Rapid preparation of tritium-labeled bile acids by enolic exchange on basic alumina containing tritiated water

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ABSTRACT When a 3-keto bile acid methyl ester was chromatographed on basic alumina inactivated with tritiated water, the enolic hydrogen atoms at C-2 and C-4 exchanged with tritium atoms. The ^{3}H-labeled keto ester was reduced with borohydride, and the resultant mixture of 3α- and 3β-hydroxy epimers was resolved by preparative thin-layer chromatography to yield a pure 2,4-^{3}H-labeled bile acid ester. Lithocholic, chenodeoxycholic, deoxycholic, and cholic acids having a specific activity of 1–10 μc/μmole were prepared from their 3-keto derivatives. The tritium label remained intact during alkaline saponification in vitro and enterohepatic cycling in vivo in human subjects.

SUPPLEMENTARY KEY WORDS exchange labeling · bile acid epimers · thin-layer chromatography · isotope fractionation

KLEIN AND KNIGHT (1) showed that ketosteroids could be exchange-labeled by column chromatography on basic alumina containing tritiated water (HTO). Further studies (2) on the stoichiometry of labeling of monoketo- and diketosteroids established that the exchange was limited to the enolic hydrogens adjacent to the keto group, and that essentially complete exchange between compound and adsorbent occurred during a single passage through an alumina column. Reduction of the keto group with metallic hydrides could be achieved without appreciable loss of label; the resultant hydroxysteroids contained four tritium atoms per keto group of the precursor, and this label was stable under strongly alkaline conditions.

Abbreviations: HTO, tritiated water; TLC, thin-layer chromatography.

We report here the successful application of this method to the preparation of tritium-labeled lithocholic, deoxycholic, chenodeoxycholic, and cholic acids—the predominant primary and secondary bile acids. The technique involves the following sequential steps (Fig. 1): preparation of a 3-keto bile acid ester by oxidation of the 3-hydroxy group; packing of a column of basic alumina previously treated with HTO; exchange labeling of the compound by passage of the keto derivative through the alumina column (Fig. 2); reduction of the keto group by treatment with borohydride; and separation of the mixture of epimers by preparative thin-layer chromatography. The product is a 2,4-^{3}H-labeled (or, more strictly, a 2,2′,4,4′-^{3}H-labeled) bile acid methyl ester.

EXPERIMENTAL PROCEDURES

Preparation of Bile Acids

3α-Epimers. Lithocholic acid (K & K Laboratories, Inc., Plainview, New York, or Nutritional Biochemicals Corporation, Cleveland, Ohio) was converted to the methyl ester and purified by adsorption chromatography on alumina columns (3, 4). Deoxycholic acid (T. Schuchardt, Munich, Germany), and cholic acid (Riedel de Haen, Hannover, Germany, or Matheson, Coleman, and Bell) were purified by crystallization (5). Chenodeoxycholic acid was prepared synthetically (4). Methyl esters were prepared by the use of an ethereal solution of diazomethane.

3-Keto Bile Acids. Methyl 3-keto-5β-cholanoate was prepared by oxidation of lithocholic acid with Kiliani's reagent (chromic oxide–concentrated sulfuric acid–water, 53:80:400 by weight) (6) followed by crystalliza-

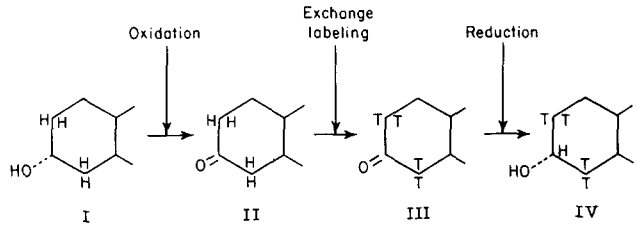

FIG. 1. The major steps in exchange labeling. The methyl ester of a 3-hydroxy bile acid (I) is oxidized to a 3-keto bile acid (II), which is exchange-labeled by passage over a column of basic alumina inactivated with HTO. The 2,4-^{3}H-3-keto bile acid (III) which is eluted from the column is reduced with borohydride to give a 2,4-^{3}H-3-hydroxy bile acid (IV) as the major product. Small amounts of the 3β-epimer are also formed during borohydride reduction; the epimer mixture is resolved by preparative TLC (see text). Only the A ring is shown.

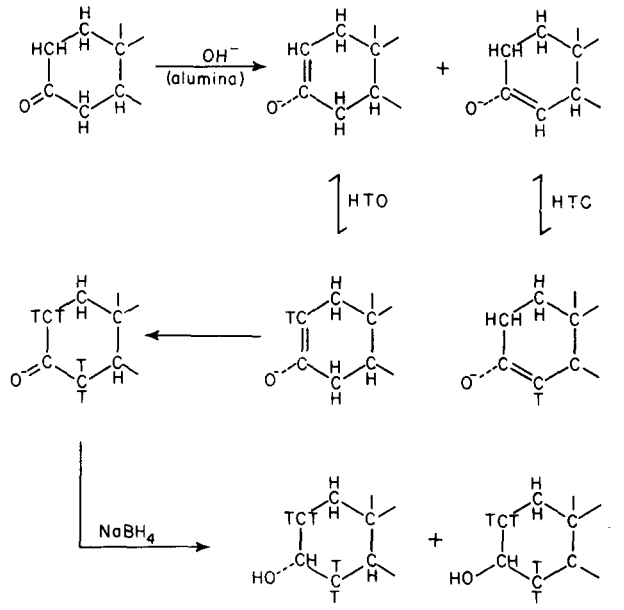

FIG. 2. The mechanism of exchange labeling of 3-keto bile acid during chromatography on basic alumina containing HTO. If full equilibration is achieved, the specific activity of the tritium on the eluted 3-keto bile acid will be equal to the specific activity of the alumina, which will be 80–90% of the specific activity of the HTO used for inactivation (2).

tion from diethyl ether, methylation (diazomethane), and crystallization of the methyl ester from ethyl acetate–heptane (mp 118°C; reported [7] 117°C). Methyl 3-keto-7α-hydroxy-5β-cholanoate was prepared by Oppenauer oxidation (8) of methyl chenodeoxycholate (4), column chromatography of the reaction mixture on alumina (3, 4), and crystallization from benzene–petroleum hydrocarbon (mp 126°C; reported [9] 125°C). Methyl 3-keto-12α-hydroxy-5β-cholanoate (mp 143°C; reported [9] 142–145°C) was donated by Dr. Erwin H. Mosbach or prepared from the methyl 3α,12α-diacetoxy-5β-cholanoate (10–12). Partial saponification, reesterification, and chromic-oxide oxidation yielded methyl 3-keto-12α-acetoxy-5β-cholanoate which, on vigorous saponification and reesterification (diazomethane), afforded the desired 3-keto compound (mp 143°C; reported [11] 144–145°C). Methyl 3-keto-7α,-12α-dihydroxy-5β-cholanoate was prepared by Oppenauer oxidation (8) of methyl cholate (4) followed by chromatography of the reaction mixture on alumina. Crystallization from ethyl acetate–heptane gave needles (mp 174°C; reported [9] 172°C).

3β-Epimers. Methyl 3β-hydroxy-5β-cholanoate was a gift of Dr. Robert Palmer. The 3β-epimers of chenodeoxycholic, deoxycholic, and cholic acids were prepared for use as chromatographic standards as described by Danielsson and associates (9).

Chromatography on Alumina

Basic alumina (aluminum oxide, basic, Merck, Brinkmann Instruments Inc., Westbury, N.Y. or alumina, basic, Woelm, Alupharm Chemicals, New Orleans, La.), 6.6 g, was placed in a 50 ml boiling flask closed with a well-lubricated stopcock (Kimax 45000) connected with vacuum tubing to a vacuum pump. A drying tube (Kimax 46035) containing thoroughly activated molecular sieves (Linde 5A, Matheson Scientific, Inc., Chicago, Ill.) was inserted between the stopcock and the vacuum pump. The adsorbent is dehydrated by heating for 1 hr at 260–300°C (oil bath) during evacuation. After the dehydration procedure, the stopcock is closed and the adsorbent allowed to cool. The stopcock is then opened slowly so that dry air enters the flask. The stopcock is then closed, and the activated adsorbent may be kept indefinitely.

HTO (5 c/ml, Nuclear-Chicago Corporation, Des Plaines, Ill.) was rapidly added to the adsorbent. 0.2 ml of HTO per 6.6 g of adsorbent corresponds to grade I (Brockmann) activity. The opening of the vial of HTO, its addition to the alumina, and all subsequent chromatographic steps must be carried out in a well-ventilated hood to avoid diffusion of vapor into the laboratory. After addition of the HTO, the vessel was tightly sealed and shaken for 3 hr to insure equilibration of hydroxyl groups. For complete equilibration of the hydroxyl groups of the alumina, a more complex procedure involving repeated cycles of dehydration and rehydration has been described (2). Although the basic alumina may be used as purchased, i.e. without any dehydration step, the specific activity of the product obtained with such a preparation will be much lower than if the single dehydration step is carried out as described.

Chromatography must be carried out with nonhydroxylic solvents to prevent tritium exchange between the solvent and the alumina. For the monohydroxy bile acids, chromatography was carried out in benzene; for dihydroxy bile acids, benzene–chloroform, 1:1 by

volume, or chloroform; and for trihydroxy bile acids, ethyl acetate. A column is prepared by pouring a slurry of the tritiated alumina (6.6 g) in the appropriate solvent into the column. We have generally used 4 × 100 mm columns, but it is unlikely that column dimensions are important. As little solvent as possible is run through the column before the sample (10–50 mg) is applied in a small volume of the same solvent used for chromatography. The keto compounds have low solubilities in pure chloroform or ethyl acetate, and it may be necessary to add a few drops of benzene to effect solution.

The mechanism of exchange labeling is shown in Fig. 2. The column was eluted at a rate not exceeding 1 ml/min with 200 ml of solvent, and fractions were collected either by means of a fraction collector or manually. Fractions were dried under a stream of dry nitrogen; this step also must be carried out in the hood. Fractions that contain keto acid may be identified by inspection, since the keto acid will crystallize or form a gum at the bottom of the vessel. Alternatively, fractions may be examined by TLC before evaporation. Isopropanol was dried with Linde 5A molecular sieves and decanted, and a saturated solution of sodium borohydride in isopropanol (about 4 g/100 ml) was prepared. 2 ml of isopropanol–borohydride was used to dissolve and transfer the keto acid to a small glass bottle, which was sealed and left for several hours. The isopropanol was then transferred, using rinses of water, to a separatory funnel. After the addition of 5 volumes of water, the solution was slowly acidified and extracted with ethyl acetate in a two-stage countercurrent extraction, the second aqueous phase being water. The pooled ethyl acetate phases were dried with sodium sulfate or calcium sulfate, decanted, and evaporated to yield the mixture of labeled epimers. In our experience, the compounds present as methyl esters do not undergo significant transesterification in the isopropanol.

TLC Separation of Epimers

The sample was dissolved in chloroform–methanol 2:1 and a few drops of ethereal diazomethane were added to the sample to insure that it was completely esterified. The sample was applied with a commercial streaker (Applied Science Laboratories Inc., State College, Pa.), and the chromatogram was developed up to four times until the bands, as observed in an iodine chamber, appeared clearly separated (Fig. 3). Resolution of the 3α- and 3β-hydroxy epimers of monohydroxy and dihydroxy bile acids is readily obtained on layers of silicic acid (Silica Gel G). The epimers of cholic acid, however, cannot be satisfactorily separated on silicic acid layers, but are well resolved when chromatographed on layers prepared from equal parts (by weight) of silicic acid and magnesium silicate (magnesium silicate for TLC, Woelm, Alupharm Chemicals). Layers of magnesium silicate alone will also separate these epimers, but the magnesium silicate layers are extremely fragile. The bands were scraped from the plate and eluted with chloroform–methanol 2:1 or 95% ethanol; the solution was concentrated and the bile acid methyl ester was extracted into ethyl acetate and washed well with water containing sodium thiosulfate to remove iodine and silicic acid. The free acid may be obtained by saponification (ethanol–1 N NaOH 1:1, overnight, room temperature) followed by dilution, acidification, and extraction of the liberated free acid into ethyl acetate or chloroform. Where salt is not an objectionable contaminant, as for administration of the product to animals, the sample may be obtained as the sodium salt in the saponification liquid by neutralization with hydrochloric acid.

RESULTS

Yield

The final yield of pure 2,4-^{3}H-labeled bile acid depends on (*a*) the specific activity of the alumina; (*b*) the degree of exchange (this is related to chromatographic conditions as well as to the intrinsic keto–enol equilibrium constant of the compound); (*c*) the completeness of the borohydride reduction; (*d*) the exchange of label with the alcohol used as solvent in the borohydride reduction; (*e*) the steric specificity of the borohydride reduction; and (*f*) the completeness of recovery after borohydride reduction and preparative TLC. The yields of radioactivity obtained with each bile acid ranged from 10 to 40 μc for 10 mg of 3-keto bile-acid precursor; specific activities of the final free bile acid ranged from 1 to 10 μc/μmole.

Fig. 4 shows a zonal scan (13) of the TLC separation of the reaction mixture after borohydride reduction of methyl 3-keto-7α-hydroxy-5β-cholanoate. The product is largely the 3α-epimer, which indicates that the steric specificity of the borohydride reduction is in favor of the equatorial configuration.

Assay of Epimer Purity by TLC

The reaction product of the borohydride reduction contains chiefly 3α-epimer, a small fraction of the 3β-epimer, and, occasionally, unreacted 3-keto bile acid. Experiments were carried out to test the completeness of separation of the 3α- and 3β-epimers by the preparative TLC step that follows the borohydride reaction. In these experiments, an aliquot of a pure 3α-fraction, obtained by preparative TLC (containing 10,000 dpm and negligible mass), was added to a reference mix-

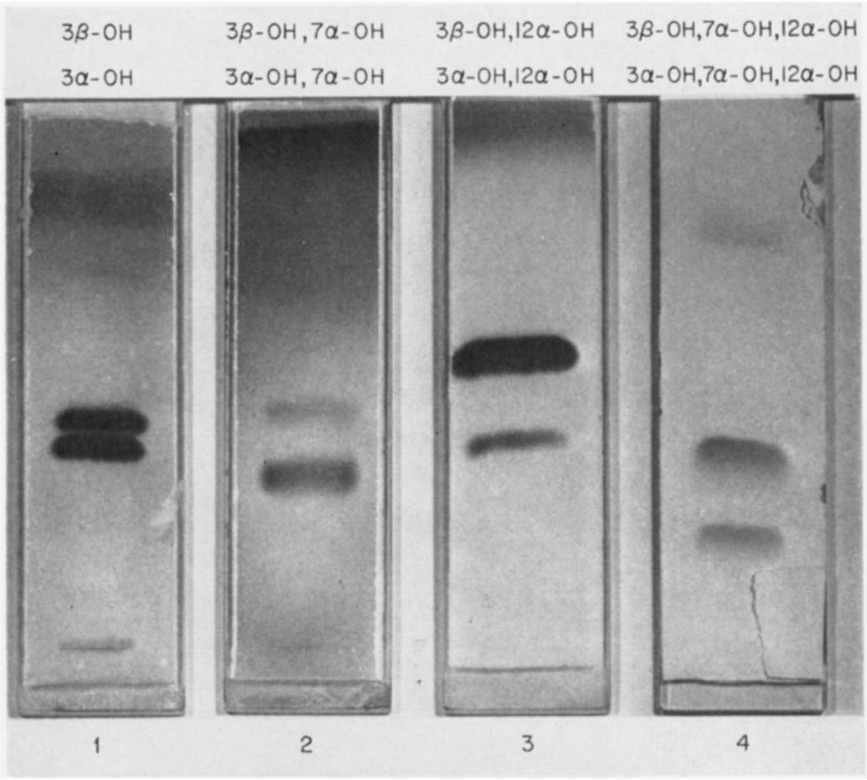

FIG. 3. TLC separation of 3α- and 3β-epimers of bile acid methyl esters. The substituents of the compounds are indicated; all chromatograms were developed twice. The adsorbent and solvent system used were as follows. Plate *1*, methyl lithocholate and 3β-epimer: silicic acid and acetone–cyclohexane, 20:80. Plates *2* and *3*, methyl chenodeoxycholate and its 3β-epimer and methyl deoxycholate and its 3β-epimer: silicic acid and acetone–benzene 30:70. Plate *4*, methyl cholate and 3β-epimer: magnesium silicate–silicic acid 1:1 and acetone–benzene 50:50. Compounds run on silicic acid plates were detected with phosphomolybdic acid, 10 g/100 ml ethanol. The methyl cholate and its 3β-epimer were detected with sulfuric acid–water 1:1.

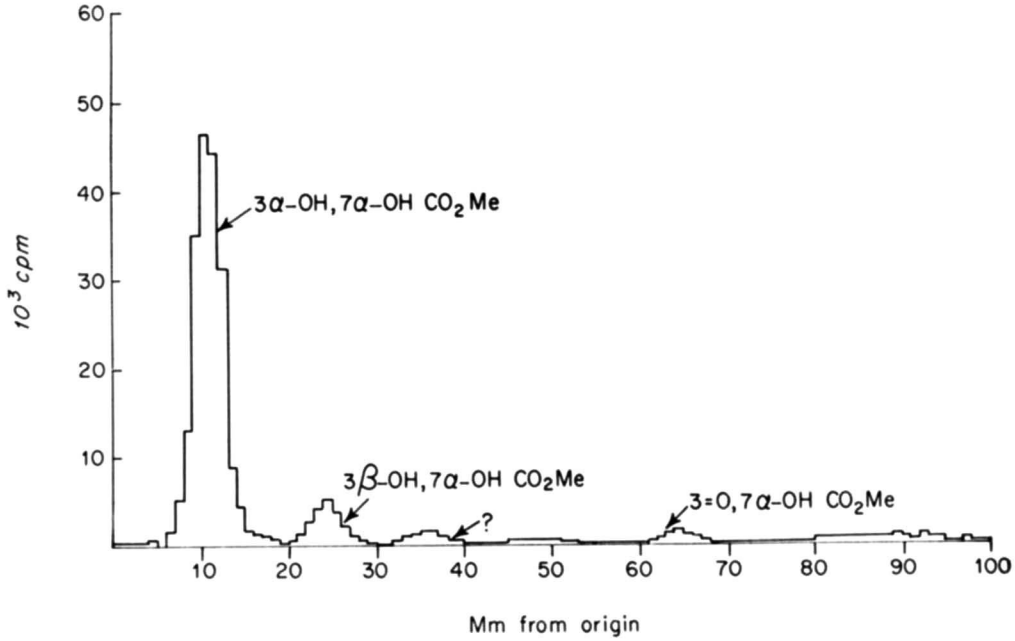

FIG. 4. Zonal scan of the reaction product of borohydride reduction of methyl 3-keto-7α-hydroxy-5β-cholanoate-2,4-^{3}H. The product is chiefly 3α-epimer; some unreduced 3-keto acid remains.

ture of nonradioactive 3α- and 3β-epimers. The reference mixture was separated by TLC, and the radioactivity in each band was determined by liquid scintillation counting. The results (Table 1) indicate that the preparative TLC step results in a product of at least 97% epimer purity.

Assay of Radiopurity and Isotope Fractionation by Cochromatography With ^{14}C-Labeled Bile Acids

The tritium-labeled compounds were chromatographically pure, by virtue of their purification by TLC. As a further test of radiopurity, each tritium-labeled bile acid was cochromatographed with its corresponding -24-^{14}C form. Methyl lithocholate mixtures were chromatographed on an alumina column and all three varieties were examined by TLC, a zonal scraper being used to divide the peak into several fractions. From the elution pattern and the $^{3}H/^{14}C$ ratio, two types of information could be derived: intrinsic radiopurity and the degree of isotope fractionation. The dispersions of the two labeled forms of lithocholic and cholic acids during chromatography were identical; this indicates similar radiopurity. The dispersion of 2,4-^{3}H-labeled chenodeoxycholic acid was less than that of the 24-^{14}C-labeled compound (14). If the logarithm of the isotope ratios is plotted against fraction number, a linear relationship that provides a further substantiation of radiopurity is obtained (15).

The increased ratios of $^{3}H/^{14}C$ in Fig. 5 indicate that isotope fractionation had occurred; tritium-labeled bile acids were adsorbed more strongly to alumina and silicic acid than the ^{14}C-labeled bile acid. The differences in mobility between the two forms were computed by previously described techniques (16) and are shown in Table 2. The degree of isotope fractionation was greater for the monohydroxy bile acid (lithocholic) than for the dihydroxy acid (chenodeoxycholic) and negligible or absent in the trihydroxy acid (cholic acid).

TABLE 1 ASSESSMENT OF EPIMER PURITY BY RECHROMATOGRAPHY

Bile Acid	β-Epimer*				Mean
	%				
Lithocholic	1.4	1.5	1.5	2.5	1.7
Chenodeoxycholic	2.2	1.6	1.0	1.5	1.6
Deoxycholic	2.5	3.0	2.5	4.8	3.2
Cholic	1.8	1.4	1.6	0.8	1.4

* (dpm with mobility of β-epimer)/(total dpm recovered) × 100.

Stability of Tritium Label in Vitro

The tritium in the final (reduced) product should not exchange in strong alkali. To test this, chenodeoxycholic acid-24-^{14}C (previously purified by preparative TLC) was added to samples of ^{3}H-labeled lithocholic, chenodeoxycholic, and cholic acids and an aliquot was taken for determination of the $^{3}H/^{14}C$ ratio (Table 3). The samples were then heated for 4 hr at 15 psi in a pressure cooker in 2 N NaOH—conditions which have been established to yield total hydrolysis of conjugated bile salts (18). After saponification, the samples were acidified and extracted three times with ethyl acetate. The

TABLE 2 ISOTOPE FRACTIONATION DURING COCHROMATOGRAPHY OF ^{14}C- AND ^{3}H-LABELED BILE ACIDS

Bile Acid	Method*	$\Delta M \pm$ SEM†	Dispersion (σ)	
			^{3}H	^{14}C
		%		
Lithocholic	Column chromatography on alumina	0.90 ± 0.03	8.13	8.15
		0.84 ± 0.03	5.89	5.82
		0.88 ± 0.01	6.63	6.64
	TLC on silicic acid	1.03 ± 0.22	2.51	2.48
	Mean	0.91 ± 0.22		
Chenodeoxycholic	TLC on silicic acid	0.38 ± 0.04	2.05	2.40
		0.35 ± 0.04	2.33	2.67
		0.49 ± 0.05	2.19	2.66
	Mean	0.41 ± 0.08		
Cholic	TLC on silicic acid	0.06 ± 0.02	2.54	2.60
		−0.05 ± 0.02	3.12	3.21
		0.04 ± 0.01	2.06	2.04
	Mean	0.02 ± 0.03		

* All compounds were run as methyl esters. The column chromatography was carried out with Woelm alumina, neutral, grade II, which was eluted with ethyl acetate–benzene 10:90. Silicic acid plates were developed three times (methyl chenodeoxycholate and methyl cholate) or once (methyl lithocholate) with acetone–benzene 30:70.

† By probit analysis, the data shown in Fig. 5 may be plotted to give a line intersecting with the abscissa at the true mean of the Gaussian distribution, the slope of which indicates the dispersion of the mean (17). The term "ΔM" is equal to (mean of ^{3}H peak − mean of ^{14}C peak)/(mean of ^{14}C peak) × 100.

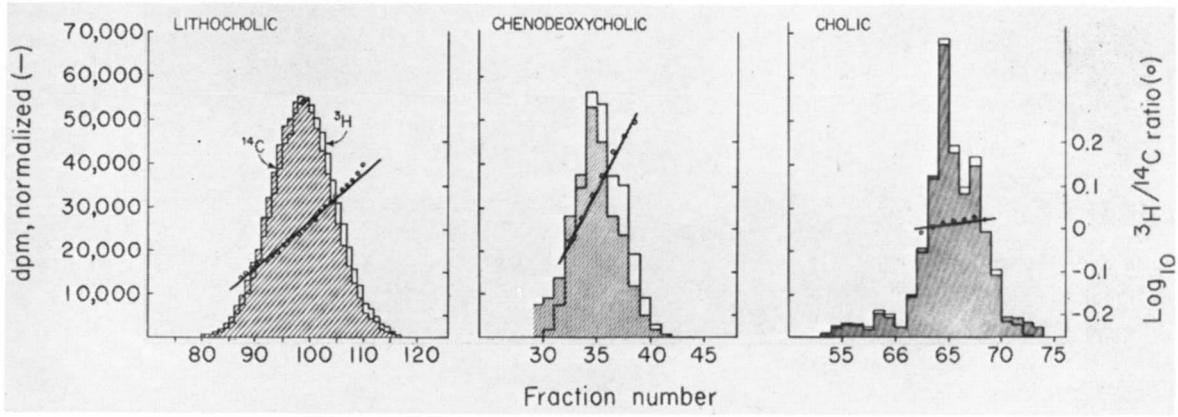

FIG. 5. Elution patterns of ^{3}H (unhatched area) and ^{14}C (hatched area) radioactivity when 2,4-^{3}H- and 24-^{14}C-labeled bile acid (as methyl esters) were cochromatographed. Methyl lithocholate was separated by column chromatography on alumina (grade II) in ethyl acetate–benzene 10:90; methyl chenodeoxycholate and methyl cholate were chromatographed on layers of Silica Gel G, the plates being developed three times in acetone–benzene 30:70. Isotope fractionation is clearly shown. Radiopurity is indicated by the linearity of the log of the ^{3}H/^{14}C ratio (15, 16). Although there is less isotope fractionation with methyl chenodeoxycholate than with methyl lithocholate (Table 2), the smaller dispersion of the chenodeoxycholate elution pattern results in a steeper slope in the ^{3}H/^{14}C ratio curve.

unchanged ^{3}H/^{14}C ratio indicates that no tritium exchanged with the hydrogen atoms present in the saponification liquor.

In an even more rigorous test, 2,4-^{3}H-labeled lithocholic, chenodeoxycholic, and cholic (50,000 dpm) acids were refluxed for 4 hr at 150°C in KOH–ethylene glycol (10 g/100 ml). After acidification and addition of an equal volume of water, the reaction mixture was extracted six times with ethyl acetate. The fact that 99% of the tritium activity was recovered again indicated that no exchange occurred under these drastic saponification conditions.

Stability of Tritium Level In Vivo

Cholic acid-2,4-^{3}H and cholic acid-24-^{14}C were mixed with chenodeoxycholic acid-2,4-^{3}H and chenodeoxycholic acid-24-^{14}C. About 10 μc of each acid was given by mouth to two healthy subjects (cases 1 and 2). 3 days later, bile samples were obtained by duodenal intubation. These samples and an aliquot of the ingested mixture were saponified, and the free bile acids, after extraction and methylation, were separated by TLC to determine the ^{3}H/^{14}C ratio (Table 4). The isotope ratio remained essentially constant after repeated enterohepatic cycling. This established the stability of the label in vivo and suggested that bile acids labeled with tritium in this manner should be valid for the study of bile acid kinetics in man.

Exchange Labeling of Other Bile Acids

To examine the exchange of the hydrogen atoms in 7-keto and 12-keto bile acids, we chromatographed a number of bile acids (10 mg) on the same alumina column. The tritium labeling of methyl 12-keto-5β-cholanoate was only 9% of that of methyl 3-keto-5β-cholanoate. However, the presence of some types of substituents also appeared to influence the tritium exchange, for methyl 3α-acetoxy-12-keto-5β-cholanoate did not

TABLE 3 STABILITY OF TRITIUM LABEL DURING ALKALINE SAPONIFICATION

Bile Acid	^{3}H/^{14}C Ratio after Saponification*
Lithocholic	0.97
	1.02
Chenodeoxycholic	1.02
Deoxycholic	1.01
	0.99
Cholic	1.00
	0.98
Mean	1.00

* The ^{3}H/^{14}C ratio of all samples before saponification was adjusted to 1.00. Each value indicates a separate sample.

TABLE 4 STABILITY OF TRITIUM LABEL DURING ENTEROHEPATIC CYCLING IN MAN

Bile Acid	^{3}H/^{14}C Ratio in Isolated Bile Acid	
	First Collection*	Second Collection
Cholic		
Case 1	1.00	0.97
Case 2	0.96	1.01
Chenodeoxycholic		
Case 1	1.07	1.08
Case 2	1.08	—†

* Collections were made on the morning and afternoon of the third day after oral administration of label, whose ^{3}H/^{14}C ratio was 1.00, to two healthy male subjects, cases 1 and 2.

† Insufficient radioactivity in sample.

take up label appreciably (0.03% that of the 3-keto-cholanoate). The monosubstituted methyl 7-keto-5β-cholanoate was not available to us, but the trisubstituted methyl 3α,12α-diacetoxy-7-keto-5β-cholanoate incorporated 3% as much tritium as the 3-ketocholanoate. Substituents also influenced the exchange of 3-keto bile acids, the following exchange rates (relative to 100 for methyl 3-keto-5β-cholanoate) being observed: methyl 3-keto-7α,12α-diacetoxy-5β-cholanoate, 130; methyl 3-keto-12α-acetoxy-5β-cholanoate, 218; methyl 3-keto-12α-hydroxy-5β-cholanoate, 5; and methyl 3-keto-7α-hydroxy-5β-cholanoate, 54. Although these results indicate that the factors determining the extent of tritium incorporation are complex, they do establish the feasibility of exchange labeling of bile acids at other than the 3 position.

COMMENT

This method may be applied to any compound possessing hydroxy groups that can be reversibly oxidized to keto groups, provided the keto groups enolize on basic alumina and the keto derivative can be eluted from the alumina with nonhydroxylic solvents.

The method, as described, can be carried out in 2 days and is thus far more rapid and economical than the Wilzbach procedure (18); the product has unequivocal radiopurity and the label appears to be remarkably stable. The specific activity of the final product is lower than that obtained by the Wilzbach procedure, and accordingly tritium-labeled bile acids prepared in this manner cannot be used for such techniques as electron microscopic radioautography. However, the availability of such labeled bile acids should facilitate metabolic studies in which the simultaneous turnover of ^{14}C- and ^{3}H-labeled bile acids is determined, or those in which tritium-labeled bile acids are added as an internal standard (19). The isotope fractionation observed during adsorption chromatography should not interfere with the use of $^{3}H/^{14}C$ ratios in experiments, provided column fractions are pooled to get the entire peak and TLC spots are completely eluted.

Early experiments, carried out in the laboratory of Dr. E. H. Ahrens, Jr., at The Rockefeller University, New York, were supported in part by Research Grant HE-6222 from the National Institutes of Health, Public Health Service; technical assistants were Joyce Lennane and Susan Stoeppler (now Mrs. Christopher Saudek).

This investigation was supported in part by Research Grant AM-6908 from the National Institutes of Health, Public Health Service to the Mayo Foundation, and by the United States Atomic Energy Commission. Mrs. Ralph (Kathleen) Rydell gave technical assistance.

Manuscript received 15 May 1968; accepted 22 July 1968.

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