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Note

Simple method for simultaneous specific activity measurements in a mixture of radiolabeled bile acids

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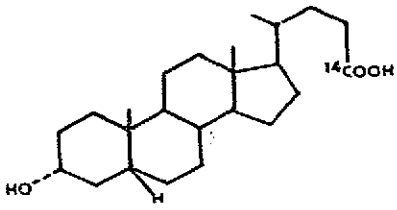
Bile acid kinetics are determined in man by measuring the decay over time of the specific activity of a bile acid after labeling its pool with radioactive tracer[1]. Conventionally, a single tracer dose of bile acid is administered intravenously and the decrease in bile acid specific activity in bile measured over five days[2]. Theoretically, the simultaneous administration of several radiolabeled bile acids should allow concurrent measurements of multiple specific activity decay curves. Practically, however, this is difficult because the separation, isolation, and quantification of bile acids of interest is often cumbersome and unsatisfactory. Such problems usually necessitate the use of different preparative methods for mass and radioactivity determinations on separate aliquots of the sample, an approach which is undesirable[2]. Alternatively, one may separate mono-, di- and trihydroxy bile acids by thin-layer chromatography (TLC) and utilize specific enzymes for the 3α- and 7α-hydroxyl groups for mass determination[3].

Because of the difficulties encountered with the available approaches, we developed a facile method for determining the specific activities of several bile acids employing one procedure on the same aliquot.

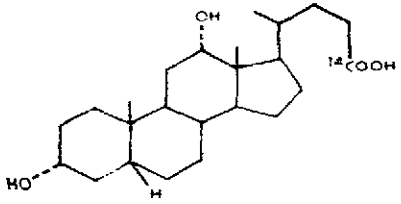
MATERIALS AND METHODS

Materials

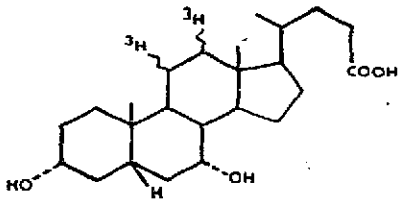
The following bile acids were used in the study: [11,12-^{3}H]-chenodeoxycholic acid, [2,4-^{3}H]cholic acid, [24-^{14}C]deoxycholic acid, [11,12-^{3}]lithocholic acid, and [24-^{14}C]ursodeoxycholic acid (Fig. 1). Precursors of chenodeoxycholic and lithocholic acid were prepared by us as described[4], and radiolabeling was performed by New England Nuclear (New Bedford, Mass., U.S.A.). [^{14}C]Cholic acid, [^{14}C]deoxycholic acid, and [^{14}C]ursodeoxycholic acid were purchased directly from New England Nuclear. Nonradioactive bile acids were crystallized to 98% purity as determined by TLC, gas–liquid chromatography and enzyme analysis, and radiolabeled bile acids had a radipurity of greater than 98% by TLC in appropriate solvents. Scintillation cocktail was prepared by mixing butyl-PBD and Biosolv (Beckman, Fullerton, Calif., U.S.A.) with scintillation grade toluene (Fisher, Itasca, Ill., U.S.A.). Analyses were performed on a Beckman Model 25 spectrophotometer and a Beckman Model 230 liquid scintillation counter.



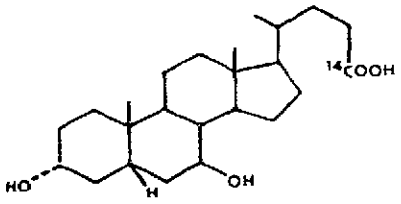
3α-hydroxy
^{14}C-LITHOCHOLIC ACID



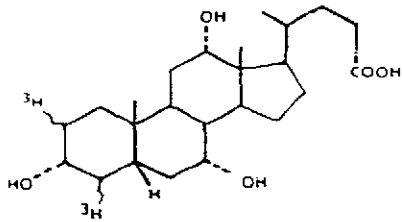
3α, 12α-dihydroxy
^{14}C-DEOXYCHOLIC ACID



3α, 7α-dihydroxy
^{3}H-CHENODEOXYCHOLIC ACID



3α, 7β-dihydroxy
^{14}C-URSODEOXYCHOLIC ACID



3α, 7α, 12α-trihydroxy
^{3}H-CHOLIC ACID

Fig. 1. Structures of radiolabeled bile acids.

Experimental procedures

The complete procedure consists of three steps: separation by TLC, mass quantification by sulfuric acid charring, and radioactivity determination by liquid scintillation counting.

Separation. 40 g of silica gel H (Brinkman, Des Plaines, Ill., U.S.A.) was mixed with 95 ml of water, and 5 × 20 cm glass plates were coated with 0.5-mm silica gel. The plates were placed in a room at 37° for 8 h and activated in an oven at 110° for 1 h prior to use. Immediately before use, the thin-layer plates were removed from the oven and scraped in a wedge-tip according to Stahl[5]. The solvent was prepared by mixing 55 ml of hexane, 35 ml of butan-2-one, 5 ml of acetic acid and 5 ml of water in a separatory funnel. The lower aqueous phase was discarded and only the upper phase used. The mixture of radiolabeled bile acids was spotted with a 5-μl Drummond "Microcaps" pipette, the plate developed, dried and developed a second time. The individual spots were located by spraying with ethanolic–sulfuric acid after drying the

plate in a 110° oven for 10 min. Individual spots were scraped into Pyrex test tubes for quantitation.

Quantitation. 2 ml of concentrated sulfuric acid were added to each of the Pyrex tubes, the silica gel and sulfuric acid mixed and heated at 200° for 15 min, and the tubes transferred to a 20° water bath for 15 sec and cooled in ice for 10 min. After cooling, 3 ml of distilled water were added to each sample, the samples mixed and cooled again in ice for 10 min. The tubes were removed, allowed to warm to room temperature for 10 min, and centrifuged for 15,000 $g \cdot$min. The supernatant was transferred to cuvettes and absorbance at 375 nm was determined. A 250-μl aliquot was transferred from the cuvettes to counting vials, 10 ml of scintillation cocktail added, and the samples counted for ten minutes using the external standard ratio method for quench correction[6].

Validation. Standard curves for both mass and radioactivity were prepared for of the individual bile acids in the following manner: individual labeled bile acids of known activity were spotted on thin-layer plates in amounts of 25, 50, 75, 100, 125 μg and the procedure outlined above followed.

RESULTS

Separation

Fig. 2 shows results of separation of radiolabeled bile acids in our chromatographic system. Radiolabeled bile acids were mixed in methanol or human bile in the

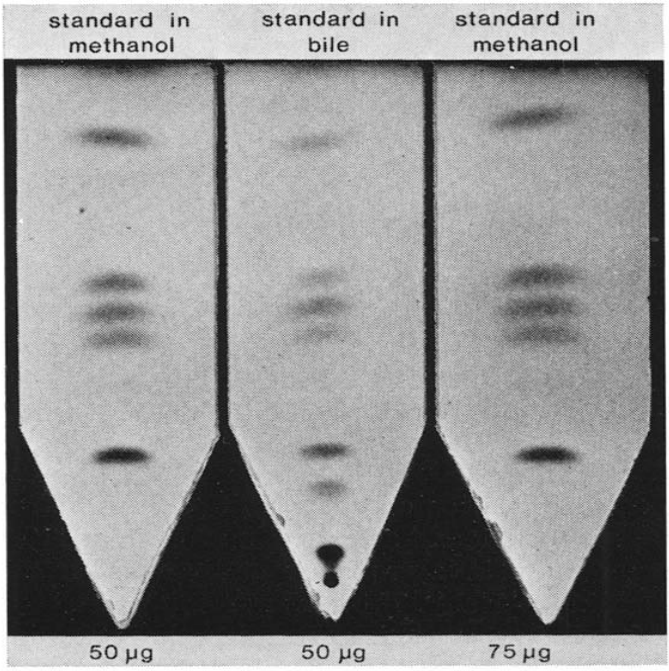


Fig. 2. Separation of radiolabeled free bile acids. 50 or 75 μg of each bile acid were added to methanol or bile, 5 μl spotted on 5 × 20 cm glass plates (0.5 mm silica gel H), and the plate developed twice in the upper phase of the solvent system described. From top to bottom: lithocholic, deoxycholic, chenodeoxycholic, ursodeoxycholic and cholic acid.

amounts indicated. With this system, the bile acids are, from top to bottom, lithocholic, deoxycholic, chenodeoxycholic, ursodeoxycholic and cholic acid. Fig. 3 shows the comigration of mass and radioactivity with the calculated R_F values. Adequate separation of all bile acids was achieved.

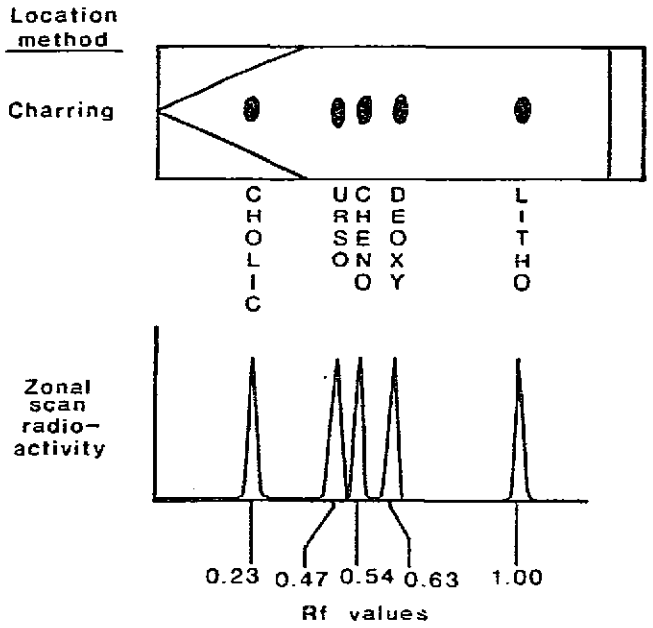


Fig. 3. Co-migration of bile acid mass and radioactivity. The upper panel shows separation of bile and identification of mass by spraying with ethanolic sulfuric acid. The lower panel shows a zonal scan of radioactivity for corresponding bile acids and their calculated R_F values relative to lithocholic acid.

Quantitation

Fig. 4. shows the absorbance of each of the individual bile acids at the different concentrations. Mean results for six experiments for each bile acid at each concentration are given. The curves are linear with a correlation coefficient (r) of 0.997–0.999 for each bile acid. The mean coefficient of variation for each concentration for all the bile acids used was $2.99 \pm 0.99\%$[7]. Fig. 5 shows radioactivity measurements from the same experiments. Again, curves are linear with a correlation coefficient of 0.995–0.999 for each bile acid.

Validation

Fig. 6 shows the effect of sulfuric acid charring on radioactivity measurements. Charring did not affect radioactivity determinations. Recovery of radioactivity from silica gel for the six experiments (mean $\pm$ S.E.M.) was $93.24 \pm 1.37\%$; recovery of mass was $95.23 \pm 0.72\%$. The correlation coefficient of radioactivity and mass was

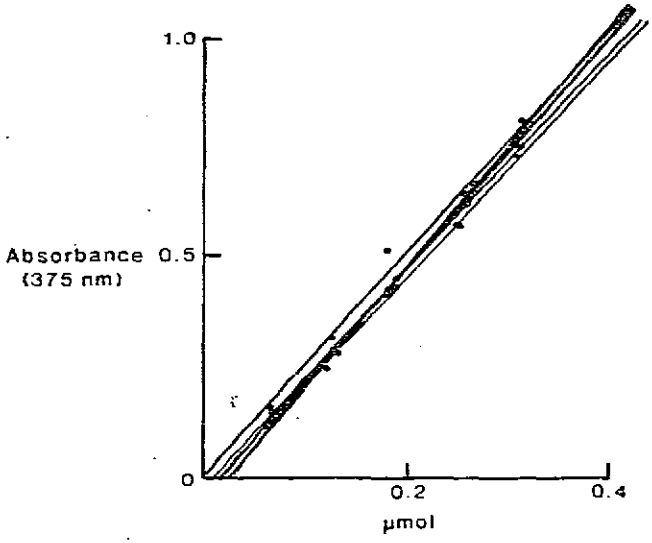


Fig. 4. Absorbance of individual bile acids at different concentrations. No attempt is made in the figure to distinguish curves for different bile acids since they are not significantly different from each other. Data are the mean for six experiments.

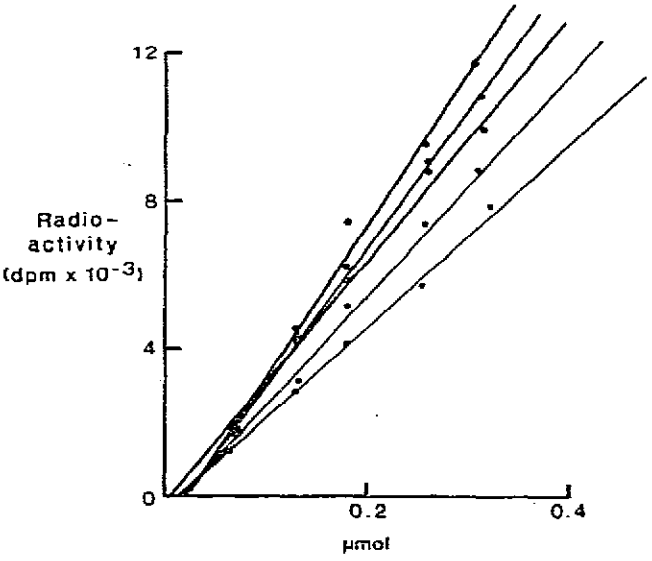


Fig. 5. Radioactivity of individual bile acids with different specific activityis at different concentrations. Note that all curves are linear and have a correlation coefficient of 0.995–0.999 for each specific activity. Data are the mean for six experiments.

0.9974 over the range of concentrations studied. Fig. 7 shows the ratio of known to measured specific activity for the five bile acids over a range of bile acid concentrations. The ratio was normalized to one and remained constant.

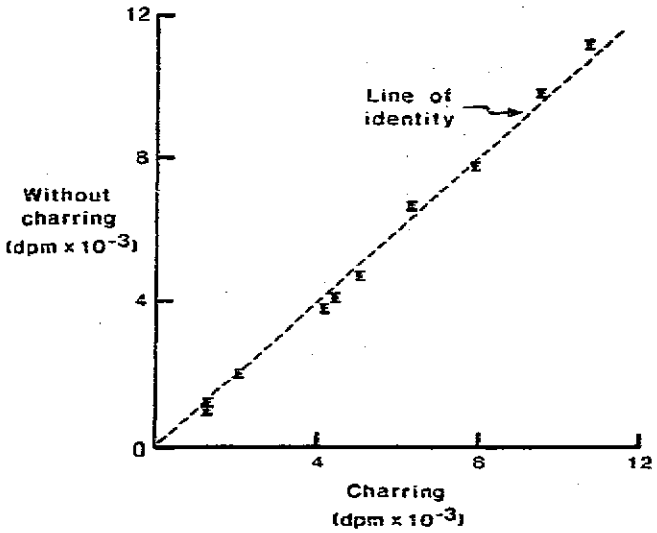


Fig. 6. Effect of acid charring on radioactivity measurements. A sample of each of the five five radiolabeled bile acids at different concentrations was divided into two aliquots. Radioactivity was determined directly on one aliquot, and, after TLC separation and mass quantitation by charring, on the other. Data are mean ± one S.D. for six experiments.

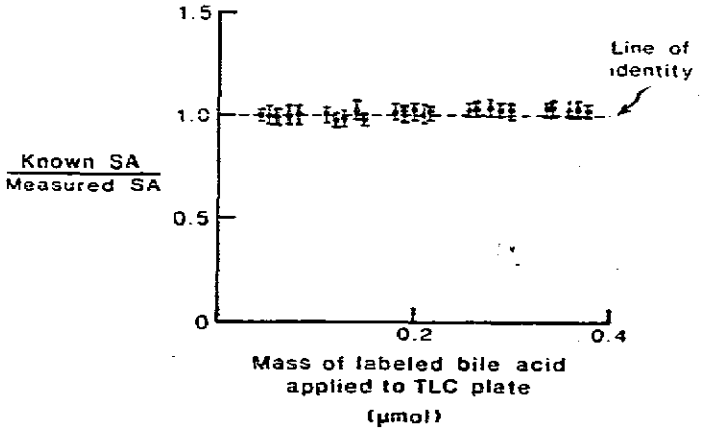


Fig. 7. Ratio of known and measured specific activities (SA) of the five bile acids studied over arange of bile acid concentrations. Data are mean ± one S.D. for six experiments.

DISCUSSION

We have developed a simple and satisfactory one-step TLC Separation of the primary, secondary, and tertiary bile acids in human bile. This separation was dependent upon equilibration of the solvent system with water, a modification to the solvent system of Petcoff[8]. In addition, our experiments have shown that the use of wedge-tipped chromatographic plates is essential for adequate bile acid separation.

We have also investigated the applicability of this technique to bile acids other than those described above. As might be expected, conjugated bile acids are not separated by this technique. 3α-Hydroxy-7-keto-5β-cholanic acid (cavicholic acid), a bacterial degradation product of chenodeoxycholic acid[9] which is subsequently converted in the liver into both chenodeoxycholic and ursodeoxycholic acids[10], also did not separate from dihydroxycholanic acids in our system.

Charring for quantitation of individual bile acids was originally reported by Kim and Kritchersky[11]. We have adopted it because of its simplicity, and have demonstrated that it is an accurate and precise technique in our analytical scheme. One concern was that charring might produce carbon particles sufficiently large to artificially lower the radioactivity measured because of self absorption within the particle[6]. Our results show this is not the case.

The major value of our analytical scheme is that it will simplify the determination of bile acid specific activities after simultaneous administration of multiple radiolabeled bile acids. In our experience, specific activities can be determined in 4 h exclusive of time necessary for liquid scintillation counting.

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