note on methodology

Determination of deoxycholic acid pool size and input rate using [24-^{13}C]deoxycholic acid and serum sampling

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Summary We have developed an isotope dilution method for determination of deoxycholic acid pool size and input rate which employs oral administration of 50 mg of [24-^{13}C]deoxycholic acid and serum sampling. The method has been validated by classical isotope dilution technique using [24-^{14}C]deoxycholic acid and bile sampling in five patients with colonic adenomas. Excellent agreement between pool sizes and input rates determined with $^{13}C/^{12}C$ isotope ratio measurements in serum and ^{14}C measurements in bile was obtained when isotope ratios were measured in the conjugated fraction of deoxycholic acid in serum. We conclude that pool size and input rate of deoxycholic acid can accurately be determined by blood sampling after oral administration of [24-^{13}C]deoxycholic acid, therewith eliminating the use of radioactive tracers and the need for bile sampling.—**Stellaard, F., G. Paumgartner, G. P. van Berge Henegouwen, and S. D. J. van der Werf.** Determination of deoxycholic acid pool size and input rate using [24-^{13}C]deoxycholic acid and serum sampling. *J. Lipid Res.* 1986. **27:** 1222–1225.

Supplementary key words bile acid metabolism • ^{13}C isotope dilution • capillary gas–liquid chromatography–mass spectrometry

Many observations related to gallstone formation (1–3), to changes of bile acid metabolism induced by cholecystectomy (4–6), and to colonic carcinomas (7,8) have led to an increased interest in the formation and colonic absorption of deoxycholic acid (DCA). DCA is the major secondary bile acid in humans and is formed by bacterial 7α-dehydroxylation of cholic acid in the colon. Alterations in DCA metabolism have usually been documented by changes in the proportions of DCA in serum or bile. Few studies have focused on measurements of DCA pool size and input rate (daily colonic absorption of newly formed DCA). These have been carried out employing the conventional Lindstedt technique (9) using [24-^{14}C]DCA and bile sampling (10). After having developed stable isotope dilution methods for the primary bile acids cholic acid (CA) and chenodeoxycholic acid (CDCA) (11), we wished to extend our methodology to studies of DCA kinetics using [24-^{13}C]DCA and serum sampling. Measurement of DCA kinetics by serum sampling poses special problems because newly formed DCA is absorbed from the colon, whereas the primary bile acids are synthesized in the liver. This means that spillover of newly formed and absorbed unconjugated DCA into the systemic circulation can take place before uptake and conjugation by the liver and mixing with the circulating pool occurs. This problem is aggravated by the fact that hepatic uptake is less efficient for unconjugated than for conjugated bile acids (12). Therefore, it was decided to measure isotopic enrichment of DCA in the conjugated fraction. Pool sizes and input rates thus obtained were compared to values determined simultaneously in bile with ^{13}C and ^{14}C isotopes. The determinations of the $^{13}C/^{12}C$ isotope ratios were measured at the University of Munich; the ^{14}C specific activity measurements were carried out at the Municipal Hospital of Arnhem. Both laboratories used their own methodologies.

METHODS

Materials and instrumentation

[24-^{13}C]DCA was purchased from Merck Sharp & Dohme, Montreal, Canada (91% ^{13}C as determined by capillary gas–liquid chromatography–mass spectrometry). SP-Sephadex was obtained from Pharmacia, Uppsala, Sweden, and DEAP-LH-20 (product name DEAP-Lipidex) was from Packard Instruments, Groningen, the Netherlands. DEAP-LH-20 columns (4 × 0.8 cm) were prepared according to Setchell and Matsui (13). All other materials and their preparations were the same as described recently (11).

Subjects

Five female patients aged 41 to 65 years with colonic adenomas documented histologically were studied. He-

Abbreviations: DCA, deoxycholic acid; CA, cholic acid; CDCA, chenodeoxycholic acid.

patobiliary or gastrointestinal disorders were excluded. They did not take any medication. In addition, eight healthy volunteers aged 25 to 40 years (five males, three females) were studied. They were all clinical personnel and were not aware of any hepatobiliary or gastrointestinal disease.

The project was approved by the Medical Ethical Committee of the Municipal Hospital of Arnhem in 1983 and informed consent was obtained from all patients and healthy volunteers.

Experimental design

At 8 PM, after a blood sample for measurement of natural abundance was drawn, patients received 5μ Ci of [24-^{14}C]DCA intravenously and 50 mg of [24-^{13}C]DCA orally in 200 ml of 0.25% sodium bicarbonate solution. On the next 4 days at 8 AM, an indwelling single-lumen nasoduodenal tube was positioned for collection of duodenal bile, and a slow cholecystokinin infusion (CCK, Kabi-Vitrum, Studsvik, Sweden, 1.2 U $\cdot$ kg^{-1} $\cdot$ hr^{-1} for 55 min) was started. In order to avoid interruption of the enterohepatic circulation of bile acids, a sample of only 1 ml of bile collected 15 min after the start of the CCK infusion was taken each morning. Seventy minutes after start of infusion, a blood sample was taken (14). Healthy volunteers received 40 mg of [24-^{13}C]DCA orally at 8 PM after a blood sample was drawn. During the next 4 days, 2 hr after a solid breakfast and 2 hr after supper, blood was collected. Blood was centrifuged and serum and bile were stored at -20°C until analysis.

Sample preparation for stable isotope ratio measurements

For isotope ratio measurements in conjugated DCA in serum, 3 ml of serum was used. After extraction of bile acids on Bond Elut C_{18} cartridges (15), the eluate (75% methanol) was passed directly through a 4 $\times$ 0.8 cm SP-Sephadex ion exchange column in the H^+ form and a 4 $\times$ 0.8 cm DEAP-LH-20 column in the acetate form (13) using a Vac-Elut^R vacuum system.

After washing the columns with 20 ml of 70% methanol, the SP-Sephadex column was removed and the DEAP-LH-20 column was washed with 9 ml of 72% ethanol. Separate elution of unconjugated and conjugated bile acids was carried out according to Setchell and Matsui (13), using 6.5 ml of 0.1 mol/L acetic acid in 72% ethanol (pH 4.0) for unconjugated and 6.5 ml of 0.15 mol/L acetic acid in 72% ethanol (pH 6.5) for conjugated bile acids. Sulfated bile acids were discarded. Conjugated bile acids were hydrolyzed enzymatically (16), methylated (17), and trimethylsilylated (13). For isotope ratio measurements in biliary DCA, 50–200 μl bile was used. Biliary bile acids were deconjugated enzymatically, extracted into diethylether, methylated, and trimethylsilylated.

Sample preparation for ^{14}C specific activity measurements

Bile acids in bile (0.5–1.0 ml) were deconjugated enzymatically and extracted using reversed phase Sep-Pak C_{18} cartridges (15,18). After methylation, DCA was isolated by thin-layer chromatography (17).

Analytical methods

^{13}C/^{12}C isotope ratio measurements were carried out using combined capillary gas–liquid chromatography–electron impact mass spectrometry and selected ion monitoring. For this purpose, bile acid methyl ester trimethylsilyl ether derivatives were separated on a capillary OV-1701 column (25 m $\times$ 0.32 mm) in a Carlo Erba, Fractovap 4160 gas chromatograph and directly introduced into a Finnigan 4000 quadrupole mass spectrometer used in the electron impact mode. The conditions used were basically the same as described recently for CA and CDCA (11). Helium served as carrier gas (0.6–0.7 kg/cm^2) and 0.5–2.0 μl of the sample dissolved in appropriate volumes of iso-octane was injected by cold on-column technique at a column temperature of 140°C. After 1 min the temperature was raised to 270°C (10°/min). After 9 min at 270°C (elution time of bile acid methyl ester trimethylsilyl ether derivatives), the temperature was raised to 290°C (10°/min) in order to elute more polar compounds from the column. The last part of the column interfacing the gas chromatograph and mass spectrometer was kept at a constant 270°C, whereas the ion source temperature was at 250°C. The electron impact ionization was carried out at an electron energy of 70 eV and an

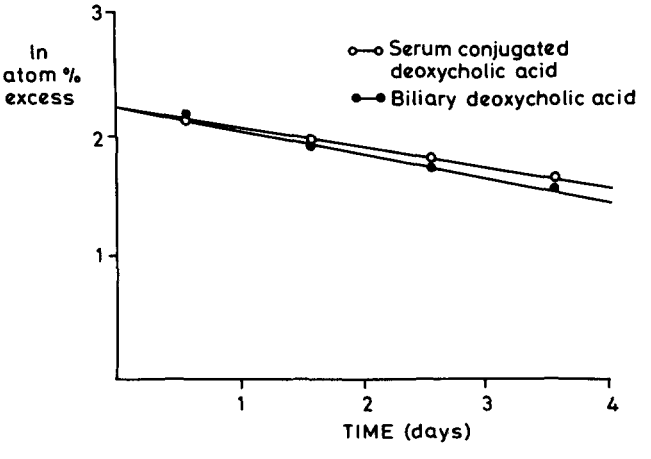

Fig. 1. Decay curves of ^{13}C/^{12}C isotope ratios in conjugated deoxycholic acid (DCA) in serum and in bile of a patient with colonic adenomas.

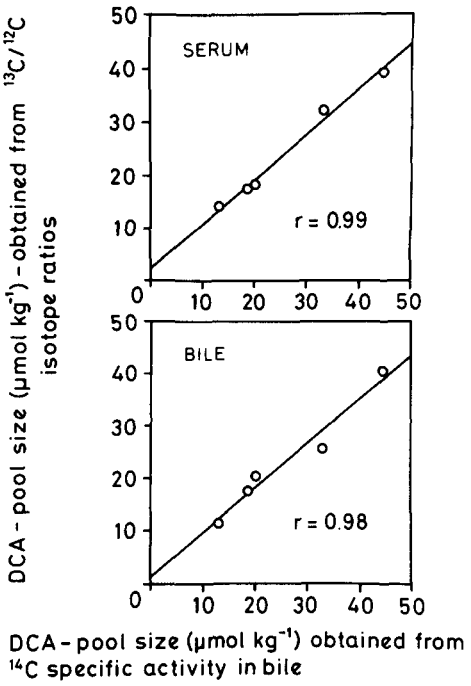

Fig. 2. Correlations of pool sizes obtained from $^{13}C/^{12}C$ isotope ratio measurements in serum DCA and biliary DCA of adenoma patients with values calculated from ^{14}C specific activity measurements of DCA in bile.

emission current of 0.3 mA. The conditions used for selected ion monitoring were adjusted to maximal sensitivity as described recently (19). Accordingly, a scan window of 1/16 amu and a scan time of 100 msec for each window were chosen.

For determination of ^{14}C specific activity of DCA in bile, DCA was quantitated by gas–liquid chromatography (20) in the DCA fraction isolated by thin-layer chromatography. The ^{14}C activity was counted by liquid scintillation counting.

Calculations

DCA input rate was calculated as the product of pool size and fractional turnover rate. Calculation of pool size and fractional turnover rate from atom percent excess, obtained from the $^{13}C/^{12}C$ isotope ratio measurements (11), and of ^{14}C specific activity decay curves was carried out using standard methods.

RESULTS

The method for $^{13}C/^{12}C$ isotope ratio measurements of DCA allowed these measurements to be carried out with coefficents of variation less than 1.5% (n = 5) for amounts above 5 pmol (2 ng) injected onto the capillary gas–liquid chromatography column (19). Examples of ^{13}C atom percent excess decay curves in conjugated serum DCA and biliary DCA of a colonic adenoma patient are shown in **Fig. 1.** The relationships between the pool sizes obtained with the different methods using either ^{13}C or ^{14}C as label in the adenoma patients are shown in **Fig. 2.** The values for the fractional turnover rates and input rates obtained in conjugated DCA in serum and those obtained in biliary DCA using ^{13}C showed good agreement with the values calculated from specific activity measurements of ^{14}C in bile (**Table 1**). Pool sizes, fractional turnover rates, and input rates measured in conjugated DCA in serum of healthy volunteers averaged 10.5 (range 4.7–27.4) $\mu mol \cdot kg^{-1}$, 0.32 (range 0.14–0.54) d^{-1}, and 3.5 (range 0.8–8.2) $\mu mol \cdot kg^{-1}$, respectively.

DISCUSSION

$^{13}C/^{12}C$ isotope ratios in the conjugated fraction of DCA in serum can be measured with sufficient accuracy to obtain decay curves of $^{13}C/^{12}C$ isotope ratios in human serum after oral administration of as little as 50 mg of [^{13}C]DCA. The results of this study demonstrate that conjugated DCA in serum and DCA in bile are in isotopic equilibrium, and comparison with measurements of ^{14}C specific activity of DCA in bile after intravenous injection of [^{14}C]DCA has shown that [^{13}C]DCA given orally is a valid marker for studies of DCA kinetics. It can be assumed that newly formed DCA, absorbed from the colon in the unconjugated form and entering the systemic cir-

TABLE 1. Fractional turnover rates of DCA from $^{13}C/^{12}C$ isotope ratio measurements in serum and bile and from ^{14}C specific activity measurements in bile of five patients with colonic adenomas

	Fractional Turnover Rate (d^{-1})			Input Rate ($\mu mol \cdot kg^{-1} \cdot d^{-1}$)		
Patient	^{13}C in Conjugated DCA in Serum	^{13}C in Biliary DCA	^{14}C in Biliary DCA	^{13}C in Conjugated DCA in Serum	^{13}C in Biliary DCA	^{14}C in Biliary DCA
1	0.17	0.20	0.19	3.0	3.5	3.2
2	0.07	0.15	0.08	2.7	6.1	3.5
3	0.27	0.36	0.32	8.7	9.2	9.8
4	0.22	0.23	0.25	3.1	2.6	2.7
5	0.32	0.24	0.22	5.9	4.9	4.4

culation before mixing with the circulating DCA pool, decreases the isotopic enrichment of total DCA in serum. The extent to which this effect occurs may vary depending on factors affecting absorption of unconjugated DCA from the large bowel. The unconjugated fraction of DCA has therefore been removed from serum prior to isotope ratio measurements.

The values for DCA pool sizes and input rates found for healthy volunteers were on the same order of magnitude as those previously obtained with [^{14}C]DCA and bile sampling in healthy young men (21). It may be concluded from our data that the methodology permits valid measurements of pool size and input rate of DCA by isotope dilution using [24-^{13}C]DCA and serum sampling.

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