Suitability of [11,12-$^3H_2$]chenodeoxycholic acid and [11,12-$^3H_2$]lithocholic acid for isotope dilution studies of bile acid metabolism in man

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Abstract Studies were carried out to assess the stability of the label in [11,12-$^3H_2$]chenodeoxycholic acid and [11,12-$^3H_2$]lithocholic acid during enterohepatic cycling and thus define the suitability of these tracers for isotope dilution studies of bile acids in man. To do this, [11,12-$^3H_2$]-chenodeoxycholic acid and [24-^{14}C]chenodeoxycholic acid were administered simultaneously to six healthy adults. The $^3H/^{14}C$ ratio of biliary chenodeoxycholic acid and the specific activity of biliary chenodeoxycholic acid were determined to permit calculation of pool size and turnover rate by the conventional isotope dilution procedure of Lindstedt (1957. *Acta Physiol. Scand.* **40:** 1). Excretion of label in urine was measured as well as the $^3H/^{14}C$ ratio of fecal bile acids. Similar studies were carried out with [11,12-$^3H_2$]lithocholic acid and [24-^{14}C]lithocholic acid.

With [11,12-$^3H_2$]chenodeoxycholic acid, 10–15% of the label was lost during the first 24 hours, after which the $^3H/^{14}C$ ratio of biliary chenodeoxycholic acid remained constant. The loss of ^3H caused a consistent overestimate of pool size by 10–15%, but since the fractional turnover rate of the two isotopes was identical, the calculated rate of synthesis was 10–15% greater when determined using ^3H. The loss of ^3H was attributed to the presence of ^3H in positions other than 11 and 12 occurring during the labeling procedure when the unsaturated precursor was exposed to carrier-free tritium gas. The $^3H/^{14}C$ ratio of feces was consistently below that of bile, suggesting bacterial removal of additional ^3H during distal intestinal passage. In contrast, [11,12-$^3H_2$]lithocholic acid lost ^3H continuously, so that by 2 days after administration, the $^3H/^{14}C$ ratio had fallen to nearly half of that of the administered mixture.

It is concluded that isotope dilution studies may be carried out with one preparation of [11,12-$^3H_2$]chenodeoxycholic acid with an error of 10–15%; possibly a more stable preparation can be prepared with a gentler tritiation procedure. One preparation of [11,12-$^3H_2$]lithocholic acid was unsatisfactory for isotope dilution studies in man.

Supplementary key words tritium labeling · bile acids · isotope dilution

Tritium-labeled bile acids facilitate the study of bile acid metabolism in man. In previous investigations in man, bile acids randomly labeled with ^3H by the Wilzbach procedure were shown to be unsatisfactory for isotope dilution studies if used without purification (1), or, even after extensive purification, to manifest some loss of label (2). We recently reported that [2,4-$^3H_4$]chenodeoxycholic acid, prepared by enolic exchange (3) was stable during enterohepatic cycling in man when compared to [24-^{14}C]chenodeoxycholic acid; however, about 30% of the label was lost into body water before fecal excretion (4). Our preparation of [2,4-$^3H_4$]cholic acid was also relatively stable during enterohepatic cycling, losing only about 5–10% of the label. However, an additional 30% of the label was lost before fecal elimination, since the $^3H/^{14}C$ ratio of stool was consistently lower than that of the administered isotope mixture. This loss occurred before or in association with the 7-dehydroxylation of cholic acid since, in bile, the $^3H/^{14}C$ ratio of deoxycholic acid was about 30% lower than that of cholic acid.

Because of these observations suggesting that all previous preparations of ^3H-labeled bile acids were only partially satisfactory for studies of bile acid metabolism in man, we sought a better ^3H label for bile acids. To this end we prepared [11,12-$^3H_2$]chenodeoxycholic and lithocholic acid by reductive tritiation of their Δ^{11}-olefinic precursors (5). In preliminary studies in man, the label of [11,12-$^3H_2$]chenodeoxycholic acid appeared to be stable during enterohepatic cycling, but

Abbreviations: [2,4-$^3H_4$]chenodeoxycholic acid, [2,2′,4,4′-$^3H_4$]-chenodeoxycholic acid; GLC, gas–liquid chromatography.

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about 10–20% of the label appeared to be lost during distal intestinal passage (5). In complementary studies, we also prepared [11,12-$^2H_2$]chenodeoxycholic acid in a similar manner and found that the deuterium in [11,12-$^2H_2$]chenodeoxycholic acid was stable during enterohepatic cycling in man (6). In this paper, we report more extensive studies on the stability in man of the label in [11,12-$^3H_2$]chenodeoxycholic and [11,12-$^3H_2$]lithocholic acid.

MATERIALS AND METHODS

Labeled bile acids

[24-^{14}C]Chenodeoxycholic acid and [24-^{14}C]lithocholic acids were obtained from International Chemical and Nuclear Corp. (ICN, Irvine, CA). They were found to have radiopurity of greater than 96% by thin-layer chromatography (7) and zonal scanning (8) and were not further purified.

[11,12-$^3H_2$]Chenodeoxycholic acid and [11,12-$^3H_2$]lithocholic acid were synthesized by New England Nuclear, Boston, MA, by catalytic reduction of 3α,7α-dihydroxy-Δ^{11}-5β-cholenic acid and 3α-hydroxy-Δ^{11}-5β-cholenic acid respectively with carrier-free tritium gas as described (5). The [11,12-$^3H_2$]chenodeoxycholic acid had a sp act of 1.5 Ci/mmol so that only a tracer dose was administered in these studies. The [11,12-$^3H_2$]chenodeoxycholic acid was purified by preparative thin-layer chromatography using a solvent system of acetone–benzene–acetic acid 30:70:1 (7). By zonal scanning (8) the compound had a purity of >98%, and when cocrystallized with [24-^{14}C]chenodeoxycholic acid, the $^3H/^{14}C$ remained constant and at 1.0 (5). The [11,12-$^3H_2$]-lithocholic acid had a similar sp act and was also purified by preparative thin-layer chromatography using a solvent system of acetone–benzene–acetic acid 15:85:1 (7) until >98% pure by zonal scanning (8). When cocrystallized with [24-^{14}C]lithocholic acid, the $^3H/^{14}C$ ratio remained constant at 1.0 (5).

Experimental design

Healthy male volunteers between the age of 20 and 25 years were studied after informed consent was obtained. A known amount of the ^{3}H-labeled and ^{14}C-labeled bile acid (as the sodium salt) was diluted to 50 ml with a sterile solution of NaCl in a volumetric flask. Aliquots were taken, in triplicate, for the determination of ^{3}H and ^{14}C radioactivities. The solution was then infused slowly via an intravenous infusion set. The tubing and the flask were rinsed three times with 25 ml of sterile saline solution and the washes were also administered. Bile samples were collected at intervals through an indwelling single lumen naso–duodenal tube positioned fluoroscopically so that its aspiration site was 5 cm distal to the ampulla of Vater. For bile collections, gallbladder contraction was evoked by a slow intravenous infusion of 75 Ivy dog units of cholecystokinin–pancreozymin (purchased from the Karolinska Institutet, Stockholm, Sweden). A sample (2–3 ml) of concentrated bile was kept and diluted with 9 volumes of isopropyl alcohol and stored at 4°C prior to analysis. The remaining bile (an average of at least 25 ml) was reinfused into the duodenum through the naso–duodenal tube.

Chenodeoxycholic acid

Six subjects received 60 μCi of [11,12-$^3H_2$]chenodeoxycholic acid and 20 μCi of [24-^{14}C]chenodeoxycholic acid; radiolabeled bile acids were administered before the evening meal on the day prior to the first bile sample collection. Bile samples were collected daily for 5 days. Twenty-four hour urine and stool collections were made for 5–7 days.

Lithocholic Acid

Three subjects received 50 μCi of [11,12-$^3H_2$]-lithocholic acid and 20 μCi of [24-^{14}C]lithocholic acid at 7:00 AM on the first day. Bile samples were then obtained at 12:00 noon, 5:00 PM and 10:00 PM that day, and in the morning and evening of the second and third days. Bile samples were obtained more frequently for lithocholic acid because of its more rapid turnover rate (9, 10). Urine was collected every 24 hours for the entire study period.

Determination of isotope ratios

Chenodeoxycholic acid kinetics and $^3H/^{14}C$ ratio in bile. A 5-ml aliquot of the isopropanol solution of bile was transferred to nickel bombs (Parr Instrument Co., Moline, IL) for alkaline hydrolysis and subsequent methylation with diazomethane and acetylation with acetic anhydride (11). The bile acid methyl acetates were dissolved in ethyl acetate; one aliquot was used for determination of mass by GLC, and a second aliquot was used for determination of radioactivity by liquid scintillation spectroscopy.

The aliquot used for radioactivity determination was evaporated to dryness, and the residue was dissolved in 10 ml of a toluene-based scintillation mixture (Omnifluor, New England Nuclear, Boston, MA) was added. Radioactivity was measured by dual channel counting of ^{3}H and ^{14}C with 12% crossover of ^{14}C into the ^{3}H window in a Beckman LS-250 liquid scintillation counter, using external standardization. All $^3H/^{14}C$ ratios were normalized by setting

to 1.0 the $^{3}H/^{14}C$ ratio of the mixture that was administered.

Gas–liquid chromatography was carried out by using a 3% cyanosilicone stationary phase (AN 600, Analabs, Hamden, CT) described previously (12). Nordeoxycholic acid was used as an internal standard.

The sp act of ^{3}H and ^{14}C for the administered bile acid was determined for each bile acid in each sample of bile, and the natural logarithm of the sp act (expressed as % dose/mmol bile acid) was plotted against time for the calculation of pool size, turnover rate, and daily synthesis rate (13, 14).

Lithocholic acid kinetics and $^{3}H/^{14}C$ ratio in bile. For the determination of bile acid mass, 2 ml of diluted bile samples were evaporated to dryness and hydrolyzed in 2 N NaOH and 50% methanol for 4 hr at 115°C with nordeoxycholic acid added as an internal standard to correct for loss during hydrolysis. After saponification, the solution was acidified to pH 1, and extracted into ether; under these conditions, complete solvolysis of sulfated lithocholate occurs, so that the mass determined by GLC represents the sum of both sulfated and unsulfated species (15). (In man, it is likely that most of the lithocholate in bile is present as the sulfated conjugate.) (9, 10, 16). For GLC, the bile acid was subsequently methylated and acetylated as described above.

For radioactivity, 5-ml aliquots of the isopropanol solution were used without further purification, since all radioactivity was assumed to be present in the four lithocholate conjugates present in bile. The aliquot was decolorized using ultraviolet light, evaporated to dryness, and redissolved in 0.5 ml of 0.05 N NaOH. Counting of radioactivity was done in a Beckman LS-250 liquid scintillation counter, as described above, after 10 ml of a toluene-based detergent scintillation cocktail (Ready-Solv VI, Beckman, Fullerton, CA) was added to each sample. The ratio of ^{3}H to ^{14}C was calculated from the radioactivity determined by liquid scintillation spectroscopy and normalized as described.

The sp act of ^{3}H- and ^{14}C-labeled lithocholic acids was determined. Kinetics were calculated in a similar fashion as that for ^{3}H- and ^{14}C-labeled chenodeoxycholic acid (see above).

$^{3}H/^{14}C$ ratio in urine. From each 24-hr urine collection, 0.5 ml of urine was taken in triplicate. After the addition of 15 ml of Ready-Solv VI (Beckman, Fullerton, CA), radioactivity was measured by liquid scintillation spectroscopy using external standardization to correct for quenching. In the studies in which subjects received [^{3}H]chenodeoxycholic acid, bile acids were absorbed from urine by adding 3 g of an ion exchange resin, Amberlyst A-26 (Mallinckrodt Chemical Works, St. Louis, MO) to a 6-ml aliquot of urine. Radioactivity in the supernatant was determined to quantitate non-bile acid radioactivity. In preliminary experiments using [^{14}C]chenodeoxycholic acid in urine, it was demonstrated that about 95% of the radioactive bile acid was bound by the resin.

$^{3}H/^{14}C$ ratio in feces. Stools were collected for the entire study period in subjects who had received labeled chenodeoxycholic acid. Stools were homogenized as described previously (17) and aliquots were sent by mail to Pemlab Co. (Brookfield, IL) for determination of $^{3}H/^{14}C$ ratios by combustion. The efficiency was calculated by combusting ^{3}H- and ^{14}C-labeled bile acid standards added to nonradioactive fecal homogenates.

Stability of label during analysis. Aliquots of the radioactive bile acid mixtures were treated by the same procedures of hydrolysis, extraction, methylation, and acetylation that were employed on the bile samples in the determination of bile acid mass. The ratios of $^{3}H/^{14}C$ were determined after each step; the ratio remained constant.

RESULTS

Chenodeoxycholic acid

Bile. The sp act curves of all subjects were similar, showing a rapid loss of 10–15% of the ^{3}H during the first 24 hr. Typical sp act decay curves are shown in **Fig. 1**. Because of the loss of the label, the intercept of the sp act decay curve with the ordinate was lower for ^{3}H than for ^{14}C, resulting in an overestimate of pool size, averaging 15%, as summarized in **Table 1**. The fractional turnover rate of the two radiolabeled bile acids was identical, so that synthesis, the product of pool size and turnover rate, was 15% lower when estimated from the ^{3}H label.

Stools and urine. The $^{3}H/^{14}C$ ratio of fecal samples was lower than that of bile, indicating additional loss of ^{3}H during intestinal passage (**Table 2**). About 6% of the ^{3}H was excreted in urine, and about half of this was present as water in urine. Thus, the loss of ^{3}H was considerably greater, since body water in man has a very slow turnover, averaging 2 weeks (18).

About 2% of the ^{14}C was excreted in urine (**Table 3**). The chemical form of this label was not characterized, and it could represent chenodeoxycholic acid, the radioimpurities present in the commercial sample, or both.

Lithocholic acid

Bile. In contrast to chenodeoxycholic acid, the loss of ^{3}H from [11,12-$^{3}H_2$]lithocholic acid continued during the entire study (**Fig. 2**). At 48 hr, the sp

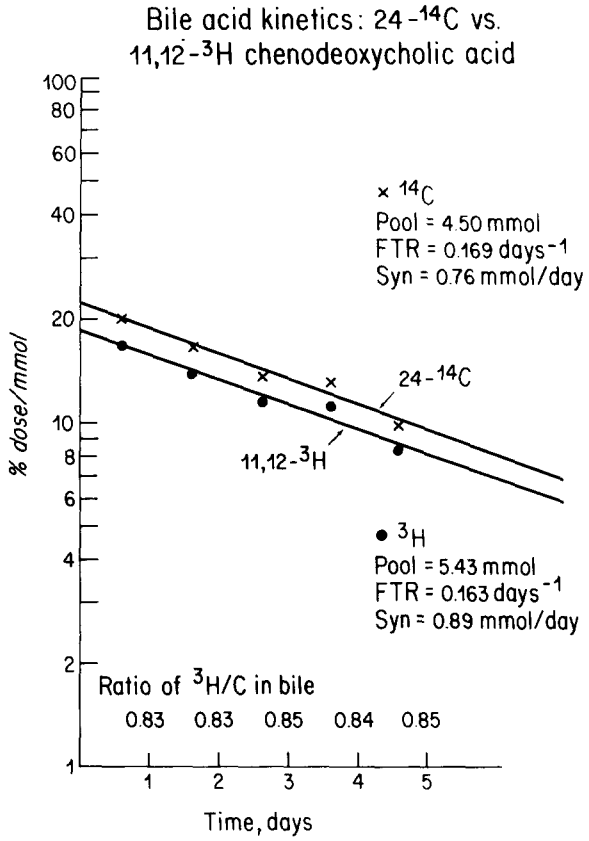

Fig. 1. Representative specific activity decay curves of chenodeoxycholic acid in bile after administration of [24-^{14}C]chenodeoxycholic acid (×) or [11,12-$^{3}H_2$]chenodeoxycholic acid (○).

act had declined to 0.6 that of [^{14}C]lithocholic acid. Because of the continuous loss of label, no attempts were made to calculate pool size and turnover rate.

Urine and Stools. About 9% of the ^{3}H was excreted in urine in the one subject who was studied (Table 2); more than half of this was excreted during the first 24 hr. Only 1.7% of the ^{14}C administered was in urine. Isotope ratios were not determined in stool, since the preparation was clearly labile during enterohepatic cycling.

DISCUSSION

Stability of label in [11,12-$^{3}H_2$]chenodeoxycholic acid

Our results indicate that [11,12-$^{3}H_2$]chenodeoxycholic acid may be used for isotope dilution studies in man with an error of less than 15% for determination of pool size and synthesis rate; further, in these studies, the labeled compound provided a valid estimate of turnover rate. The rapid, small, and consistent loss of ^{3}H during the first 24 hr is most reasonably explained by the presence of ^{3}H at a site other than the 11,12-position. Our preparation was prepared by exposure to carrier-free tritium gas and, under these conditions, migration of ^{3}H atoms may well have occurred. Our previous studies (6) which indicated that the label in [11,12-$^{2}H_2$]chenodeoxycholic acid is stable supports this belief.

We prepared material with the highest possible sp act since we wished to use the preparation for our radioimmunoassay of conjugates of chenodeoxycholic acid (19). We speculate that preparation of [11,12-$^{3}H_2$]chenodeoxycholic acid using lower sp act tritium gas would yield a product in which the ^{3}H is sufficiently stable to give valid results when used in isotope dilution studies of bile acid metabolism in man.

Additional loss of label occurred after the tracer was lost from the enterohepatic circulation, presumably by bacterial desaturation of the B-ring. Desaturation by bacteria of the A-ring of steroids (20) and bile acids (21) is well documented in in vitro studies. In man, A-ring desaturation must occur in view of our previous studies showing loss of ^{3}H from [2,4-$^{3}H_4$]-labeled bile acids (4). However, C-ring desaturation of bile acids has not been demonstrated previously. Since unsaturated bile acids have not been detected in fecal bile acids (22), resaturation is likely to occur during colonic transit (23).

TABLE 1. Chenodeoxycholic acid kinetics: comparison of results obtained with [24-^{14}C]- or [11,12-$^{3}H_2$]chenodeoxycholic acid

	Pool Size			Fractional Turnover Rate			Synthesis Rate		
Subject	^{14}C	^{3}H	% Diff.	^{14}C	^{3}H	% Diff.	^{14}C	^{3}H	% Diff.
	mmol			*per day*			*mmol/day*		
Jn	4.50	5.43	+18.7	0.169	0.163	−3.6	0.76	0.89	+15.8
Aj	3.66	4.26	+15.2	0.178	0.175	−1.7	0.65	0.75	+14.3
Gs	2.52	3.01	+17.7	0.174	0.172	−1.2	0.44	0.52	+16.7
Je	2.37	2.69	+12.6	0.163	0.165	+1.2	0.39	0.44	+12.0
Gk	2.36	2.65	+11.6	0.216	0.207	−4.3	0.51	0.55	+7.5
Te	1.75	2.01	+13.8	0.619	0.610	−1.5	1.08	1.23	+13.0
Mean	2.86	3.34	+14.93	0.25	0.25	−1.85	0.64	0.73	+13.22
± SE	± 0.42	± 0.52	± 1.15	± 0.07	± 0.07	± 0.79	± 0.1	± 0.12	± 1.34

Stability of label in [11,12-$^3H_2$]lithocholic acid

[11,12-$^3H_2$]Lithocholic acid, in contrast to [11,12-$^3H_2$]chenodeoxycholic acid rapidly lost its label, precluding its use for isotope dilution studies in man. Lithocholic acid is excreted in bile largely as sulfated conjugates (9, 10, 16), which are poorly reabsorbed from the small intestine in man (10). In the distal intestine, the unsulfated conjugates are desulfated and deconjugated, and the liberated lithocholic acid is in part reabsorbed to enter the enterohepatic circulation (10). Thus, lithocholate is unique among the major primary and secondary bile acids in that the majority of that which is absorbed has already been exposed to bacteria in the distal intestine, whereas most other bile acids (cholic, chenodeoxycholic, and deoxycholic) are absorbed from the small intestine without undergoing deconjugation (24, 25). This could be the explanation for the rapid and continuous loss of ^3H during enterohepatic cycling; alternatively, or in addition, lithocholate may be more susceptible to bacterial biotransformation than chenodeoxycholate.

TABLE 2. Ratios of $^3H/^{14}C$ radioactivity in bile and feces in subjects given [24-^{14}C]- or [11,12-$^3H_2$]chenodeoxycholic acids

Subject	Day	$^3H/^{14}C$ Ratios[a] Bile	Feces
Jn	1	0.83	0.79
	2	0.83	0.74
	3	0.85	0.76
	4	0.84	0.77
	5	0.85	0.77
Aj	1	0.86	
	2	0.86	
	3	0.88	0.86
	4	0.87	
	5	0.87	0.86
			0.79
Gs	1	0.84	
	2	0.84	
	3	0.83	
	4	0.85	0.84
	5	0.84	0.80
Je	1	0.88	
	2	0.87	0.85
	3	0.87	0.85
	4	0.88	0.77
	5	0.87	0.76
			0.78
Gk	1	0.90	
	2	0.88	0.85
	3	0.88	0.83
	4	0.88	0.86
	5	0.88	0.81
	6	0.85	0.81
	7	0.90	0.82
Te	1	0.89	
	2	0.89	0.84
	3	0.89	
	4	0.89	0.86
	5	0.89	
	6	0.92	
	7	0.92	0.76

[a] The ratio of isotopes administered was normalized to 1.0.

TABLE 3. Urinary excretion of radioactivity after intravenous administration of [24-^{14}C]- or [11,12-$^3H_2$]-labeled bile acids, % dose[a]

Chenodeoxycholic Acid Subject	Normal Location of Isotope 11,12-$^3H_2$	24-^{14}C
Jn	3.9	1.1
Aj	4.7	2.4
Gs	4.3	3.6
Je	10.4	1.9
Gk	4.9	1.7
Te	5.7	1.8
Mean ± SE	5.65 ± 1.0	2.1 ± 0.35

[a] Data for lithocholic acid are not presented for individual subjects since the 11,12-^3H label was clearly unstable. Average urinary loss of radioactivity for three subjects was 8.8% for ^3H and 1.7% for ^{14}C, when expressed as % dose.

Desiderata

The present studies are incomplete in that they suggest, but do not prove, that a preparation of

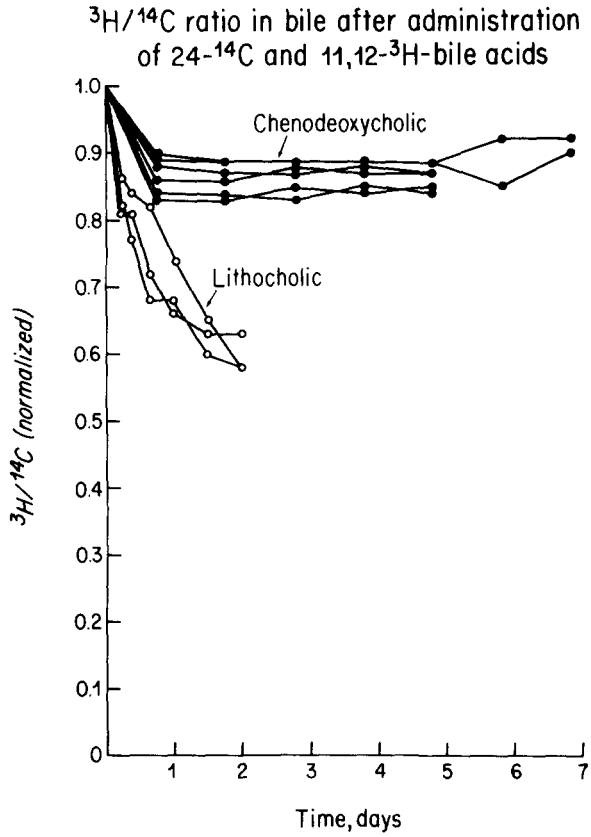

Fig. 2. Time course of $^3H/^{14}C$ ratio of chenodeoxycholic acid (●) or lithocholic acid (○) in bile after simultaneous administration of a mixture of [24-^{14}C] and [11,12-$^3H_2$]-labeled bile acids.

[11,12-$^{3}H_2$]chenodeoxycholic acid can be prepared that is suitable for isotope dilution studies in man. The major advantage of [11,12-$^{3}H_2$]chenodeoxycholic acid is that this radiolabeled bile acid can be prepared much more cheaply and with much higher sp act than [2,4-$^{3}H_4$]- or [24-^{14}C]chenodeoxycholic acid. Clearly, the [11,12-$^{3}H_2$]lithocholic acid cannot be used. It would seem prudent to find methods for inserting ^{3}H into the side chain of the bile acid molecule, since alterations in ring substituents by bacteria are common, whereas the side chain of bile acids is considered to remain intact during intestinal transit.

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