Tritiated bile acids: problems and recommendations

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Summary Kinetic studies of cholic and chenodeoxycholic acids were carried out in three patients by simultaneous intravenous administration of appropriate pairs of ^{3}H- and ^{14}C-labeled compounds. The results obtained indicated two sources of error: chemical impurity and loss of tritium by biological exchange. Precautions are listed for use of tritiated bile acids in studies of pool sizes and turnover rates.

Supplementary key words [2,4-^{3}H]cholic acid · [random-^{3}H]chenodeoxycholic acid · purity · tritium exchange · kinetics

This report describes certain difficulties that we have encountered in the use of tritiated bile acids in studies of bile acid metabolism in man. It is our belief that, by so reporting, we may spare other workers the same mishaps.

The problems have been of two types: (*a*) the well-known (but often disregarded) question of the chemical purity of compounds obtained from commercial sources and (*b*) the exchange of tritium with hydrogen that may occur in the biological system being tested. It is obvious that kinetic data obtained through use of tritiated bile acids that are either impure or that lose tritium by exchange in the course of the experiment can lead to erroneous conclusions.

Methods

Labeled bile acids. Randomly labeled [^{3}H]chenodeoxycholic, [2,4-^{3}H]cholic, [24-^{14}C]cholic, and [24-^{14}C]chenodeoxycholic acids were purchased from New England Nuclear Corp., Boston, Mass. All labeled bile acids were carried through the entire isolation procedure we use (1) for determination of specific activity in biological samples. This procedure involves saponification under pressure, extraction after acidification, methylation, and thin-layer chromatography; in no case was there significant loss of isotope due to chemical exchange.

The randomly labeled chenodeoxycholic acid was prepared by the Wiltzbach method (2) and the 24-^{14}C-labeled bile acids by the method of Bergström, Rottenburg, and Voltz (3). The [2,4-^{3}H]cholic acid was prepared by exposure of the methylated 3-ketone derivative of cholic acid to $^{3}H_2O$ on an alumina column, according to the method of Hofmann, Szczepanik, and Klein (4). The radioactive ketonic ester is eluted from the column and reduced with sodium borohydride; during this reduction process, 3α-hydroxy and 3β-hydroxy epimers of cholic acid are formed. The supplier then subjected this mixture of 3α- and 3β-hydroxy compounds to TLC on silica gel G, using the solvent system isooctane–isopropyl ether–isopropyl alcohol–acetic acid 2:1:1:1; the material having the same R_F as the 3α-hydroxy epimer of cholic acid was recovered and prepared for distribution to purchasers. The supplier stated that all four materials were pure by gas–liquid chromatography; we confirmed that each sample showed a single GLC peak with the appropriate relative retention on a 1% HiEff-8BP column (Applied Science Laboratories, State College, Pa.).

Patients. Small samples of bile were obtained repeatedly from three women (A.R., 56 yr; L.A., 26 yr; and C.P., 60 yr), recently subjected to cholecystectomy, 6–10 wk after surgery; in all cases the operation was performed for treatment of cholesterol cholelithiasis. Their postoperative courses were uncomplicated, and they were maintained at constant body weight on ward diets after the third postoperative week. None of the patients displayed any evidence of intestinal disease, and in none was there evidence of biliary tract infection, at or subsequent to surgery. Repeated aerobic and anaerobic cultures of bile were uniformly negative.

Administration of labeled bile acids. A known amount of radioactive bile acid in 2–4 ml of ethanol was pipetted into a drip bottle containing 250 ml of physiological saline plus 30 ml of 7.5% sodium bicarbonate. This infusion mixture was administered intravenously over a 45-min period. After the infusion, the bottle and tubing were thoroughly rinsed with ethanol in order to recover the unadministered isotopic material and thus to calculate by difference the administered dose. All infusions were given in the morning after a 14-hr fasting period.

Chemical methods. Bile acids were isolated for determination of their specific activity by methods previously described (1). Key steps included the separate isolations of cholic, chenodeoxycholic, and deoxycholic acids by TLC on silica gel G using isooctane–ethyl acetate–acetic acid–*n*-butanol 70:35:10.5:10.5 as a developing system, GLC of the trimethylsilyl ethers on 1% HiEff-8BP at 225°C, and counting of ^{3}H and ^{14}C radioactivity in a Packard scintillation counter (model 3380, and AAA model 544 automatic window adjustment for minimizing crossover), in which the counting efficiency at the time of these experiments was 32.5% for ^{3}H and 43.0% for ^{14}C.

Chemical impurity. The sample of [2,4-^{3}H]cholic acid was subjected to TLC, according to Hofmann et al. (4), to ascertain whether the desired 3α-hydroxy compound was contaminated by the 3β-hydroxy epimer formed in the tritiation procedure. When only 85% of the total radioactivity was found in the 3α band and 15% in the 3β band, an inquiry to the supplier elicited the information that they had performed their preparative TLC on silica gel G without addition of magnesium silicate, an omission known (4) to give a poor separation of 3α- and 3β-hydroxy cholic acids.

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Abbreviations: TLC, thin-layer chromatography; GLC, gas–liquid chromatography.

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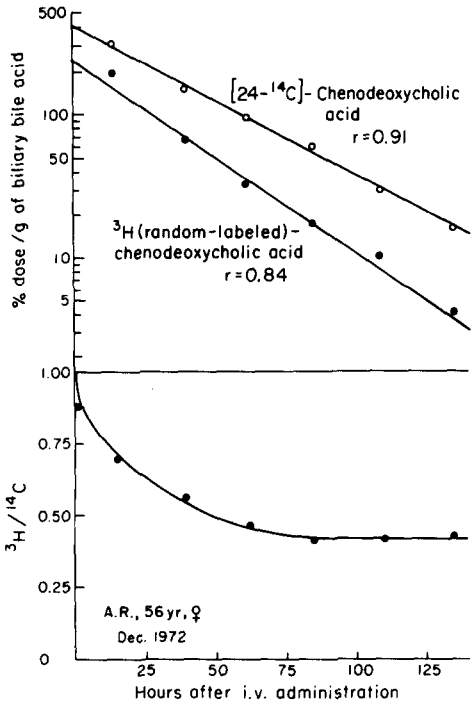

Fig. 1. *Top,* semilogarithmic plot of percentage of dose per gram of biliary bile acid vs. time after simultaneous i.v. administration of [24-^{14}C]chenodeoxycholic acid (*open circles*) and [random-^{3}H]chenodeoxycholic acid (*solid circles*) to patient A.R. *Bottom,* the same data plotted as ^{3}H/^{14}C ratios.

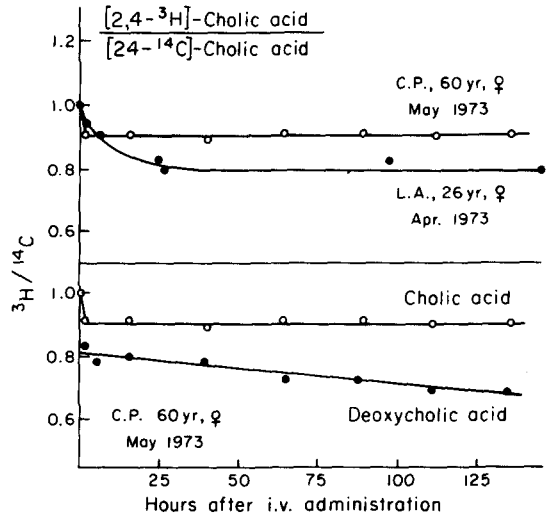

Fig. 2. *Top,* specific activity–time course data after simultaneous i.v. administration of [24-^{14}C]cholic acid and [2,4-^{3}H]cholic acid to patient C.P. (*open circles*) and patient L.A. (*solid circles*), plotted in terms of ^{3}H/^{14}C ratios. Patient L.A. received the tritiated product as received from the supplier; patient C.P. was given the same product after removal of the 3β-OH epimer by TLC. *Bottom,* ^{3}H/^{14}C ratios in cholic and deoxycholic acids in patient C.P., demonstrating the continued loss of tritium by biological exchange during the transformation of cholic acid to deoxycholic acid.

Results

Biological exchangeability. In order to test whether tritium was lost from either of the two tritiated bile acids by exchange in vivo, double-label experiments were performed (*a*) by simultaneous administration of [^{3}H]chenodeoxycholic acid (randomly labeled) and [24-^{14}C]chenodeoxycholic acid and (*b*) by simultaneous administration of [2,4-^{3}H]cholic acid (before and after removal of the 3β epimer as described above) and [24-^{14}C]cholic acid.

The first experiment is plotted in **Fig. 1.** At the top, the time-course decay in specific activity is shown for the two isotopically labeled chenodeoxycholic acids as a semilog plot of percentage of administered dose per gram of biliary chenodeoxycholic acid; at the bottom, the ratio of ^{3}H to ^{14}C in biliary chenodeoxycholic acid is plotted against time after normalization of all data to a ratio of 1:1 in the administered dose. It is seen that at the first sampling interval (1 hr) the ^{3}H specific activity was well below that of ^{14}C, and the values continued to diverge until 60 hr, after which they became approximately parallel; the ratio plot strikingly demonstrates the loss by exchange of about 55% of the tritium during the initial 60 hr. It is more than likely that these effects were due to enolization of chenodeoxycholic acid during its enterohepatic circulation, oxidoreduction of its 3-hydroxyl group, and consequent loss of tritium atoms around that hydroxyl group, as suggested by the experiments of Kallner (5) on deoxycholic acid.

Calculations of pool size and daily turnover of [^{3}H]chenodeoxycholic acid were carried out according to Lindstedt (6). There were considerable discrepancies between these results and those calculated similarly for [^{14}C]chenodeoxycholic acid: for pool sizes, 419 and 238 mg, respectively, and for daily synthesis, 303 and 141 mg/day. In these comparisons the ^{14}C and ^{3}H data were fitted by computer to single regression lines: the ^{14}C slope had a correlation coefficient of 0.91, and that of the ^{3}H slope was 0.84. However, it is obvious from the ^{3}H/^{14}C ratios that the data plotted in Fig. 1 (*top*) for [^{3}H]chenodeoxycholic acid did not describe a one-phased curve and thus that the calculation of pool size and turnover rate with the Lindstedt (6) equations is inappropriate. Had the loss of tritium by exchange been smaller, the inappropriateness of this calculation would not have been so apparent, of course.

The second experiment is shown in **Fig. 2** (*top*), in which the in vivo loss of label from [2,4-^{3}H]cholic acid was tested before and after removal of the 3β-hydroxy epimer by two successive TLC purifications in the optimal solvent system (4). (Regrettably, the two experiments could not be performed in the same patient.) The loss of ^{3}H was more extensive and slower in patient L.A., given the mixture of epimers, than in patient C.P., given the 3α-hydroxy epimer alone. It is evident that, even after removal of the 3β-hydroxy epimer, there was a 9% loss of ^{3}H from [2,4-^{3}H]cholic acid within the first 2 hr after administration, after which time the decay curves were strictly parallel, with half-lives of 24 hr. The effect of this 9% loss on calculations of pool size and turnover rate of cholic acid was correspondingly small, but nevertheless real: for pool sizes, 704 and 645 mg, and for synthesis rates, 488 and 447 mg/day. The 20% loss of ^{3}H encountered when the mixture of [^{3}H]cholic acid epimers was

used would, of course, have caused larger errors if kinetic calculations had been based on the ^3H curves alone.

A second type of isotope loss is demonstrated in the experiment shown in Fig. 2, *bottom,* in which the ^3H and ^{14}C specific activities of deoxycholic acid, the major secondary product formed in the intestine by bacterial 7α-dehydroxylation, were compared. Whereas there was no further loss of ^3H from cholic acid (the precursor) after 2 hr, there was an ever greater loss of ^3H from deoxycholic acid (the product) at 2 hr; thereafter, further losses of ^3H continued throughout the course of the experiment and by 130 hr amounted to 30% of the [^3H]cholic acid initially administered.

These two sets of observations with [2,4-^3H]cholic acid suggest to us that some labeled impurity was present in the sample even after removal by TLC of the 3β-hydroxy epimer, and that this impurity was rapidly removed within the first 2 hr by some biological process that we could not duplicate in the laboratory purification of the sample prior to its administration. Secondly, the experiment suggests that the loss of ^3H from deoxycholic acid was of a different nature because it was greater in extent than that from its precursor, cholic acid, and proceeded continuously throughout the observation period. Although we are not prepared to propose a chemical mechanism to explain the phenomenon, it appears that ^3H in position 2 or 4 must become exchangeable as 7α-dehydroxylation takes place in the intestine (5).

Our experience has led us to believe that studies carried out with [2,4-^3H]cholic acid should be interpreted with caution and that it would be advisable to encourage the syntheses of other tritiated compounds in which chemical purification can be assured and biological loss of tritium avoided. Very similar conclusions have been reached by Hofmann and his colleagues at Mayo Clinic (7): they noted a 10% loss of tritium from [2,4-^3H]cholate but no tritium loss from [2,4-^3H]chenodeoxycholate.

Recommendations

As a result of these experiences, we suggest that the following precautions be observed by workers using ^3H-labeled bile acids:

(*1*) Avoid the use of *randomly* tritiated bile acids in kinetic studies altogether. Although reports by distinguished colleagues have been based on data obtained with these compounds as recently as 1973 (8–13), Vlahcevic (14) has repeatedly cautioned against reliance on these materials and has successfully measured pool sizes and turnover rates of cholic and chenodeoxycholic acids by simultaneous oral administration of the 24-^{14}C-labeled acids.

(*2*) Assure that no readily exchangeable isotope is present in a *specifically* tritiated compound by measuring its recovery after carrying it through the entire laboratory procedure to be used in determining its specific activity in the definitive biological experiment.

(*3*) Rigorously test the chemical purity of the compound, using effective TLC procedures for isolation of epimers, and in at least one biological system (such as we have shown in this report) demonstrate that the 24-^{14}C-labeled compound and the specifically labeled ^3H compound are handled identically within the first few hours.

(*4*) Rigorously test whether any loss of tritium takes place by exchange in the biological system under study, even when loss by chemical exchange cannot be demonstrated in the laboratory.

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