good resolution but with run times of over 20 min (6). We used a 30 m column coated with SE30 and a temperature program to achieve quicker run times and still maintain resolution of bile acid peaks.

The Hewlett-Packard benchtop mass spectrometer (model #5970) provided superior performance in the analysis of the isotope ratios of serum bile acids. During the course of our studies, we have used four different mass spectrometers: *1)* Biospect quadrupole, *2)* Finnigan MAT 212, *3)* VG Micromass 16, *4)* a Hewlett-Packard, model 5970

benchtop quadrupole. Since the Biospect quadrupole and Finnigan MAT 212 were used in the early phases of our studies, no direct comparisons with the other instruments were possible. The VG Micromass 16 was a magnetic sector scanner and could not provide sufficiently precise or reproducible measurements for our isotope dilution studies. On the other hand, measurements made with the HP-5970 were highly precise and reproducible. In addition, the latter instrument has been essentially maintenance-free (down time of only 2 weeks in over 2 years of analyses). Thus, of the instruments used, the HP-5970 provided superior performance.

Analysis of serum bile acid kinetics

Because of the low concentrations of bile acid in the serum of healthy humans, GLC–MS and stable isotopes are required for measurement of bile acid kinetics from serum. For example, Hofmann and Cummings (2) estimated that dilution of 10 μCi of a radiolabeled bile acid into a 5-g pool would result in only 2 DPM/ml plasma.

Tateyama et al. (8) were the first to measure bile acid kinetics from serum. However, they had to administer large amounts of isotope (200 mg of [11,12-^{2}H]CDCA) and still could not demonstrate equality in measurements made from serum and bile. We had reported that the measurement of kinetics from serum was possible with 25–50 mg of isotope but our results were inconsistent (9, 10). Although only three healthy subjects were completely studied with both bile and serum measurements, Stellard et al. (6) showed that kinetics from serum and bile for both CDCA and CA were similar. Our results corroborate and extend their findings.

We found that, after administration of isotopes, bile acids in serum and bile are in isotopic equilibrium in intact healthy subjects. In addition, serum bile acids from fasting subjects exhibit isotope ratios similar to bile acids from serum obtained after gallbladder contraction. The data imply that, although bile acids may be stored in the gallbladder during an overnight fast, they are not "static". There is mixing of the entire bile acid pool even during gallbladder bile storage; i.e., bile acid that enters the gallbladder mixes completely with bile acid that is present in the gallbladder. Bile acid that exits the gallbladder is a product of this process. Therefore, isotope ratios in fasting bile, stimulated bile, fasting serum and stimulated serum are equal.

Timing of serum sampling is crucial to accurate measurement of kinetics. For example, the level of CA in fasting serum is very low (25, 26). Since the accuracy of the measurements of isotope ratios depends on the amount of CA in the sample, accurate measurements of CA can be made only when serum levels are increased such as occurs after gallbladder contraction. Our data show that maximum levels of serum bile acid are achieved after the noon or evening meal in patients ingesting regular meals, suggesting that serum should be sampled at that time.

The peak in serum bile concentration was not related to emptying of bile from the gallbladder. The gallbladder emptied within 2 hr of breakfast, had not refilled appreciably at the initiation of the noon or evening meal, and little bile was evacuated from the gallbladder after the noon or evening meal. Yet, peak serum concentrations occurred after the latter meals. Perhaps nutrients returning to the liver from the intestine via portal blood after these larger meals impaired hepatic bile acid uptake causing more bile acid to spill into the systemic circulation.

The DCA pool can be estimated from the ratio of 370 ions of DCA and CDCA. The DCA:CDCA ratio in serum approached that in bile only after stimulation of gallbladder contraction and cycling of the bile acid pool. However, the DCA:CDCA ratio was still higher in serum even after stimulation of gallbladder contraction. It remains to be determined whether DCA pool size is more accurately estimated from stimulated samples of serum or bile. As others observed in previous studies (27, 28), we found that the relative proportion of DCA is increased in serum during fasting. Thus, the DCA pool size is overestimated by our method if serum is sampled in the fasting state.

Steady-state kinetics measured by dual isotope technique

The method of Vantrappen et al. (4), was modified for stable isotopes and serum sampling. The motivation for development of this method was to reduce the sample number required for the measurement of serum bile acid kinetics. By reducing sample number, the time required for sample processing and GLC–MS analysis would be reduced. As a result, the overall costs of measuring bile acid kinetics by GLC–MS and serum sampling would be less. We validated the dual isotope technique for CDCA using [24-^{13}C]CDCA and [11,12-^{2}H]CDCA by showing that kinetics obtained from serum and bile were essentially the same. At the time of these validation studies the only available stable isotope for CA was the [24-^{13}C]compound. Thus, it remains to be determined whether CA kinetics can be measured from serum by the dual isotope technique. If complete bile acid kinetics can be measured, the dual isotope technique offers several advantages: *1)* only a small amount of blood is required (ideal for pedatric studies); *2)* patient comfort is enhanced; and *3)* sample preparation and GLC–MS time are reduced by 80%.

In summary, we have developed techniques for the purification of bile acids from normal human serum and for the accurate measurement of their isotope ratios by GLC–MS. Superb sample purification was achieved. As a result of that and the use of a new benchtop quadrupole mass spectrometer, bile acid kinetics from normal healthy individuals were accurately determined from serum samples. In addition, a dual isotope method to measure kinetics from a single sample of serum has been developed. This latter method significantly reduces the time and expense required to do kinetic analyses.

APPENDIX A

To accurately measure the steady state kinetics of CDCA by dual isotope technique, one must clearly resolve the two administered stable isotopes of CDCA from each other. The base ion of unlabeled CDCA is 370; of [24-^{13}C]CDCA, 371; and of [11,12-^{2}H]CDCA, 372. However, [24-^{13}C]CDCA is also enriched in the 372 ion and [11,12-^{2}H]CDCA in the 371 ion. These "cross-ion contributions" to the base ions of each isotope must be corrected by use of simultaneous equations. The degree of cross-ion contribution is dependent upon the completeness of isotopic labeling during the synthesis of the compound, the isotopic abundance of the atoms comprising the molecule, and the characteristics of fragmentation of the compound within a given mass spectrometer at a given ionization potential. When the label on a given compound is in a stable position and the fragmentation pattern is constant, one can correct for cross-ion contributions. As shown in **Fig. 8,** the enrichment in both 371/370 and 372/370 ratios for both isotopes is linearly related to the molar ratio of labeled to unlabeled CDCA. Thus, for both isotopes there is a constant relationship between the 371/370 and 372/370 ratios. For [24-^{13}C]CDCA this relationship is given by,

$$\Delta IR\ 372/370 = (a)\ (\Delta IR\ 371/370) \qquad Eq.\ 6)$$

where a is a constant. For [11,12-^{2}H]CDCA this relationship is given by,

$$\Delta IR\ 371/370 = (b)\ (\Delta IR\ 372/370) \qquad Eq.\ 7)$$

where b is a constant. The values of a and b for the isotopes used in this study were 0.30 and 0.20, respectively.

Thus, the equations describing the isotope ratios, A and B, from a given sample, are,

$$A = x + by \qquad Eq.\ 8)$$
$$B = ax + y \qquad Eq.\ 9)$$

where A = ΔIR 371/370 of the sample, B = ΔIR 372/370 of the sample, x = ΔIR 371/370 from [24-^{13}C]CDCA, and y = ΔIR 372/370 from [11,12-^{2}H]CDCA. By rearranging equations 8 and 9, the following expressions for x and y are obtained,

$$x = [(A/b) - B]/[(1/b) - a] \qquad Eq.\ 10)$$

and

$$y = B - ax \qquad Eq.\ 11)$$

The sample isotope ratios (A and B) are measured and x and y are calculated. The molar ratios of [24-^{13}C]CDCA to unlabeled CDCA and [11,12-^{2}H]CDCA to unlabeled CDCA are determined from calibration curves (Fig. 8).

CDCA kinetics are then calculated from molar ratios by the following equations,

$$MR_{t_1} = MR_0 e^{-kt_1} \qquad Eq.\ 12)$$

and

$$mr_{t_2} = mr_0 e^{-kt_2} \qquad Eq.\ 13)$$

where MR_{t_1} = the molar ratio of [11,12-^{2}H]CDCA to unlabeled CDCA at t_1, t_1 is the time from administration of [11,12-^{2}H]CDCA to obtaining the sample, MR_0 = the molar ratio of [11,12-^{2}H]CDCA to unlabeled CDCA at t_0, t_0 is the time the isotope was administered, mr_{t_2} and mr_0 are the corresponding molar ratios for [24-^{13}C]CDCA, k is the fractional turnover rate, and t_2 is the time from administration of [24-^{13}C]CDCA to obtaining the sample. Equal amounts of both isotopes are given, both isotopes are diluted by the same pool, and they are given exactly 1 day apart. Therefore, $MR_0/mr_0 = 1$ and $t_2 - t_1 = 1$. Thus, equations 12 and 13 can be simplified to,

$$FTR = k = Ln\left(\frac{MR_{t_1}}{mr_{t_2}}\right) \qquad Eq.\ 14)$$

where FTR is the fractional turnover rate.

MR_0 is determined from equation 12 by appropriate substitutions for MR_t and k. Pool size and synthesis are then calculated according to equations 2 and 3 (see text).

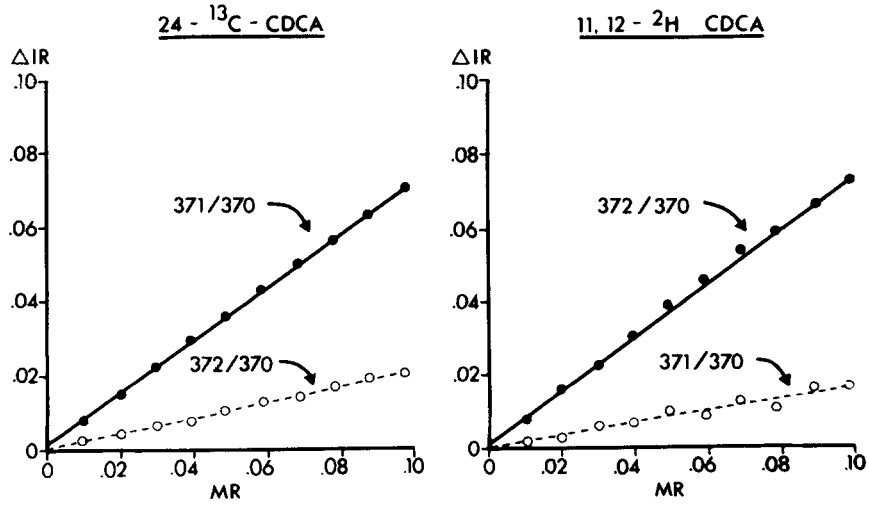

Fig. 8. Calibration curves describing the relationships of both the major and minor ions for both isotopes. The major ion of [24-^{13}C]CDCA is m/z 371 and that of [11,12-^{2}H]CDCA is m/z 372. The minor ion of [24-^{13}C]CDCA is m/z 372 and that of [11,12-^{2}H] is m/a 371. The standard deviations of the measurments of isotope ratios for each compound were all less than 0.0018.

APPENDIX B

P I. Table of isotope ratios for the validation study of the single isotope technique: raw data

			(M + 1)/M Isotope Ratios[a]							
			CDCA				CA			
	t(d)		Serum		Bile		Serum		Bile	
Subject	Serum	Bile[b]	Mean	SD	Mean	SD	Mean	SD	Mean	SD
1	0	--	.3125	.0013	--	--	.3670	.0006	--	--
	.61	--	.3589	.0011	--	--	.4006	.0022	--	--
	1.03	.73	.3571	.0007	.3572	.0003	.3987	.0014	.3993	.0022
	2.03	1.76	.3529	.0006	.3516	.0002	.3937	.0018	.3980	.0003
	2.84	2.68	.3495	.0013	.3454	.0002	.3886	.0008	.3911	.0009
	3.82	3.68	.3428	.0005	.3432	.0002	.3840	.0008	.3829	.0010
	4.03	--	.3419	.0005	--	--	.3837	.0025	--	--
2	0	--	.3120	.0002	--	--	.3616	.0009	--	--
	1.07	.94	.3489	.0004	.3489	.0001	.3929	.0024	.3934	.0021
	2.02	1.90	.3426	.0007	.3416	.0003	.3827	.0023	.3853	.0008
	3.10	--	.3371	.0011	--	--	.3769	.0011	--	--
	4.17	4.08	.3313	.0008	.3329	.0005	.3717	.0003	.3721	.0001
	4.48	--	.3319	.0002	--	--	.3711	.0008	--	--
	5.08	--	.3305	.0007	--	--	.3695	.0014	--	--
3	0	--	.3108	.0001	--	--	.3676	.0013	--	--
	1.11	.90	.3771	.0005	.3736	.0006	.4298	.0008	.4337	.0010
	2.04	1.90	.3559	.0030	.3640	.0003	.4159	.0021	.4188	.0002
	3.25	--	.3635	.0006	--	--	.4123	.0002	--	--
	4.13	4.07	.3533	.0001	.3458	.0043	.4051	.0015	.3916	.0005
	4.96	4.84	.3400	.0011	.3376	.0016	.3881	.0024	.3879	.0013
4	0	--	.3126	.0012	--	--	.3636	.0031	--	--
	1.29	1.04	.3555	.0008	.3574	.0008	.3901	.0001	.3903	.0028
	2.15	2.11	.3487	.0014	.3448	.0016	.3806	.0024	.3801	.0018
	3.16	3.04	.3415	.0001	.3416	.0001	.3782	.0011	.3768	.0026
	4.20	4.07	.3345	.0006	.3360	.0008	.3719	.0012	.3686	.0005
	4.40	--	.3349	.0011	--	--	.3737	.0005	--	--
5[c]	0	--	.3167	.0021	--	--	.3509	.0003	--	--
	1.23	1.00	.3637	.0008	.3640	.0001	.4009	.0032	.4150	.0060
	2.25	1.96	.3569	.0014	.3534	.0001	.3939	.0041	.4012	.0009
	2.44	--	.3616	.0011	--	--	.3913	.0017	--	--
	3.13	2.98	.3526	.0021	.3515	.0005	.3862	.0033	.3835	.0105
	3.30	--	.3553	.0019	--	--	.3816	.0015	--	--
	4.27	3.98	.3491	.0011	.3487	.0007	.3710	.0027	.3871	.0001

[a]The (M + 1)/M isotope ratio of CDCA equals the area of the 371 ion divided by the area of the 372 ion; for CA it is the area of the 459 ion divided by the area of the 458 ion.

[b]Bile was only obtained after administration of isotopes. Thus, the natural abundance determination from serum was used as the natural abundance level for bile. The natural abundance isotope ratio determinations for serum were within 0.0030 of the isotope ratios of pure standards of CDCA and CA.

[c]The VG Micromass 16 was used in the analysis of these samples. Note the difference in the natural abundance isotope ratios and the greater variability in measurements when compared to the four previous studies, all of which were analyzed by the HP #5970.

P II. Table of isotope ratios for the validation study of the dual isotope technique: raw data

		CDCA Isotope Ratios[a]							
		371/370				372/370			
		Serum		Bile		Serum		Bile	
Subject	Type[b]	Mean	SD	Mean	SD	Mean	SD	Mean	SD
A	Base	.3094	.0004	--	--	.0513	.0002	--	--
	F	.3538	.0003	--	--	.0913	.0000	--	--
	S	.3507	.0002	.3479	.0003	.0923	.0018	.0909	.0001
B[c]	Base	.3417	.0009	--	--	.0588	.0005	--	--
	F	.3614	.0018	--	--	.0732	.0001	--	--
	S	.3597	.0016	.3581	.0002	.0714	.0003	.0708	.0001
C	Base	.3096	.0011	--	--	.0518	.0003	--	--
	F	.3518	.0008	.3513	.0012	.0916	.0003	.0920	.0003
	S	.3504	.0007	.3497	.0002	.0909	.0002	.0919	.0001
D	Base	.3146	.0011	--	--	.0525	.0009	--	--
	F	.3365	.0016	.3331	.0012	.0733	.0011	.0726	.0010
	S	.3311	.0002	.3346	.0011	.0712	.0000	.0737	.0005
E	Base	.3112	.0007	--	--	.0510	.0003	--	--
	F	.3554	.0018	.3617	.0005	.0970	.0021	.0987	.0008
	S	.3541	.0023	.3606	.0018	.0924	.0002	.0989	.0008
F	Base	.3098	.0002	--	--	.0507	.0002	--	--
	F	.3454	.0010	.3458	.0006	.0830	.0004	.0846	.0007
	S	.3441	.0015	.3449	.0010	.0840	.0006	.0851	.0003
G	Base	.3112	.0003	--	--	.0514	.0001	--	--
	F	.3389	.0007	.3395	.0006	.0709	.0002	.0730	.0008
	S	.3319	.0017	.3365	.0011	.0701	.0007	.0724	.0003
H	Base	.3113	.0003	--	--	.0521	.0003	--	--
	F	.3330	.0007	.3290	.0018	.0708	.0002	.0711	.0004
	S	.3309	.0011	.3310	.0007	.0704	.0009	.0702	.0003
I	Base	.3117	.0004	--	--	.0529	.0004	--	--
	F	.3920	.0011	.3915	.0002	.1194	.0007	.1184	.0001
	S	.3894	.0008	.3901	.0005	.1180	.0010	.1165	.0008
J	Base	.3116	.0010	--	--	.0515	.0008	--	--
	F	.3318	.0006	.3307	.0002	.0704	.0006	.0693	.0010
	S	.3312	.0011	.3297	.0005	.0712	.0002	.0687	.0004

[a]The data represent the "raw" isotope ratios, prior to subtraction of natural abundance and correction for cross ion contributions of administered isotopes.

[b]Isotope ratios are compared between bile and serum for samples obtained after an overnight fast (F) and 2 hr after stimulation of gallbladder contraction (S). Base represents the natural abundance isotope ratios for CDCA from samples of subject's sera obtained prior to the administration of isotopes.

[c]The samples from subject B were analyzed by the VG Micromass 16. Note difference in natural abundance measurements compared to the other subjects. The samples from all other subjects were analyzed by the HP #5970.

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