Validation of [22,23-^{3}H]cholic acid as a stable tracer through conversion to deoxycholic acid in human subjects

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Summary Bile acids labeled with ^{3}H on the sterol nucleus lose a substantial fraction of label during enterohepatic cycling and conversion to secondary bile acids. We tested the isotopic stability of a side-chain ^{3}H label, [22,23-^{3}H]cholic acid in humans. The ^{3}H-labeled compound was administered simultaneously with [24-^{14}C]cholic acid to four healthy volunteers. Duodenal bile was collected daily for 5 days after isotope administration to determine the ratio of ^{3}H/^{14}C in bile acids. Urine was collected to determine loss of radioactivity by this route. Cholic acid and deoxycholic acid were isolated from biliary bile acids by thin-layer chromatography after deconjugation with cholylglycine hydrolase. The ratio of ^{3}H/^{14}C in cholic acid and deoxycholic acid remained constant and identical to that of the administered mixture in all subjects, indicating stability of the ^{3}H label during enterohepatic cycling. Cumulative loss of ^{3}H in urine averaged only 1.2% of administered dose and was identical to loss of ^{14}C (average 1.3%) indicating little if any transfer of ^{3}H from bile acid to body water. Deconjugation of biliary bile acids by alkaline hydrolysis resulted in 15–20% loss of ^{3}H label, consistent with known base-catalyzed exchange of α-carbon protons on carboxylic acids. We conclude that [22,23-^{3}H]cholic acid is a biologically stable, and therefore reliable, isotopic tracer of cholic acid in humans during enterohepatic cycling including conversion to deoxycholic acid, provided deconjugation is performed enzymatically. Because the 22,23-^{3}H label can be inserted into most C_{24} bile acids, it appears the best way to tag ^{3}H-labeled bile acids for metabolic studies. **–Duane, W. C., C. D. Schteingart, H-T. Ton-Nu, and A. F. Hofmann**. Validation of [22,23-^{3}H]cholic acid as a stable tracer through conversion to deoxycholic acid in human subjects. *J. Lipid Res.* 1996. **37:** 431–436.

Supplementary key words bile acids and salts • bile • enterohepatic circulation • urine • dehydroxylation • radioactive bile acids

note on methodology

Much of our understanding of bile acid metabolism has been achieved by studies using isotopically tagged bile acids as tracers. Bile acids labeled at the C-24 position with ^{14}C or ^{13}C have been considered the most reliable because of biological stability of the label.

In addition, efforts have been made repeatedly to develop biologically stable ^{3}H- or ^{2}H-labeled bile acids to enable simultaneous determination of tracer kinetics of multiple bile acids or of the steroid and amino acid moieties of conjugated bile acids. Cholic acid labeled with ^{3}H or ^{2}H in the 2,4-position (1, 2) and chenodeoxycholic acid labeled in the 1,2-position (1, 2) or 11,12-position (3, 4) have been used in a number of metabolic studies. Although these labels appear superior to ^{3}H randomly labeled bile acids prepared by the Wilzbach procedure (5, 6), they still have not behaved perfectly in validation studies. With both [2,4-^{3}H]cholic acid (2) and [11,12-^{3}H]chenodeoxycholic acid (4), a 10–15% loss of the ^{3}H or ^{2}H label has been observed in human studies. Still more disturbing is that further loss of ^{3}H occurs during conversion of each of these bile acids to its 7-deoxy derivative by colonic bacteria. For 2,4-^{3}H-labeled bile acids this loss was to be expected because 7-dehydroxylation proceeds via a $\Delta^{4,6}$-3-oxo intermediate (7). Loss of ^{3}H from the 11,12-^{3}H-position in the conversion of chenodeoxycholic acid to lithocholic acid does not have a ready explanation. Finally when [11,12-^{3}H]lithocholic acid has been administered to humans, ^{3}H has been lost extremely rapidly (4), again for no known reason.

During enterohepatic cycling in vertebrates, the sterol nucleus of bile acids is subject to a wide variety of modifications including hydroxylation–dehydroxylation, desaturation during the dehydroxylation process, and oxidoreduction with or without epimerization; these changes resulting from both hepatic and bacterial enzymes (8–12). In contrast, except for conjugation–deconjugation and α-oxidation of α-hydroxy bile acids (13), the side-chain is not modified. We therefore postulated some years ago (4) that positioning a ^{3}H or ^{2}H label on the side-chain, rather than the sterol nucleus, would produce a biologically stable tracer. To test this hypothesis, we have now performed, and report here, experiments that examine the stability of the ^{3}H label in [22,23-^{3}H]cholic acid during enterohepatic cycling in healthy human subjects.

METHODS

Chemicals

[22,23-^{3}H]cholyltaurine had been prepared earlier as described (14). Briefly, triformylcholic acid was brominated on the 23-position with N-bromosuccinimide in trifluoroacetic acid/trifluoroacetic anhydride. The product was esterified with diazomethane, and bromine

Abbreviations: DPM, disintegrations per minute; SA, specific activity.

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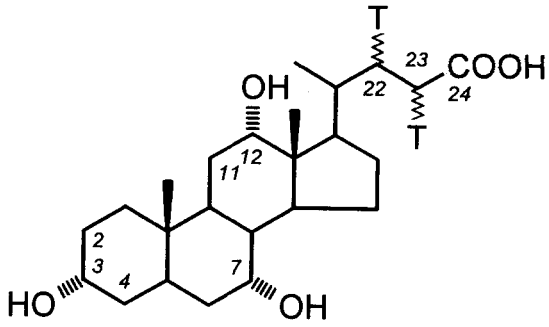

Fig. 1. Chemical structure of [22,23-^{3}H]cholic acid.

was eliminated by heating at 120°C in hexamethylphosphoramide. Alkaline hydrolysis afforded Δ^{22}-cholic acid which was conjugated with taurine using the method of Tserng, Hachey, and Klein (15) to give Δ^{22}-cholyltaurine. This was reduced with pure tritium gas with 5% Pd on charcoal as catalyst (National Tritium Facility, Berkeley, CA) and purified by reversed phase thin-layer chromatography to give [22,23-^{3}H]cholyltaurine with a specific activity of 58 Ci/mmole.

By ^{3}H-NMR (320 MHz, [^{1}H] decoupling, in d_4-methanol) (16) the tritium atoms were found to be located exclusively on C-22 and C-23. The ^{3}H-NMR spectrum showed two pairs of doublets with an integrated ratio of 60:40 corresponding to the products of addition of tritium from either side of the double bond. The most intense pair of doublets was located at 2.086 and 1.756 ppm, chemical shifts characteristic of one H-23 and H-22, respectively, in bile acids (17, 18). The signals appeared as doublets with a coupling constant J = 7.3 Hz, indicating the presence of exactly one tritium atom on each carbon with a defined configuration. This was the expected result because in catalytic hydrogenations the two hydrogen atoms are added from the same side of the double bond (*cis* addition). The corresponding signals for the least abundant pair of doublets (J = 5.7 Hz) appeared at 2.230 and 1.320 ppm, characteristic of the other H-23 and H-22, respectively, in bile acids (17, 18). The absence of any other ^{3}H signals in the spectrum indicated that there was no label scrambling or double bond epimerization during the reduction step.

Approximately 100 μCi of [22,23-^{3}H]cholyltaurine was diluted with unlabeled cholyltaurine (Sigma Chemical, St. Louis, MO) to yield a specific activity of about 50 mCi/mmole and deconjugated with cholylglycine hydrolase by the method of Batta, Salen, and Shefer (19). The resulting [22,23-^{3}H]cholic acid was extracted into chloroform–methanol and purified by preparative thin-layer chromatography on silicic acid with a mobile phase of isooctane–ethyl acetate–acetic acid 5:5:1. [24-^{14}C]cholic acid was obtained commercially (New England Nuclear, Boston, MA) and checked for radioisotopic purity by thin-layer chromatography. Structure of the labeled compound is shown in **Fig. 1.**

Study procedures

We studied four healthy male volunteers ranging in age from 39 to 68 years. All were in good health as judged by previously published criteria (20). All had biliary ultrasonography demonstrating absence of gallstones. Informed consent was obtained from each subject, and all study procedures were approved by the committee overseeing use of human subjects in research at the Minneapolis VA Medical Center.

In the evening, after a light supper, each subject was given an oral sodium bicarbonate solution containing both [24-^{14}C]cholic acid and [22,23-^{3}H]cholic acid. Subjects 1 and 2 (Table 1) received 6.49 μCi [24-^{14}C]cholic acid and 13.0 μCi [22,23-^{3}H]cholic acid. Subjects 3 and 4 received 5.05 μCi [24-^{14}C]cholic acid and 11.1 μCi [22,23-^{3}H]cholic acid. For the 5 subsequent days, gallbladder bile was collected via peroral duodenal tube using cholecystokinin octapeptide to stimulate gallbladder contraction as previously described (21). Urine was quantitatively collected for the 5 days following administration of isotope.

Biliary bile acids present in aliquots of duodenal bile were deconjugated using cholylglycine hydrolase (19). To test whether the method of deconjugation influenced the ^{3}H/^{14}C ratio of the resulting unconjugated bile acids, a second aliquot of bile from two subjects was deconjugated by alkaline hydrolysis in 1.25 N NaOH at 121°C and 15 psi for 30 min. In both cases, the deconjugated bile acids were acidified and the unconjugated bile acids were partitioned into the chloroform phase of a chloroform–methanol extraction system. Cholic acid and deoxycholic acid were then separated by thin-layer chromatography as previously described (20, 21). A small portion of each band eluate was analyzed by gas–liquid chromatography of the methyl ester acetate derivatives (20, 21) to assure identity and purity. An aliquot of each eluate was analyzed for radioactivity by liquid scintillation counting using appropriate settings to permit simultaneous quantitation of ^{14}C and ^{3}H. A separate aliquot of each eluate was analyzed for bile acid mass by automated enzymatic assay based on 3α-hydroxysteroid dehydrogenase (20, 22, 23).

Volume of urine output was measured daily, and aliquots were assayed for ^{3}H and ^{14}C radioactivity by liquid scintillation counting. Outputs of urinary radioactivity for each of the 5 days after isotope administration were summed and expressed both as cumulative total output of radioactivity in disintegrations per minute (DPM) and as percent of administered isotope (Table 1).

RESULTS

Figure 2 shows the time course of the ratio of $^3H/^{14}C$ radioactivity in cholic acid (upper panel) and deoxycholic acid (lower panel) for samples deconjugated enzymatically. The ratio of $^3H/^{14}C$ in the isolated bile acid has been normalized by dividing by the ratio of $^3H/^{14}C$ in the administered mixture. The value for each individual subject is shown. It is evident that this normalized isotopic ratio remained constant during the 5 days after isotope administration at a value essentially equal to 1.00 for both bile acids in all subjects. Thus, the ^3H label remained intact in cholic acid during enterohepatic cycling and was not removed during the multiple steps involved in bacterial 7-dehydroxylation of cholic acid to form deoxycholic acid.

Table 1 summarizes the recovery of radioactivity in urine. Cumulative 5-day urinary excretion of isotope was similar in all subjects and quite small, being 1.2% for ^3H and 1.4% for ^{14}C. This magnitude of loss of radioactivity from the bile acid pool as urinary bile acids is in agreement with published values (2, 4). Moreover, the identical recovery of ^3H and ^{14}C radioactivity suggests that all urinary ^3H radioactivity was in the form of bile acids. Had an appreciable amount of ^3H been lost from bile acid to body water, a disproportionate amount of ^3H, compared to ^{14}C, would have been excreted in the urine.

In two subjects, biliary bile acids were also deconjugated by alkaline hydrolysis. For both cholic acid and deoxycholic acid, this procedure resulted in 15–20% lower ^3H specific activities compared to hydrolysis using cholylglycine hydrolase, indicating loss of ^3H radioactivity induced by the alkaline procedure (**Table 2**). In contrast, ^{14}C specific activities determined after alkaline hydrolysis were indistinguishable from those after enzymatic hydrolysis.

TABLE 1. Total urinary excretion of radioactivity for 5 days after simultaneous administration of [22,23-^3H]cholic acid and [24-^{14}C]cholic acid

Subject	DPM[a]		Percent[b]	
	^3H	^{14}C	^3H	^{14}C
1	273,000	178,300	0.95	1.24
2	394,000	229,000	1.36	1.59
3	445,000	174,000	1.80	1.55
4	145,800	75,100	0.59	0.67
Mean			1.17	1.26

[a]Total radioactivity in disintegrations per minute (DPM) excreted over 5 days.
[b]Percentage of administered isotope excreted over 5 days.

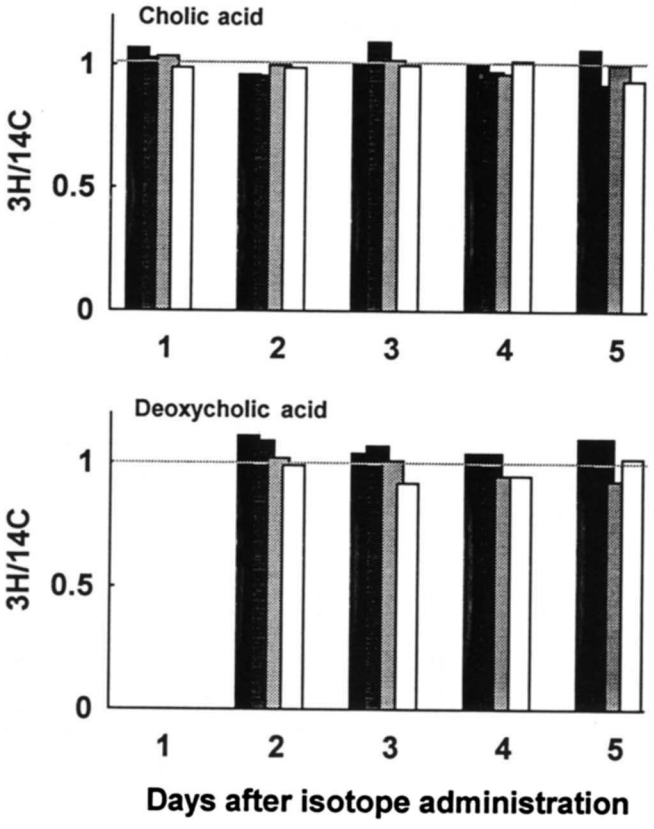

Fig. 2. Time course in four human subjects of the ratio of $^3H/^{14}C$ radioactivity in cholic acid (top panel) and deoxycholic acid (bottom panel) as a function of days after simultaneous administratin of [22,23-^3H]cholic acid and [24-^{14}C]cholic acid. Bile acids were isolated from bile following enzymatic econjugation. Experimental data have been normalized by dividing the ratio of $^3H/^{14}C$ measured in each bile acid by the ratio of $^3H/^{14}C$ in administered material. Individual ubjects are indicated by bar pattern: subject 1, white; subject 2, light gray; subject 3, dark gray; and subject 4, black. Amount of radioactivity in deoxycholic acid on day 1 was not yet sufficient to permit accurate measurement.

DISCUSSION

These data indicate that in healthy humans [22,23-^3H]cholic acid is a biologically stable tracer of cholic acid during enterohepatic cycling including conversion to deoxycholic acid. To the best of our knowledge, no previously tested tritiated primary bile acid has been shown to be stable during the multiple bacterial and hepatic biotransformations that occur during enterohepatic cycling. These results are consistent with much previous data indicating that, except for deconjugation, the bile acid side-chain in humans is unaffected by intestinal bacteria (9). At least for carbons 22 and 23, we found no apparent proton exchange in vivo.

The labeling procedure described here can be applied to any natural C_{24} bile acid with an unsubstituted side-chain because nuclear hydroxy substitutents are not altered by the labeling process. In contrast, labeling at the C-11 and C-12 positions requires preparation of the

TABLE 2. Specific radioactivities (SA) of cholic acid and deoxycholic acid after either alkaline or enzymatic hydrolysis in two subjects

	Day	Akaline Hydrolysis			Enzymatic Hydrolysis		
		^{3}H SA	^{14}C SA	^{3}H/^{14}C	^{3}H SA	^{14}C SA	^{3}H/^{14}C
		dpm/μmol			*dpm/μmol*		
Cholic acid							
Subject 1							
	1	9210	5510	1.67	9520	4810	1.98
	2	6840	4080	1.68	7810	3950	1.98
	3	5580	3270	1.71	6380	3190	2.00
	4	3940	2380	1.66	4490	2200	2.04
	5	2010	1175	1.71	3020	1680	1.80
	Mean	5520	3280	1.68	6240	3170	1.96
Subject 2							
	1	6760	3940	1.72	8280	4000	2.07
	2	5880	3440	1.71	6780	3380	2.01
	3	5190	3050	1.70	5610	2740	2.05
	4	4080	2430	1.68	4440	2360	1.88
	5	3040	1797	1.69	3450	1716	2.01
	Mean	4990	2930	1.70	5710	2840	2.00
Deoxycholic acid							
Subject 1							
	2	3180	2060	1.54	4060	2040	1.99
	3	4040	2580	1.57	5050	2750	1.84
	4	4430	2680	1.65	5410	2850	1.90
	5	2690	1580	1.70	3210	1578	2.03
	Mean	3580	2220	1.62	4430	2300	1.94
Subject 2							
	2	1166	648	1.80	1359	665	2.04
	3	2120	1239	1.71	2500	1236	2.02
	4	2480	1454	1.71	2840	1496	1.89
	5	4280	2670	1.60	5440	2910	1.87
	Mean	2510	1500	1.70	3030	1577	1.96

Δ^{11} intermediate which removes a C-12 hydroxy group. This, this label cannot be used for cholic acid or other C-12 hydroxy bile acids. Nonetheless, additional studies are required to document the suitability of the 22,23-^{3}H label for other natural bile acids. A method for preparing 11α,12α,22ε,23ε-$^{2}H_4$-labeled chenodeoxycholic acid and ursodeoxycholic acid has also been reported by Lai, Byon, and Gut (24).

A minor disadvantage of the 22,23-^{3}H label noted in our study was loss of some tritium during alkaline hydrolysis (Table 2). The α-protons of carboxylic acids are known to undergo exchange in alkaline conditions (25) suggesting that loss of tritium during alkaline deconjugation occurred mainly from the 23-position. Thus, in the workup of samples from metabolic studies using 22,23-^{3}H-labeled bile acids, enzymatic deconjugation must be used.

Studies of bile acid metabolism are complicated not only by the variety of bile acids produced both by hepatic synthesis and bacterial modification, but also because these bile acids are present in both conjugated and unconjugated forms (26–28). Characterization of this system in vivo using isotopic tracer methods is often greatly facilitated by using several simultaneously administered isotopic tracers. Simultaneous administration of [24-^{14}C]cholic acid and [24-^{14}C]chenodeoxy-

cholic acid is commonly done in order to measure synthesis rate of the two primary bile acids in humans (21, 29–31). However, this approach neglects conversion of primary bile acids to secondary bile acids, a process which at least for cholic acid appears important in pathogenesis of cholesterol cholelithiasis (32, 33). Fully accounting for these conversions would require a third and fourth tracer, which heretofore has not been feasible because of biological instability of tritiated bile acids. The present study indicates that a method is now available for preparing tritiated C_{24} bile acids that should faithfully trace bile acid metabolism during enterohepatic cycling.

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