methods

Measurement of parameters of cholic acid kinetics in plasma using a microscale stable isotope dilution technique: application to rodents and humans

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Abstract **A stable isotope dilution method is described that allows measurement of cholic acid (CA) kinetics, that is, pool size, fractional turnover rate (FTR), and synthesis rate in mice, rats, and humans. Decay of administered [2,2,4,4-$^2H_4$]CA enrichment was measured in time in 50-μl plasma samples by gas-liquid chromatography/electron capture negative chemical ionization-mass spectrometry, applying the pentafluorobenzyl-trimethylsilyl derivative. The kinetic data expressed species-dependent differences. The CA pool sizes were 16.8 ± 2.1, 10.6 ± 1.2, and 2.4 ± 0.7 μmol/100 g body weight for mice, rats, and humans, respectively. The FTR values were 0.44 ± 0.03, 0.88 ± 0.10, and 0.46 ± 0.14 per day for mice, rats, and humans. The corresponding synthesis rates were 7.3 ± 1.6, 9.3 ± 0.1, and 1.0 ± 0.2 μmol/100 g body weight per day. The human data agreed well with literature data obtained by conventional isotope dilution techniques. For rats and mice these are the first reported isotope dilution data. The method was validated by confirmation of isotopic equilibrium between biliary CA and plasma CA in the rat. Its applicability was demonstrated by the observation of increased CA FTR and CA synthesis rate in rats fed cholestyramine, which is known to increase fecal bile acid excretion. The presented stable isotope dilution method enables the determination of CA kinetic parameters in small plasma samples. The method can be applied in unanesthetized rodents with an intact enterohepatic circulation and may also be valuable when studying the development of human neonatal bile acid kinetics.**—Hulzebos, C. V., L. Renfurm, R. H. Bandsma, H. J. Verkade, T. Boer, R. Boverhof, H. Tanaka, I. Mierau, P. J. J. Sauer, F. Kuipers, and F. Stellaard. **Measurement of parameters of cholic acid kinetics in plasma using a microscale stable isotope dilution technique: application to rodents and humans.** ***J. Lipid Res.*** **2001.** 42: **1923–1929.**

Supplementary key words bile acid metabolism • fractional turnover rate • mass spectrometry • pool size • synthesis

The active secretion of bile acids into the canaliculus generates bile flow and biliary secretion of phospholipids and cholesterol (1). Apart from their role in bile secretion, bile acids enhance the intestinal absorption of dietary fats and cholesterol. The conversion of cholesterol to bile acids is of crucial importance for maintenance of cholesterol homeostasis (2). Cholic acid (CA) is a primary bile acid in humans as well as in rodents and comprises 30% to 50% (humans) or 50% to 80% (rodents) of the total bile acid pool. CA pool size, fractional turnover rate (FTR), and synthesis rate are the kinetic parameters that allow description of its production and conservation in the body. Isotope dilution techniques applying radioactive or stable isotopes (3–7) have been accepted as the preferred method to study bile acid kinetics in vivo and have contributed significantly to the present knowledge of bile acid (patho)physiology in humans (8–14). The techniques allow for the simultaneous determination of the CA pool size, FTR, and synthesis rate without interruption of the enterohepatic circulation.

Several novel animal models, including transgenic and knockout models, offer the opportunity to gain further insight in the regulation of bile acid metabolism as well as in the metabolic action of bile acids. Different methods have been used to measure parameters of bile acid kinetics in experimental animals, that is, analysis of the intestinal bile acid content as a reflection of the pool size (15, 16) and the "washout" method to estimate pool size and synthesis rate (17, 18). The fecal balance method is commonly applied to determine the total bile acid synthesis rate in

Abbreviations: CA, cholic acid; FTR, fractional turnover rate; GLC-MS, gas liquid chromatography-mass spectrometry; PFB-TMS, pentafluorobenzyl-trimethylsilyl.

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rodents (19). Finally, the activities or expression levels of cholesterol 7α-hydroxylase and sterol 27-hydroxylase, the rate-limiting enzymes in the classic and alternative pathway of bile acid synthesis, respectively, are often used as a reflection of bile acid synthesis rates (20, 21). However, none of the described methods is suitable to measure all relevant parameters of bile acid metabolism (i.e., pool size, FTR, and synthesis rate) simultaneously in vivo in animals with an intact enterohepatic circulation and most of the methods cannot be used repeatedly in the same animal. These disadvantages are theoretically circumvented by application of an isotope dilution procedure using blood sampling. However, current stable isotope approaches require 5-ml blood samples to obtain accurate measurements of isotope enrichment, which precludes use in rodents and in small humans (infants and neonates).

Therefore, we have developed a microscale stable isotope dilution procedure for CA, which allows for isotope enrichment measurements in small volumes of plasma. The method chosen is based on gas liquid chromatography (GLC)/electron capture negative chemical ionization-mass spectrometry (MS) applied to the pentafluorobenzyl-trimethylsilyl (PFB-TMS) derivative of CA (6). The technique was applied in healthy humans to enable comparison with literature data obtained by the conventional stable isotope technique with plasma sampling. To demonstrate isotopic equilibrium in the bile acid pool, CA kinetic data were determined simultaneously in plasma and bile of male unanesthetized Wistar rats with an exteriorized enterohepatic circulation (22). The applicability of the procedure was tested in male Wistar rats after manipulation of CA kinetics by cholestyramine treatment, which is anticipated to enhance CA turnover (23). The technique was also applied in male mice to be able to evaluate species dependency of CA metabolism. The studies reported herein show that the method allows for the measurement of bile acid kinetic parameters in rodents and, because of the small blood volumes, may also be useful in human neonates and children.

MATERIALS AND METHODS

Materials

2,2,4,4-Tetradeuterated CA ([$^2H_4$]CA; isotopic purity, 98%) was obtained from Isotec (Miamisburg, OH). Cholylglycine hydrolase from *Clostridium perfringens* (*welchii*) was purchased from Sigma (St. Louis, MO). Pentafluorobenzylbromide was purchased from Fluka Chemie (Buchs, Neu-Ulm, Switzerland). All other chemicals and solvents used were of the highest purity commercially available.

Animals and diets

Male Wistar rats (Harlan Laboratories, Zeist, The Netherlands) of ~350 g and male FVB mice (breeding colony at the Animal Facility of the Academic Medical Center of Amsterdam) of ~30 g were used in the experiments. The animals were housed in a light- and temperature-controlled facility. They had free access to tap water and standard lab chow, or, when indicated, to chow supplemented with 1% (w/w) cholestyramine (Sigma). Experimental protocols were approved by the Ethical Committee for Animal Experiments, Faculty of Medical Sciences (University of Groningen, Groningen, The Netherlands).

Human studies

Experimental protocols for studies in human volunteers were approved by the Ethical Committee of the University Hospital Groningen and were performed with the informed consent of the participants.

Experimental procedures

Experiment 1: Measurement of [$^2H_4$]CA kinetics in humans. CA kinetic parameters were measured in six healthy volunteers taking a normal Western-type diet (two females, four males; age, 25–58 years). Fifty milligrams of [2,2,4,4-$^2H_4$]CA was administered orally in 200 ml of 0.5% $NaHCO_3$ (5). Basal blood samples were collected before $^2H_4$ administration. Subsequently postprandial blood samples were taken twice daily for the next 3 days. Blood was centrifuged and plasma was stored at −20°C until analysis.

Experiment 2: Comparison of CA kinetics determined in rat plasma and bile. Three rats were individually housed and equipped with permanent catheters in the bile duct, duodenum, and heart, using techniques described previously (22). Catheters in bile duct and duodenum were connected to maintain an intact enterohepatic circulation. Animals were allowed to recover from surgery for 5 days. On day 5, 7 mg of [$^2H_4$]CA in a total volume of 250 μl of 0.5% $NaHCO_3$ in PBS was slowly administered as a bolus via the duodenal catheter. The amount of labeled CA administered was estimated to equal ~10% of the endogenous bile acid pool (15). Blood samples (250 μl) were collected via the heart catheter 1, 3, 4.5, 6, 9, 12, 21, 24, 48, 72, and 96 h after administration of [$^2H_4$]CA. Bile (50 μl) was sampled simultaneously with the blood samples from the bile duct catheter at ~2 min. After bile sampling, the enterohepatic circulation was immediately restored. Blood was centrifuged at 4,000 rpm for 10 min. Plasma and bile samples were stored at −20°C until analysis.

Experiment 3: Effects of cholestyramine treatment on CA kinetics in intact rats. To characterize the effects on CA kinetics of 1% (w/w) cholestyramine added to the diet of rats, 12 rats were individually housed and equipped with a heart catheter (33). They were randomly divided in two groups of six animals each. One group had free access to normal chow (controls), and the other group received normal chow supplemented with 1% (w/w) cholestyramine for 7 days. On day 5, 5 mg of [$^2H_4$]CA in a total volume of 250 μl of 0.5% $NaHCO_3$ in PBS solution was slowly administered through the heart catheter. Blood samples (250 μl) were collected in heparinized test tubes via the heart catheter before and 3, 4.5, 6, 12, 24, 30, and 48 h after injection of [$^2H_4$]CA. Blood was centrifuged at 4,000 rpm for 10 min. Plasma samples were stored at −20°C until analysis. Feces were collected in 24-h fractions during the experiment to determine fecal bile acid output as an independent reflection of bile acid synthesis, assuming steady state conditions.

Experiment 4: Measurement of [$^2H_4$]CA kinetics in mice. To determine bile acid kinetics in mice, six male mice were housed individually. On day 7, 210 μg of [$^2H_4$]CA in a solution of 0.5% $NaHCO_3$ in PBS was slowly injected intravenously via the penile vein under halothane anesthesia. At 6, 23, 48, and 72 h after administration (n = 3) or at 14, 34, 48, and 72 h (n = 3), blood samples (225 μl) were collected by tail bleeding under halothane anesthesia. Samples used for baseline isotope abundance measurements were obtained by heart puncture from a separate group of mice. Blood was collected in microhematocrit tubes containing heparin and centrifuged to obtain plasma. After centrifugation (4,000 rpm for 10 min) plasma was stored at −20°C until analysis.

Analytical techniques

Preparation of plasma and bile samples for isotope analysis. Plasma and bile samples were prepared for GLC-MS analysis as described previously (24).

Gas-liquid chromatography-electron capture negative chemical ionization-mass spectrometry. All analyses were performed on a Finnigan SSQ7000 quadrupole GLC-MS instrument (Finnigan MAT, San Jose, CA). Gas-liquid chromatographic separation was performed on a 30 m × 0.25 mm column, with a film thickness of 0.25 μm (DB-5MS; J&W Scientific, Folsom, CA). One to 3 μl of the final solution of derivatized bile acids was injected in the splitless mode at an injector temperature of 290°C and a column temperature of 150°C. The column temperature was then programmed to remain at 150°C for 1 min, to rise to 315°C at a rate of 30°C/min, and then to remain at 315°C for 14 min. The interface temperature was 290°C. The ion source was operated in the negative ion chemical ionization mode at 150°C, applying methane as the moderating gas at a source pressure of 1,600 mTorr. Isotope ratios were determined in the selected ion monitoring mode on *m/z* 623.3 (M0) and 627.3 (M4) for CA.

Optimal GLC-MS conditions were assessed by examining the effects of mass spectrometric and selected ion monitoring parameters. Settings were adjusted for each set of samples to obtain at least 30 data points on the gas chromatographic peak and a peak intensity for the M0 cholate peak at *m/z* 623.3 between 1×10^6 and 5×10^7 instrument units. Within this range stable isotope ratio values of M1/M0, M2/M0, M3/M0, and M4/M0 were obtained. Injection volume and electron multiplier voltage were adjusted to obtain base peak intensities at the defined intensity plateau, assuring accurate and reproducible M4/M0 isotope ratios. Because bile acid concentrations are different in bile and plasma and in plasma samples of different species, results have always been obtained under these standardized conditions.

Fecal bile acids. Total bile acid concentrations were measured in an aliquot of freeze-dried homogenized feces and determined by an enzymatic fluorimetric assay, as described previously (25).

Calculations

Isotope dilution technique. The area ratio M4/M0 is calculated after computerized integration of peak areas of M4 CA and M0 CA in the mass chromatograms for *m/z* 627.3 and 623.3, using LCQuan software (Finnigan MAT). Enrichment is defined as the increase of M4/M0 after administration of $[^2H_4]$CA and expressed as the natural logarithm of the atom% excess (ln APE) value (26). The decay of ln APE over time was described by linear regression analysis. From this linear decay curve the FTR and pool size of CA were calculated. The FTR (per day) equals the slope of the regression line. The pool size (micromoles per 100 g body weight) is determined according to the formula:

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

where D is the administered amount of label, b is the isotopic purity, and *a* is the intercept on the y axis of the ln APE-versus-time curve. The CA synthesis rate (micromoles per 100 g body weight per day) is determined by multiplying pool size and FTR (5).

Statistics

Values represent means ± SD for the indicated number of humans and animals per group.

Using SPSS (Chicago, IL) version 8.0 statistical software, the significance of differences was calculated by using the two-tailed Student's *t*-test for normally distributed paired data or a Mann-Whitney U test for data that were not normally distributed. $P < 0.05$ was considered significant. For the assessment of agreement between the measurements in bile and plasma, the Bland-Altman approach was used (27).

RESULTS

Accuracy and precision of M4/M0 isotope abundance measurements

Table 1 summarizes the measurements of M4/M0 natural abundance of CA and the corresponding variations in plasma and bile of rats, and in plasma of mice and humans. The theoretical natural isotope abundance based on the elemental and isotopic composition of the selected $[M\text{-}PFB]^-$ ion is also shown. All measured abundance values were within 20% of the theoretical value, which is considered to be accurate at the abundance level of 1.95%. The precision was good for CA in the rat and human specimens (SD < 0.1%), but for unknown reasons precision was less for mouse plasma. There were small but consistent differences in the natural abundance (M4/M0) of plasma CA between the different species. There was close agreement between the measurements of natural abundance in plasma and bile in rats.

Linearity of $[^2H_4]$CA enrichment measurements

To assess the linearity of the analyses, M4/M0 area ratio values in CA calibration standards containing 0, 0.02, 0.04, 0.06, 0.08, and 0.1 molar ratios of $[^2H_4]$CA, respectively, were determined. A Pearson's correlation coefficient of >0.99 was obtained. The slope (1.09) was close to one and the intercept (0.09) was close to 0. The coefficients of variation of the M4/M0 area ratios in the calibration samples of $[^2H_4]$CA/$[^2H_0]$CA ranged from 0.2% to 1.2%.

CA kinetics in humans

In applying the technique to the group of healthy human volunteers administered a normal Western-type diet the CA pool size averaged 2.4 ± 0.7 μmol/100 g body weight. The FTR and synthesis rate values were 0.46 ± 0.14 per day and 1.0 ± 0.2 μmol/100 g body weight per day, respectively (**Table 2**). Data were in close agreement with those described earlier for human volunteers, using a stable isotope dilution technique (6) and applying larger plasma samples and GLC combined with electron impact

TABLE 1. Natural abundance of CA M4/M0 in rats, mice, and humans as measured by gas-liquid chromatography/electron capture negative chemical ionization-mass spectrometry, using the pentafluorobenzyl-TMS derivative

Species	Matrix	Natural M4/M0 Abundance
		%
Rat	Plasma	1.69 ± 0.03
	Bile	1.69 ± 0.09
Mouse	Plasma	2.36 ± 0.44
Human	Plasma	1.76 ± 0.03
Theoretical value		1.95

Values represent means ± standard deviation (n = 3 or 4) per group.

TABLE 2. Pool sizes, fractional turnover rates, and synthesis rates of CA obtained by [$^2H_4$]CA isotope enrichment measurements in plasma of rats, mice, and humans

Species	Pool Size	FTR	Synthesis Rate
	μmol/100 g body weight	*per day*	*μmol/100 g body weight per day*
Rat	10.6 ± 1.2	0.88 ± 0.10	9.3 ± 0.1
Mouse	16.8 ± 2.1	0.44 ± 0.03	7.3 ± 1.6
Human	2.4 ± 0.7	0.46 ± 0.14	1.0 ± 0.2

Values represent means ± standard deviation (n = 6) for all species.

mass spectrometry (CA pool size, 2.8 ± 1.0 μmol/100 g body weight; FTR, 0.44 ± 0.13 per day; synthesis rate, 1.2 ± 0.5 μmol/100 g body weight per day).

Comparison of kinetic data obtained in rat bile and plasma

CA isotope enrichments between 1% and 60% were obtained in bile and plasma samples of rats collected between 3 and 96 h after administration of label. Linear regression analysis between plasma data and bile data yielded a slope of 1.02, an intercept of 0.6%, and an r^2 value of 0.9931 (**Fig. 1A**). Analysis by the Bland-Altman method showed that the mean difference between enrichments found in plasma and bile was +1.3% (range, −1.0 to +3.6%) over the whole range of values (Fig. 1B), indicating a slight overestimation of enrichment in plasma compared with bile. This difference was not dependent on the degree of enrichment. This phenomena may reflect the dilution of biliary [^{2}H]CA by newly synthesized unlabeled CA molecules at the time of sampling.

CA decay curves measured simultaneously in plasma and bile of rats exhibited first-order kinetics (**Fig. 2**). The average linear regression correlation coefficients were 0.9973 and 0.9975 for [$^2H_4$]CA decay in plasma and bile, respectively. Mean values of the intercepts and slopes of the ln APE-versus-time curves determined in plasma and bile were not different. In accordance with this observation, also the CA pool sizes (mean difference, plasma − bile: −1.0 μmol/100 g body weight), FTR (−0.01 per day), and synthesis rates (−0.5 μmol/100 g body weight per day) determined via plasma sampling and bile sampling, respectively, were similar. However, the absolute values for pool size, FTR, and synthesis rates obtained in this experiment have no physiological meaning because they are strongly affected by the removal of bile during the experiment. Bile sampling reflects a transient interruption of the enterohepatic circulation and approximately 5% of the CA pool is removed each time.

CA kinetics in intact rats and the effects of cholestyramine feeding

The CA pool size, FTR, and synthesis rate determined in rats with intact enterohepatic circulation not affected by catheterization and bile sampling are shown in Table 2.

The CA pool size was slightly (−21%) but significantly reduced in cholestyramine-treated rats (8.4 ± 1.9 vs. 10.7 ± 1.2 μmol/100 g body weight; $P < 0.05$). As expected, the

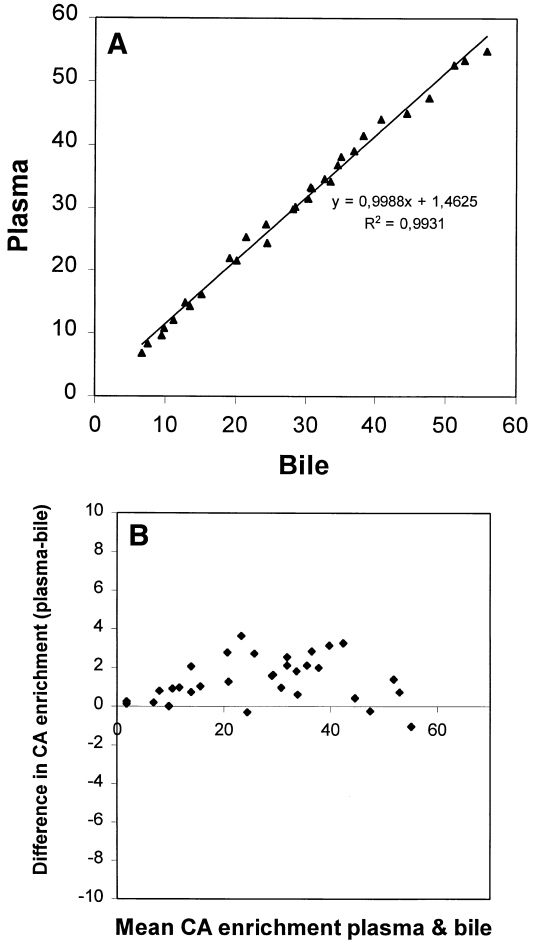

Fig. 1. Comparison of molar [$^2H_4$]CA enrichment in plasma and bile of male Wistar rats 3–96 h after intravenous injection of label. A: Expressed as linear regression. B: Expressed as a Bland-Altman plot.

FTR and synthesis rate of CA were significantly higher in rats treated with cholestyramine: 1.89 ± 0.29 versus 0.88 ± 0.11 per day ($P < 0.005$) and 15.7 ± 3.4 versus 9.3 ± 0.1 μmol/100 g body weight per day ($P < 0.05$), respectively.

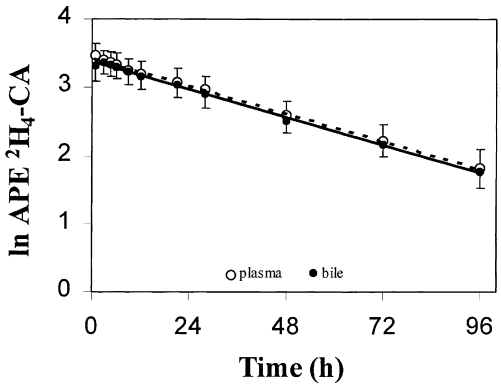

Fig. 2. Decay of [$^2H_4$]CA in plasma (open circles, dashed trend line) and bile (closed circles, solid trend line) after intravenous injection into male Wistar rats. Values are expressed as means ± SD (n = 3 per time point).

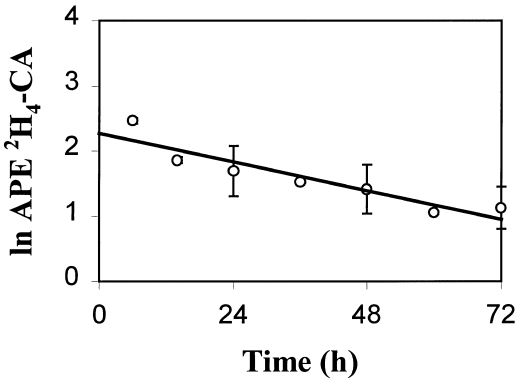

Fig. 3. Decay of [$^2H_4$]CA in plasma after intravenous injection into male FVB mice. Values are expressed as means ± SD (n = 3 per time point).

In line with this result, an approximately 2.5-fold increase in the total fecal bile acid excretion was observed in the cholestyramine-treated group (26.6 ± 3.1 vs. 10.9 ± 2.2 μmol/100 g body weight per day; $P < 0.05$).

CA kinetics in mice

Also in male FVB mice kept on regular lab chow the decay of label enrichment showed first-order kinetics (**Fig. 3**). Kinetic data obtained are shown in Table 2. CA pool sizes and synthesis rates were similar in rats and mice, but markedly higher in these rodents than in humans. In contrast, the FTR was markedly lower in mice compared with rats.

DISCUSSION

We have developed and evaluated a stable isotope dilution method using small plasma samples, applicable for determination of bile acid kinetics in vivo in humans as well as in rodents. To the best of our knowledge, this is the first report describing a stable isotope dilution technique applied in vivo in rodents with an intact enterohepatic circulation. Application of this technique allows for determination of CA pool size, FTR, and synthesis rate in the intact, unanesthetized animal and circumvents the specific disadvantages of previously used methods outlined in the introduction. The washout method requires biliary drainage for estimation of bile acid synthesis and bile acid pool size. This method is invasive, requires laparotomy, and cannot be repeated in the same animal. Moreover, anesthesia is most often required, which influences intestinal motility and therefore may reduce intestinal bile acid absorption (22, 28). Estimation of the pool size via measurement of intestinal bile acid content can obviously be performed only after killing the animal. In experiments involving the fecal balance method, feces are collected for several days and the daily fecal bile salt excretion is measured. Under steady state conditions, fecal bile acid excretion corresponds to hepatic bile acid neosynthesis. In this way the method provides data for bile acid synthesis. However, no information about bile acid pool size and FTR is obtained. Although the procedure is principally harmless and can be repeatedly performed, sample preparation is laborious, and requires determination of the extraction efficiency, while the presence of unidentified fecal metabolites may affect the outcome (19, 29). Also, to ensure that all feces are collected a stool marker is necessary as well as individual housing of the animals in metabolic cages. Measurement of cholesterol 7α-hydroxylase and sterol 27-hydroxylase expression and/or activity requires sampling of hepatic tissue. In addition, the degree of change in enzyme activity does not by definition reflect the actual change in bile acid synthesis (30). The isotope dilution method is the accepted method to determine bile acid pool sizes, FTR, and synthesis rates in humans. Development of stable isotope technology and mass spectrometry enabling isotope enrichment measurements in plasma has greatly improved the applicability of the method in adult humans. However, the conventional electron impact MS technique applied to the methyl TMS derivative lacks sensitivity to allow measurements in sufficiently small plasma samples to allow application in human neonates and in rodents. From previous studies, it is known that application of electron capture negative chemical ionization to PFB-TMS derivates of bile acids yields high sensitivity (24).

Indeed, by applying this derivatization technique, the blood sample size could be strongly reduced without loss of quality of measurement. The isotope ratio measurements for the natural isotope abundance resulted in values near the theoretical natural abundance value that were highly reproducible. This is a basic prerequisite because natural abundance values are subtracted from the enriched values obtained after administration of labeled CA. Small but consistent differences were found between values obtained in rats, mice, and humans. The largest difference is found in mouse plasma. Because of the larger interindividual variation this difference is not statistically significant. Because the intraindividual variation is smaller, these differences do not affect the measurement of isotope enrichment. The method is accurate in measuring enrichments, as could be demonstrated by the high degree of linearity of the calibration curve and the low coefficients of variation of the measurements.

The protocol for stable isotope dilution kinetic measurements in humans is well established. We describe the first experiments in rodents using the dosage of label and time schedule as described. On the basis of this experience it can be concluded that the time schedule of 72 h is well chosen for rodents. Also, the dose of 210 μg of [$^2H_4$]CA is recommendable for mice because the calculated enrichment at time point 0 is below 10%, whereas at 72 h the enrichment is still above 1%. It is recommended that the dose of [$^2H_4$]CA in rats be lowered to 2.5 mg to meet the same criteria as described for the mouse.

The data obtained for CA kinetic parameters in humans fitted well within the range of data reported in stable isotope dilution studies applying plasma samples or bile samples (3–6). This indicates that downscaling of plasma sample sizes and adaptation of the sample preparation procedure and mass spectrometry technique did not affect the final outcome, that is, values for pool size, FTR, and synthesis rate.

To demonstrate that, also in rodents, isotope enrichment values measured in plasma (<1% of the total pool) reflect those determined in the enterohepatic pool (>99% of the total pool), the [$^2H_4$]CA enrichment was measured simultaneously in bile and plasma of unanesthetized rats. The natural isotope abundance was similar in the two matrices, that is, bile and plasma. A small but consistent difference in enrichment was found independent of the enrichment itself. This difference cannot be explained by inadequate isotopic equilibration, but may be due to the steady influx of newly synthesized and unlabeled CA from the hepatocyte. No significant differences were found between the decay of label enrichment in bile and plasma. Isotopic equilibrium was thus quickly achieved after administration of [$^2H_4$]CA. Moreover, no significant differences in CA pool size, synthesis rate, and FTR calculated from plasma and bile measurements were found. Therefore, in analogy to studies of humans (5), CA kinetics can be determined reliably by stable isotope enrichment measurements in plasma of rats. Interestingly, CA kinetic data obtained in rats in which bile collections were performed were clearly different from the data in rats without bile collections. This may be explained by the pool depletion due to bile sampling. In retrospect, we could have diminished this effect by collecting smaller bile samples. In humans Duane (31) compared different techniques for measurement of bile acid synthesis in hypertriglyceridemia patients and control subjects. The techniques included isotope dilution using radioactive isotopes for CA and chenodeoxycholic acid, fecal bile acid excretion, and the release of $^{14}CO_2$ after [26-^{14}C]cholesterol administration. They found systematically higher values when the isotope dilution technique was used, applying radioactive tracers and bile sampling. The authors hypothesized that artificial, cholecystokinin-induced gallbladder contraction used for bile collection induces bile acid synthesis. This puts further emphasis on the strength of our isotope dilution method, which does not require bile sampling.

To determine whether manipulation of bile acid kinetics can be detected by this method, an experiment was conducted in which cholestyramine was added to the diet of rats. Cholestyramine is a resin known to bind bile acids in the small intestine, increasing the turnover of the bile acid pool as well as bile acid synthesis and cholesterol synthesis (23). In line with these data, cholestyramine feeding of rats in this study resulted in an increased CA synthesis rate and FTR. This observation is also in accordance with the increased total fecal bile acid output in the cholestyramine-treated rats compared with the control group and it demonstrates that manipulations of bile acid kinetics in vivo in rats can easily be detected via application of this stable isotope procedure. Despite strongly increased fecal bile acid loss, CA pool size was only slightly reduced. This demonstrates the capability of the system to upregulate CA synthesis under an induced condition of increased fecal loss. From our data no direct comparison can be made between CA synthesis data obtained with the isotope dilution technique and the fecal loss data for total bile acids. Total bile acid excretion is composed not only of CA metabolites but also of metabolites derived from α, β, and ω muricholic acids.

This study shows, to the best of our knowledge, the first data on CA kinetics in mice with an intact enterohepatic circulation. It appears that the CA pool size in male FVB mice, when expressed relative to body weight, is somewhat larger than that of male Wistar rats whereas CA synthesis rates are comparable in both species. The difference in pool size may be explained by a more efficient conservation of the CA pool in the mouse as FTR values in mice were almost 2-fold lower than in rats. This difference in FTR may be caused by the fact that, in contrast to rats, mice have a gallbladder. Obviously, it should be realized that there may be considerable strain differences in bile acid metabolism between different strains of mice (17, 32), which must be taken into account when comparing data from different studies.

In general, a direct comparison with literature data on bile acid kinetics in rodents is hampered by the large variation in reported values (15–19, 33–39). For example, previous estimates of the total bile acid pool size vary over a range of 12–60 μmol/100 g body weight in rats and 13–89 μmol/100 g body weight in mice. Strain, sex, and size differences between the animals, different dietary regimens, as well as the applied method may all account for the observed quantitative differences in total bile acid pool size. When taking into account that CA comprises 50–80% of the total bile acid pool in rodents, and assuming identical kinetics for all bile acids, our measurements lead to mean estimates of total bile acid pool sizes of about 15 μmol/100 g body weight in male Wistar rats and 25 μmol/100 g body weight in FVB mice. These values are in the lower range of data reported in the literature. Data on CA synthesis rates specifically are scarce for rodents because most studies report data on total bile acid synthesis rates.

Comparison of CA kinetics in rodents and humans obtained by the same methodology now reveals for the first time that rodents have a much larger CA pool size (about three to seven times) than human adults when expressed relative to body weight. Second, the FTR of CA is similar in mice and humans (0.4–0.5 per day), implying that the pool of CA is renewed by synthesis in about 48 h. CA synthesis in rodents is seven to nine times higher than in humans. Rodents also have a higher rate of cholesterol synthesis than humans when data are related to body size (40). Although the physiological consequences thereof are beyond the scope of this article, a higher bile acid synthesis may contribute to the relative resistance of rodents against diet-induced hypercholesterolemia.

In conclusion, the development and validation of a microscale stable isotope dilution technique for measurement of CA kinetics have been presented. It allows for use of small plasma volumes and reliable determination of CA kinetics in plasma of humans and small experimental animals with an intact enterohepatic circulation. The method can be used repeatedly in the same animal. In particular, the development of genetically modified mouse models with altered cholesterol and bile acid metabolism has generated a wealth of applications for this technique. In addi-

tion, this microscale method provides reliable data in adult humans and may well be applicable in human neonates and small children permitting study of the early development of human bile acid metabolism.

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REFERENCES

1. Hofmann, A. F. 1999. Bile acids: the good, the bad, and the ugly. *News Physiol. Sci.* **14:** 24–29.
2. Vlahcevic, Z. R., W. M. Pandak, and R. T. Stravitz. 1999. Regulation of bile acid biosynthesis. *Gastroenterol Clin. North Am.* **28:** 1–25.
3. Lindstedt, S., and A. Norman. 1957. The turnover of cholic acid in man. *Acta Physiol. Scand.* **40:** 1–9.
4. Watkins, J. B., D. Ingall, P. A. Szczepanik, P. D. Klein, and R. Lester. 1973. Bile salt metabolism in the newborn. Measurement of pool size by stable isotope technique. *N. Engl. J. Med.* **288:** 431–434.
5. Stellaard, F., M. Sackmann, T. Sauerbruch, and G. Paumgartner. 1984. Simultaneous determination of cholic and chenodeoxycholic acid pool sizes and fractional turnover rates in human serum using ^{13}C-labeled bile acids. *J. Lipid Res.* **25:** 1313–1319.
6. Stellaard, F., M. Sackmann, F. Berr, and G. Paumgartner. 1987. Simultaneous determination of pool sizes and fractional turnover-rates of deoxycholic acid, cholic acid, and chenodeoxycholic acid in man by stable isotope dilution with ^{2}H and ^{13}C labels and serum sampling. *Biomed. Environ. Mass Spectr.* **14:** 609–611.
7. Koopman, B. J., F. Kuipers, C. M. A. Bijleveld, J. C. van der Molen, G. T. Nagel, R. J. Vonk, and B. G. Wolthers. 1988. Determination of cholic and chenodeoxycholic acid pool sizes and fractional turnover rates by means of stable isotope dilution technique, making use of tetradeuterated cholic acid and chenodeoxycholic acid. *Clin. Chim. Acta.* **175:** 143–156.
8. Watkins, J. B., P. A. Szczepanik, J. B. Gould, P. D. Klein, and R. Lester. 1975. Bile salt metabolism in the human premature infant. Preliminary observations of pool size and synthesis rate following prenatal administration of dexamethason and Phenobarbital. *Gastroenterology.* **69:** 706–713.
9. Watkins, J. B., A. L. Järvenpää, P. Szczepanik van Leeuwen, P. D. Klein, D. K. Rassin, G. Gaull, and N. C. Raiha. 1983. Feeding the low-birth weight infant. V. Effects of taurine, cholesterol, and human milk on bile acid kinetics. *Gastroenterology.* **85:** 793–800.
10. Kern, F., Jr., G. T. Everson, B. DeMark, C. McKinley, R. Showalter, W. Erfling, D. Z. Braverman, P. Szczepanik-van Leeuwen, and P. D. Klein. 1981. Biliary lipids, bile acids, and gall bladder function in the human female: effects of pregnancy and the ovulatory cycle. *J. Clin. Invest.* **68:** 1229–1242.
11. Kern, F., Jr., G. T. Everson, B. DeMark, C. McKinley, R. Showalter, D. Z. Braverman, P. Szczepanik-Van Leeuwen, and P. D. Klein. 1982. Biliary lipids, bile acids, and gallbladder function in the human female: effects of contraceptive steroids. *J. Lab. Clin. Med.* **99:** 798–805.
12. Pauletzki, J., F. Stellaard, and G. Paumgartner. 1989. Bile acid metabolism in human hyperthyroidism. *Hepatology.* **9:** 852–855.
13. Berr, F., F. Stellaard, E. Pratchke, and G. Paumgartner. 1989. Effects of cholecystectomy on the kinetics of primary and secondary bile acids. *J. Clin. Invest.* **83:** 1541–1550.
14. Berr, F., E. Pratschke, S. Fischer, and G. Paumgartner. 1992. Disorders of the bile acid metabolism in cholesterol gallstone disease. *J. Clin. Invest.* **90:** 859–868.
15. Fisher, M. M., G. Kakis, and I. M. Yousef. 1975. Bile acid pool in Wistar rats. *Lipids.* **11:** 93–96.
16. Norman, A., and J. Sjövall. 1958. On the transformation and enterohepatic circulation of cholic acid in the rat: bile acids and steroids 68. *J. Biol. Chem.* **233:** 872–885.
17. Schwarz, M., D. W. Russell, J. M. Dietschy, and S. D. Turley. 1998. Marked reduction in bile acid synthesis in cholesterol 7α-hydroxylase deficient mice does not lead to diminished tissue cholesterol turnover or to hypercholesterolaemia. *J. Lipid Res.* **39:** 1833–1843.
18. Oude Elferink, R. P. J., R. Ottenhof, M. van Wijland, J. J. M. Smit, A. H. Schinkel, and A. K. Groen. 1995. Regulation of biliary lipid secretion by mdr2 P-glycoprotein in the mouse. *J. Clin. Invest.* **95:** 31–38.
19. Kinugasa, T., K. Uchida, M. Kadowaki, H. Takase, Y. Nomura, and Y. Saito. 1981. Effect of bile duct ligation on bile acid metabolism in rats. *J. Lipid Res.* **22:** 201–207.
20. Russell, D. W., and K. D. R. Setchell. 1992. Bile acid biosynthesis. *Biochemistry.* **31:** 4737–4749.
21. Chiang, J. Y. L. 1991. Reversed-phase high-performance liquid chromatography assay of cholesterol 7α-hydroxylase. *Methods Enzymol.* **206:** 483–491.
22. Kuipers, F., R. Havinga, H. Bosschieter, G. P. Toorop, F. R. Hindriks, and R. J. Vonk. 1985. Enterohepatic circulation in the rat. *Gastroenterology.* **88:** 403–411.
23. Gustafsson, B. E., B. Angelin, K. Einarsson, and J-A. Gustafsson. 1978. Influence of cholestyramine on synthesis of cholesterol and bile acids in germfree rats. *J. Lipid Res.* **19:** 972–977.
24. Stellaard, F., S. A. Langelaar, R. M. Kok, and C. Jakobs. 1989. Determination of plasma bile acids by capillary gas-liquid chromatography-electron capture negative chemical ionization mass fragmentography. *J. Lipid Res.* **30:** 1647–1652.
25. Murphy, G. M., B. H. Billing, and D. N. Baron. 1970. A fluorimetric and enzymatic method for the estimation of serum total bile acids. *J. Clin. Pathol.* **23:** 594–598.
26. Campbell, I. M. 1974. Incorporation and dilution values: their calculation in mass spectrally assayed stable isotopes labeling experiments. *Bioorg. Chem.* **3:** 386–397.
27. Bland, J. M., and D. G. Altman. 1983. Measurement in medicine: the analysis of method comparison studies. *Statistician.* **32:** 302–317.
28. Gustafsson, B. E., and A. Norman. 1969. Bile acid absorption from the caecal contents of germfree rats. *Scand. J. Gastroenterol.* **4:** 585–590.
29. Setchell, K. D., A. M. Lawson, N. Tanida, and J. Sjövall. 1983. General methods for the analysis of metabolic profiles of bile acids and related compounds in feces. *J. Lipid Res.* **24:** 1085–1099.
30. Smit, M. J., A. M. Temmerman, R. Havinga, F. Kuipers, and R. J. Vonk. 1990. Short- and long-term effects of biliary drainage on hepatic cholesterol metabolism in the rat. *Biochem. J.* **269:** 781–788.
31. Duane, W. C. 1997. Measurement of bile acid synthesis by three different methods in hypertriglyceridemic and control subjects. *J. Lipid Res.* **38:** 183–188.
32. Fuchs, M., F. Lammert, D. O. Wang, B. Paigen, M. C. Carey, and D. E. Cohen. 1998. Sterol carrier protein 2 participates in hypersecretion of biliary cholesterol during gallstone formation in genetically gallstone-susceptible mice. *Biochem. J.* **336:** 33–37.
33. Turley, S. D., M. Schwarz, D. K. Spady, and J. M. Dietschy. 1998. Gender-related differences in bile acid and sterol metabolism in outbred CD-1 mice fed low- and high-cholesterol diets. *Hepatology.* **28:** 1088–1094.
34. Uchida, K., Y. Nomura, M. Kadowaki, H. Takase, K. Takano, and N. Takeuchi. 1978. Age-related changes in cholesterol and bile acid metabolism in rats. *J. Lipid Res.* **19:** 544–552.
35. Uchida, K., I. Okuno, H. Takase, Y. Nomura, M. Kadowaki, and N. Takeuchi. 1978. Distribution of bile acids in rats. *Lipids.* **13:** 42–48.
36. Kuipers, F., J. M. van Ree, M. H. Hofker, H. Wolters, G. I. In't Veld, R. Havinga, R. J. Vonk, H. M. G. Princen, and L. M. Havekes. 1996. Altered lipid metabolism in apolipoprotein E-deficient mice does not affect cholesterol balance across the liver. *Hepatology.* **24:** 241–247.
37. Eyssens, H. J., G. G. Parmentier, and J. A. Mertens. 1976. Sulfated bile acids in germ-free and conventional mice. *Eur. J. Biochem.* **66:** 507–514.
38. Uchida, K., H. Mizuno, K. Hirota, K. Takeda, N. Takeuchi, and Y. Ishikawa. 1983. Effects of spinasterol and sitosterol on plasma and liver cholesterol levels and biliary and fecal sterol and bile acid excretions in mice. *Jpn. J. Pharmacol.* **33:** 103–112.
39. Uchida, K., S. Makino, and T. Akiyoshi. 1985. Altered bile acid metabolism in nonobese, spontaneously diabetic (NOD) mice. *Diabetes.* **34:** 79–83.
40. Dietschy, J. M. 1984. Regulation of cholesterol metabolism in man and other species. *Klin. Wochenschr.* **62:** 338–345.