

Simultaneous determination of cholic acid and chenodeoxycholic acid pool sizes and fractional turnover rates in human serum using ^{13}C -labeled bile acids

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Abstract A method has been developed for simultaneous determination of pool sizes and fractional turnover rates (FTR) of chenodeoxycholic acid (CDCA) and cholic acid (CA) in man by $^{13}\text{C}/^{12}\text{C}$ isotope ratio measurements of bile acids in serum after oral administration of 20–50 mg of [$^{24}\text{-}^{13}\text{C}$]-labeled bile acids. $^{13}\text{C}/^{12}\text{C}$ isotope ratio measurements were performed by capillary gas-liquid chromatography/electron impact mass spectrometry. CA and CDCA kinetics in serum measured by this method exhibited first order kinetics and permitted calculation of pool size and FTR of CA and CDCA. The validity of the measurements in serum was tested by simultaneous measurements in bile in three healthy volunteers and in five patients with various hepatobiliary disorders (three patients with cirrhosis, one with cholecystectomy and sphincterotomy, and one with sphincterotomy only). No consistent differences were found between the pool sizes and FTR's obtained from serum and bile. In a total of five healthy volunteers bile acid kinetics were measured in serum. The values found for the pool sizes and FTR's of CA and CDCA in these subjects were in excellent agreement with data reported in the literature based on ^{14}C or ^3H measurements in bile. ■ The pool sizes (mean \pm SD) of CDCA and CA were 32.6 ± 9.9 and $31.8 \pm 16.0 \mu\text{mol} \cdot \text{kg}^{-1}$, respectively. The corresponding values for the FTR's were 0.24 ± 0.13 and $0.48 \pm 0.22 \text{ d}^{-1}$. These data demonstrate that pool sizes and fractional turnover rates of cholic and chenodeoxycholic acid can be measured simultaneously by blood sampling after oral administration of the respective ^{13}C -labeled bile acids.—Stellaard, F., M. Sackmann, T. Sauerbruch, and G. Paumgartner. Simultaneous determination of cholic acid and chenodeoxycholic acid pool sizes and fractional turnover rates in human serum using ^{13}C -labeled bile acids. *J. Lipid Res.* 1984. 25: 1313–1319.

Supplementary key words capillary gas-liquid chromatography • mass spectrometry

Measurements of pool size and fractional turnover rate (FTR) of the primary bile acids cholic acid (CA) and chenodeoxycholic acid (CDCA) in man have been carried out by many investigators using the isotope dilution technique introduced by Lindstedt in 1957 (1).

For this purpose a tracer dose of ^{14}C - or ^3H -labeled bile acids is administered intravenously or orally and the exponential decay of the specific activity is measured in duodenal bile samples collected daily for up to 9 days. In order to modify the method for studies in pregnancy (2) and newborns (3, 4), Klein and co-workers introduced stable isotope labeled ^{13}C and ^2H bile acids and the measurement of isotope ratios in bile. These measurements were carried out with gas-liquid chromatography-mass spectrometry-isotope ratiometry. A Stable Isotope Ratiometer Multiple Ion Detector (SIRMID) was developed in order to monitor the ^{13}C and ^{12}C isotopes and accurately measure the isotope ratios after subtraction of background noise (5). Isobutane-chemical ionization mass spectrometry was used for the fragmentation of methyl ester acetate derivatives (6). DeMark and his colleagues (7, 8) finally described a modified method for measurement of isotope ratios of bile acids in serum using NH_3 -chemical ionization. The application, however, was limited to the measurement of natural abundance. So far, only abstracts have appeared, describing the measurement of serum kinetics of CDCA (9, 10) and CA (11) in small numbers of patients and no reports of simultaneous determination of CDCA and CA kinetics in serum have been published. Moreover, only limited data (9, 11) exist on the comparison of the bile acid kinetics measured in serum with the kinetics measured in bile. Recently, a method for $^{13}\text{C}/^{12}\text{C}$ isotope ratio measurements of CDCA in serum by capillary gas-liquid chromatography and electron impact mass spectrometry has been developed in our laboratory (12) and has now been adjusted for simultaneous measurements of CDCA

Abbreviations: FTR, fractional turnover rate; CA, cholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; EI, electron impact; TMS, trimethylsilyl; GLC, gas-liquid chromatography.

and CA kinetics. The measurements of the pool sizes and FTR's by this method in serum were validated against simultaneous measurements in bile in healthy volunteers and in patients with various hepatobiliary disorders. This comparison not only tests the validity of the measurements, but also proves that, in healthy subjects and in patients with cholecystectomy, sphincterotomy, or cirrhosis, the bile acids in the systemic and the enterohepatic circulation are in equilibrium.

MATERIALS AND METHODS

Subjects

Five healthy volunteers (four females and one male) age 25–34 years were studied. None had evidence of hepatobiliary or intestinal disease, neither did they take any medication. Conventional liver tests (serum bilirubin, alkaline phosphatase, AST) as well as serum bile acids were within normal limits. In addition, five patients with hepatobiliary disorders were studied: one with cholecystectomy and sphincterotomy, one with sphincterotomy only, and three with cirrhosis of the liver with mild to moderate signs of cholestasis (Table 1). All patients were in stable condition as judged by the results of the liver tests before and during the study period. Patients as well as healthy volunteers were on a free diet. All subjects gave their informed consent prior to the study.

Experimental design

In the morning an indwelling single-lumen nasoduodenal tube was positioned under radiological control in the third part of the duodenum and remained in this position during the study period of 3.5 days. When the tube was tolerated, blood and bile samples were taken for measurements of the natural abundance of CA and CDCA at 8 PM, and 50 mg of [24-¹³C]-labeled cholic acid and 50 mg of [24-¹³C]-labeled chenodeoxycholic acid were administered orally in 200 ml of 0.25%

sodium bicarbonate solution. The two sphincterotomized patients received only 20 mg of each labeled compound because it has been shown previously that sphincterotomized patients have a smaller bile acid pool (13). Thereafter, blood and bile samples were collected simultaneously, at 12-hr intervals (once after an overnight fast and once in the evening) for 3.5 days. In addition, in two patients with cirrhosis as well as in two healthy volunteers, blood samples were also taken 1.5 hr after injection of cholecystokinin. Fasting duodenal bile samples were obtained in all patients and in three healthy volunteers after stimulation of gallbladder contraction by a slow intravenous injection of cholecystokinin (CCK, Kabi Diagnostika, Nyköping, Sweden). Patients with cirrhosis and elevated bilirubin (subjects 4 and 5) received 0.5, all others received 1.0 Ivy-dog units of CCK per kg body weight. In the evening, duodenal content was aspirated without stimulation of the gallbladder. One to two ml of the fluid was collected in sterile tubes and frozen at once. Simultaneously, 15 ml of blood was taken from an antecubital vein, centrifuged, and the serum was then stored at –20°C until further analysis.

Materials

[24-¹³C]Cholic acid and [24-¹³C]chenodeoxycholic acid were purchased from Merck, Sharp and Dohme, Canada. The degree of labeling was 90% as determined by mass spectrometry. Bond Elut reverse-phase octadecylsilane bonded silica cartridges (100 mg) and a Vac-Elut vacuum system were obtained from Analytichem International, England. The cartridges were washed with 5 ml of methanol and 5 ml of water. Lipidex 1000 as a methanol suspension was purchased from Packard Instruments (Groningen, The Netherlands), slurried into a column with a bedsize of 4 × 1 cm, and washed with 20 ml of 0.01 N HCl. Cholyglycine hydrolase from *Clostridium perfringens* (Welchii), 90 units/mg of protein, was obtained from Sigma, St. Louis, MO. Pyridine p.a. was

TABLE 1. Clinical data of patients studied

Subject	Sex	Age	Body weight	Diagnosis	Total Serum Bilirubin ^a	Serum AST ^b	Serum γ -Glutamyl-transferase ^c	Serum Alkaline Phosphatase ^d	Serum Bile Acids ^e
		yr	kg		mg/dl		I.U./liter		μ mol/liter
1	F	75	76	Cholecystectomy + sphincterotomy	0.9	8	7	96	10.8
2	M	67	60	Sphincterotomy	2.0	9	7	137	3.9
3	M	43	61	Cirrhosis of the liver	1.2	13	68	154	28.9
4	F	48	51	Cirrhosis of the liver	5.2	21	38	108	82.9
5	M	62	78	Cirrhosis of the liver	7.1	73	124	322	130.8

^a Normal values < 1.0 mg/dl.

^b Normal values < 18 I.U./liter.

^c Normal values < 18 I.U./liter (F), < 28 I.U./liter (M).

^d Normal values < 200 I.U./liter.

^e Normal values < 9 μ mol/liter (enzymatic photometric assay for 3 α -hydroxy-bile acids, Merckotest®, Merck, Darmstadt, Germany).

refluxed over barium oxide and distilled (14). All other solvents and reagents were of analytical grade.

Analytical methods

Sample preparation. Two ml of serum was diluted with 8 ml of 0.1 N NaOH and the bile acids bound to protein were cleaved by incubation at 64°C for 15 min (15). Bile acids were extracted by absorption on a 100 mg Bond Elut octadecylsilane bonded silica cartridge (16). After washing the cartridge with 5 ml of 0.1 N NaOH and 5 ml of water, the bile acids were eluted with 6 ml of 75% methanol. Interfering substances were then removed by an extraction with 6 ml of n-hexane after acidification of the eluate to pH 3–4. The eluate was taken to dryness with nitrogen at 60°C and solvolysis was carried out according to the method of Parmentier and Eyssen (17) which achieves practically complete solvolysis of 3-sulfates. After neutralization and evaporation of the solvent, the glycine and taurine conjugates were hydrolyzed enzymatically for 2 hr at 37°C using cholyglycine hydrolase (18). After acidification to pH 3–4, the aqueous solution was passed through a 4 × 1 cm Lipidex 1000 column prepared according to Setchell and Matsui (15). Thereafter, the column was washed with 15 ml of 0.01 N HCl and 20 ml of water and the deconjugated bile acids were eluted with 10 ml of 75% methanol. The solvent was evaporated under nitrogen at 60°C. The methylation of the bile acids was carried out according to Ali and Javitt (19) using 2,2-dimethoxypropane and the trimethylsilyl ethers were formed with pyridine–hexamethyldisilane–trimethylchlorosilane 3:2:1 (v/v) at 60°C under nitrogen for 15 min (15). The sample was taken to dryness and dissolved in 25–50 μl of isoctane for healthy subjects and in up to 500 μl for patients with liver disease. Total recovery over the whole procedure of sample preparation was 80% for cholic acid and 92% for chenodeoxycholic acid. Duodenal bile samples (50–500 μl) were deconjugated as described above for serum. After acidification to pH 1, the aqueous phase was extracted three times with an equal volume of diethylether. Thereafter, methylation and trimethylsilylation were carried out as described above.

Gas-liquid chromatography. The methyl ester TMS ether derivatives were separated by gas-liquid chromatography on a 25 m × 0.33 mm CP Sil 19 CB column (Chrompack, Middelburg, The Netherlands), which consists of a chemically bonded OV-1701 coating on a fused silica capillary column. Helium was used as carrier gas ($p = 0.8 \text{ kg/cm}^2$). The gas chromatograph used was a Carlo Erba, Fractovap 4160, equipped with a LT 430 temperature programmer. The sample was introduced by on-column injection at 140°C oven temperature using secondary injector cooling. After passage of the

solvent through the column, the secondary cooling was turned off and the oven temperature was programmed up to 270°C at 10°/min. The temperature remained constant at 270°C for 8 min, after which it was raised to 300°C at 10°/min to elute late-eluting substances from the column.

Mass spectrometry. The fused silica capillary column was inserted directly into the ion source of a Finnigan 4021 mass spectrometer/data system combination, which was used in the electron impact mode (70 eV). The interfacing part of the column was heated at 230°C. The source temperature used was 180°C. The scanning conditions were as follows. A Selected Ion Monitoring (SIM) program was used monitoring four pairs of ions, each pair being characteristic for mono-, di-, and trihydroxy bile acids (7, 20). Of these, m/z 370, 371 were suitable for measuring isotope ratios for CDCA and, when of interest, deoxycholic acid (DCA), and m/z 458, 459 for CA. The remaining pairs can be used to monitor lithocholic acid and ursodeoxycholic acid when these are of interest for other reasons. After determination of the exact mass centers, a mass range of 125 mmu was chosen around the peak center and this mass range was scanned for 50 msec. A total scan time of 451 msec was therefore required. As the bottom peak widths were about 10 seconds, approximately 20 scans could be acquired for each eluting bile acid. These scans were used to calculate an average $^{13}\text{C}/^{12}\text{C}$ isotope ratio. The natural abundance was measured in a serum sample obtained immediately before administration of the label.

Calculations. The $^{13}\text{C}/^{12}\text{C}$ isotope ratios were converted into the atom percent excess values according to Campbell (21):

$$\text{atom \% excess} = \frac{R - R_0}{1 + (R - R_0)} \times 100\%$$

where R represents the isotope ratio and R_0 the natural abundance.

The ln atom % excess-time curve was calculated using linear regression analysis. The pool size was determined using the equation:

$$\text{pool size} = \frac{D \times b \times 100}{e^a} - D,$$

where a represents the intercept on the ordinate, b the degree of labeling of the administered marker, and D the dose of the marker. The FTR equals the slope of the regression line. The synthesis rate was calculated by multiplying pool size and FTR. As proposed by Hofmann and Hoffman (22), the pool sizes were expressed as $\mu\text{mol kg}^{-1}$, the FTR's as d^{-1} , and the synthesis rates as $\mu\text{mol kg}^{-1}\text{d}^{-1}$. Results were expressed as means \pm standard deviation.

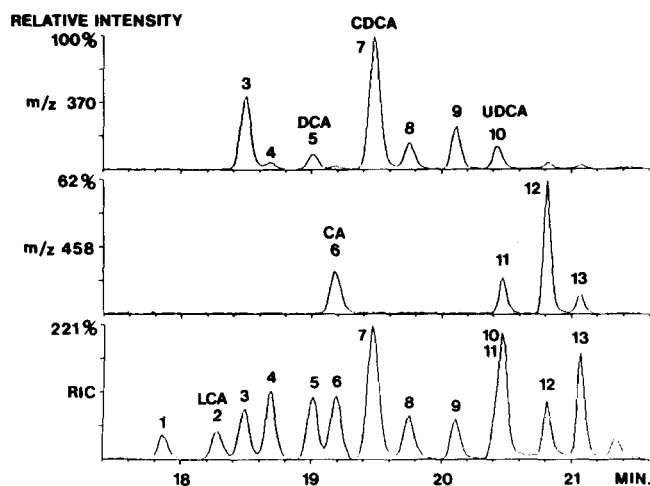


Fig. 1. Separation of the methyl ester TMS-ether derivatives of bile acid standards by GLC-mass spectrometry using a fused silica 25 m \times 0.32 mm OV-1701 column under the conditions described in the text; 1. iso-lithocholic, 2. lithocholic, 3. iso-chenodeoxycholic, 4. iso-deoxycholic, 5. deoxycholic, 6. cholic, 7. chenodeoxycholic, 8. 3 β -hydroxy-5-cholenic, 9. hyodeoxycholic, 10. ursodeoxycholic, 11. ursocholic, 12. hyocholic, 13. β -muricholic acid. RIC: reconstructed ion chromatogram.

RESULTS

The separation of bile acids on the CP Sil 19 CB column under the conditions used in this study is shown in **Fig. 1**. Baseline separation of CDCA and CA was essential, as CA has an important mass fragment with m/z 368, the ($M + 2$) isotope of which can interfere with the CDCA measurements in case of overlap. None of the bile acids found in human serum interfered with CDCA or with CA. When the characteristic ions for

CDCA and CA in serum were monitored, the mass chromatograms for the ^{12}C and ^{13}C isotopes shown in **Fig. 2** were obtained. For both CDCA and CA about 20 scans were available for calculation of the average isotope ratios. The reproducibility of the isotope ratio measurements is shown in **Table 2**. Even in the 0.1–1.1 μM range, coefficients of variation for isotope ratio measurements were 1.1% for cholic acid and 0.8% for chenodeoxycholic acid when the same sample was measured repeatedly. Looking at all volunteers included in this study, a coefficient of variation less than 1.5% was found for the measurements of natural abundance. The deviation of the natural abundance in serum from that in bile did not exceed 1.8% for CA and 1.5% for CDCA.

CA and CDCA decay curves measured in serum exhibited first order kinetics (**Fig. 3**). Linear regression correlation coefficients greater than 0.90 were obtained for both bile acids in all subjects. No consistent differences in isotope ratios were found between serum samples collected in the fasting state and those obtained 1.5 hr after CCK stimulation of the gallbladder.

No consistent differences were found between the pool sizes and FTR's obtained from serum and from bile (**Fig. 4** and **Fig. 5**). Linear regression correlation coefficients of 0.99 (pool size) and 0.97 (FTR) were obtained for CA, 0.99 and 0.96 for CDCA. Combining the data for CA and CDCA resulted in similar regression lines. The deviation of the CA pool measured in serum from that measured in bile ranged from -11.4% to $+11.8\%$ ($1.1 \pm 9.3\%$). The corresponding deviation for the FTR ranged from -12.3% to $+26.7\%$ ($4.0 \pm 11.1\%$), except for one healthy volunteer whose

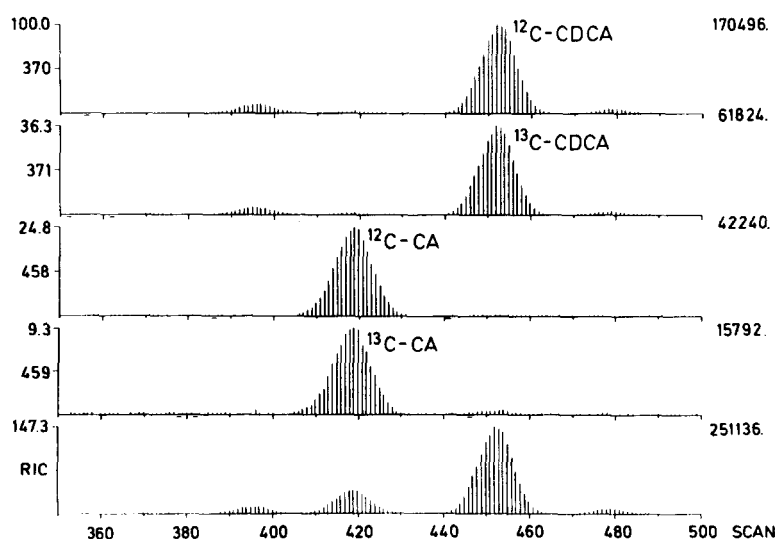


Fig. 2. Mass chromatograms of the ^{12}C and ^{13}C contributions of chenodeoxycholic acid and cholic acid measured in serum of a healthy volunteer.

TABLE 2. Intra-individual and inter-individual variation (c.v.) of ^{13}C -natural abundance measurements in cholic acid (m/z 459/458) and chenodeoxycholic acid (m/z 371/370) in serum and bile (means \pm SD)

	Cholic Acid		Chenodeoxycholic Acid	
	Bile ^a	Serum ^a	Bile ^a	Serum ^a
Intra-individual variation	0.3643 \pm 0.0010	0.3604 \pm 0.0008	0.3204 \pm 0.0004	0.3177 \pm 0.0006
c.v.	0.3%	0.2%	0.1%	0.2%
	0.3633 \pm 0.0009	0.3674 \pm 0.0010	0.3167 \pm 0.0007	0.3212 \pm 0.0012
c.v.	0.3%	0.3%	0.2%	0.4%
Inter-individual variation	0.3649	0.3640	0.3200	0.3250
	0.3678	0.3745	0.3269	0.3290
	0.3643	0.3616	0.3219	0.3172
	0.3678	0.3659	0.3212	0.3166
	0.3618	0.3609	0.3175	0.3167
	0.3643	0.3604	0.3204	0.3177
	0.3633	0.3674	0.3167	0.3212
	0.3570	0.3612	0.3232	0.3233
	0.3639 \pm 0.0035	0.3645 \pm 0.0048	0.3199 \pm 0.0043	0.3208 \pm 0.0046
c.v.	1.0%	1.3%	1.3%	1.4%

^a Means of three determinations.

FTR's were 0.28 d⁻¹ and 0.10 d⁻¹ in serum and bile, respectively. The deviation of the CDCA pool measured in serum from that measured in bile ranged from -16.1% to +17.2% (2.3 \pm 10.0%). The corresponding deviation for the FTR ranged from -19.1% to 15.8% (1.7 \pm 14.1%).

The kinetic values measured in serum of five healthy volunteers are shown in Fig. 6. The pool sizes for CA (31.8 \pm 16.0 $\mu\text{mol/kg}$) and for CDCA (32.6 \pm 9.9 $\mu\text{mol/kg}$) were about equal. The FTR's for CA (0.48 \pm 0.22 d⁻¹) were larger than the corresponding values for CDCA (0.24 \pm 0.13 d⁻¹). As the synthesis rates are calculated from FTR and pool size, the mean synthesis rate for CA (13.3 \pm 4.9 $\mu\text{mol kg}^{-1} \text{d}^{-1}$) was also higher than that for CDCA (7.0 \pm 3.6 $\mu\text{mol kg}^{-1} \text{d}^{-1}$).

DISCUSSION

The serum sample preparation and the OV-1701 capillary GLC column used in this study separate CA

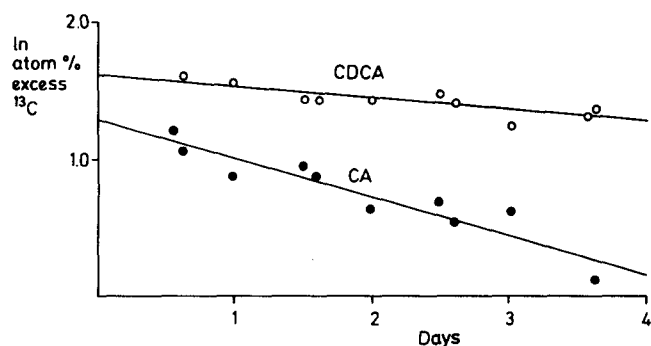


Fig. 3. Decay curves of ^{13}C -labeled chenodeoxycholic acid and cholic acid determined in serum of a healthy volunteer.

and CDCA from other bile acids or substances that could interfere when $^{13}\text{C}/^{12}\text{C}$ isotope ratios are measured in human serum. The narrow GLC peaks establish sufficient peak intensity to create good peak shapes for both the ^{12}C and ^{13}C contributions of CA and CDCA. In some instances using fasting serum, isotope ratios were measured in cholic acid peaks representing serum concentrations as low as 0.1 $\mu\text{mol/l}$. In these cases measurements were carried out on 5–10 ng of bile acid injected onto the GLC column. The reproducibility of the isotope ratio measurements (coefficient of variation < 1%) is sufficient to obtain a good description of the decay curve even when the fractional turnover rate is

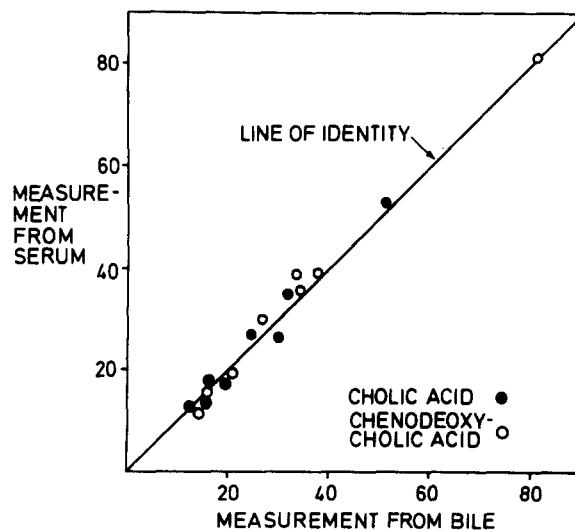


Fig. 4. Correlation between the pool sizes obtained from measurements in serum and measurements in bile for all subjects studied. CDCA: $y = 0.70 + 1.01x$, $r = 0.9936$, $n = 8$; CA: $y = -0.69 + 1.04x$, $r = 0.9857$, $n = 8$.

low. This is demonstrated in Fig. 3 where the CDCA curve has a slope of 0.08 d^{-1} .

The excellent agreement between the pool sizes obtained from serum and those obtained from bile, shows that the ^{13}C -label mixes well with the endogenous bile acid pool, which is distributed over the enterohepatic and the systemic circulation. It also demonstrates that both circulations can be described as one compartment. This conclusion is supported by the fact that in all but one subject the FTR's measured in serum and bile were about equal. These findings are in agreement with the recently described physiological pharmacokinetic model for the metabolism and enterohepatic circulation of bile acids described by Hofmann et al. (23). Only in one healthy volunteer was a discrepancy between the CA-FTR in serum and bile observed. Quantitation of the serum CA concentration ($0.12 \mu\text{mol/l}$) showed that in this subject only approximately 0.01% of the total CA pool was located in the systemic circulation in the fasting state. For the other healthy volunteers this value amounted to approximately 0.1%.

Cholecystectomy and/or sphincterotomy in the patients studied did not affect the agreement between the values obtained from serum and from bile. This confirms the idea that a more continuous enterohepatic flux of bile acids in cholecystectomized patients, and even more in cholecystectomized and sphincterotomized patients, will not inhibit but rather favor mixing of the label with the endogenous pool. Also, the patients with liver disease showed good agreement of bile acid pool sizes and FTR's obtained from serum and from bile, which means that in these patients bile acid exchange freely between systemic and enterohepatic circulation.

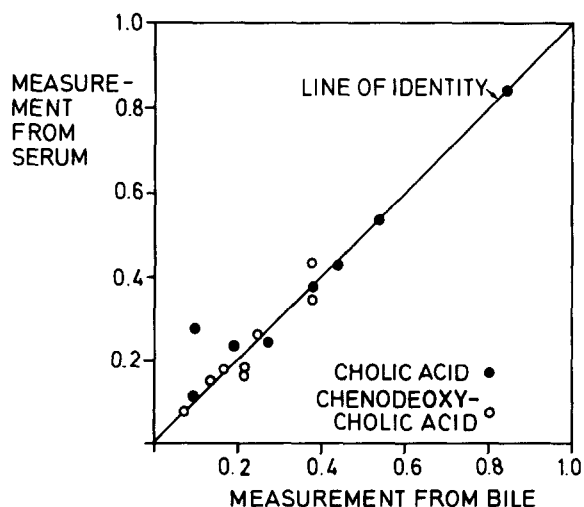


Fig. 5. Correlation between the fractional turnover rates obtained from measurements in serum and measurements in bile for all subjects studied. CDCA: $y = -0.01 + 1.06x$, $r = 0.9630$, $n = 8$; CA: $y = 0.07 + 0.88x$, $r = 0.9675$, $n = 8$.

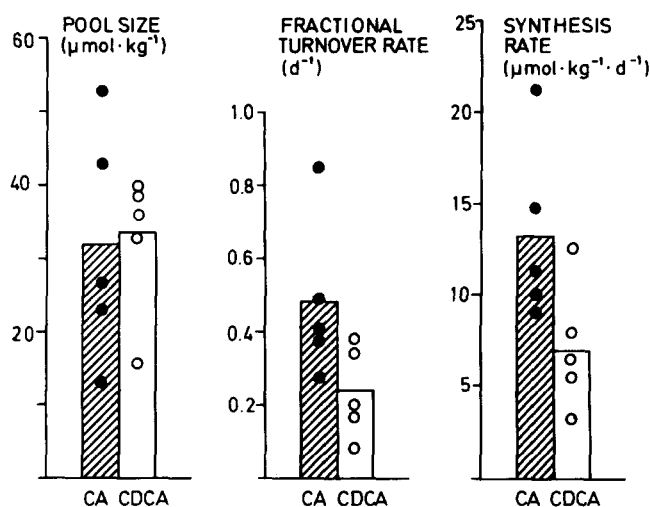


Fig. 6. Pool sizes, fractional turnover rates, and synthesis rates of chenodeoxycholic acid and cholic acid measured in serum of healthy volunteers ($n = 5$).

The values found for the pool sizes, FTR's, and synthesis rates of CA and CDCA in healthy volunteers are in agreement with data reported in the literature based on ^{14}C or ^3H measurements in bile (24, 25). This finding that CA and CDCA pool sizes were about equal is consistent with present concepts about the bile acid metabolism in man (25). It is also generally accepted that CDCA is better preserved in the enterohepatic circulation than CA, because of a more effective passive absorption in the upper small intestine (26, 27). Therefore, the FTR and synthesis rate of CDCA are thought to be smaller than those of CA. This phenomenon is also reflected in our data.

In conclusion, the data document that pool sizes and fractional turnover rates of the primary bile acids, cholic acid and chenodeoxycholic acid, can be measured simultaneously by blood sampling after administration of the respective nonradioactive ^{13}C -labeled bile acids. The method is not only applicable in healthy subjects, but also in patients without gallbladder and/or sphincter of Oddi function. Its validity could also be demonstrated in patients with chronic liver disease associated with mild to moderate cholestasis. ■■

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