Evaluation of the 3α-hydroxysteroid dehydrogenase assay for ursodeoxycholic acid, and 7 oxo- and 12 oxo- bile acids

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Summary 3α-Hydroxysteroid dehydrogenase (SDH) quantifies ursodeoxycholic acid, 3α,12α-diol-7-one-5β-cholanoic acid, 3α-ol-7-one-5β-cholanoic acid, and 3α,7α-diol-12-one-5β-cholanoic acid in a manner similar to the more commonly measured bile acids, exemplified by taurocholic acid. The type of oxygen function at the 7 or 12 position and its orientation at the 7 position has no effect on the rate of reaction of the enzyme. Discrepancies in the glycine/taurine ratios of patient intestinal aspirates containing ursodeoxycholic acid and oxo bile acids, obtained by SDH and gas–liquid chromatographic methods are not a result of the enzyme assay procedure.—**Haeffner, L. J., S. J. Gordon, J. S. Magen, and O. D. Kowlessar.** Evaluation of the 3α-hydroxysteroid dehydrogenase assay for ursodeoxycholic acid, and 7 oxo- and 12 oxo- bile acids. *J. Lipid Res.* 1980. **21:** 477–480.

Supplementary key words taurocholic acid · free and conjugated bile acids

Intestinal aspirates from patients with various gastrointestinal disorders have been investigated for their bile acid content by different methods in our laboratory. During these investigations an observation was made that a discrepancy existed between glycine/taurine (G/T) ratios calculated by enzymic assay employing 3α-hydroxysteroid dehydrogenase from *Pseudomonas testosteroni* (SDH) and by gas–liquid chromatographic (GLC) analysis (1). This discrepancy did not occur when aspirates from normal healthy volunteers were studied. The patient samples were characterized by *a*) no or low deoxycholic acid; *b*) measurable quantities of ursodeoxycholic acid; and *c*) measurable amounts of the oxo bile acid(s), 3α,12α-diol-7-one-5β-cholanoic acid and/or 3α-ol-7-one-5β-cholanoic acid. Although a recent paper by Turley and Dietschy (2) evaluated a 3α-hydroxysteroid dehydrogenase assay for determining the concentration of bile acids, oxo bile acids and ursodeoxycholic acid were not included. The present study was under-

Abbreviations: G/T, glycine/taurine; SDH, 3α-hydroxysteroid dehydrogenase; TLC, thin-layer chromatography; GLC, gas–liquid chromatography.

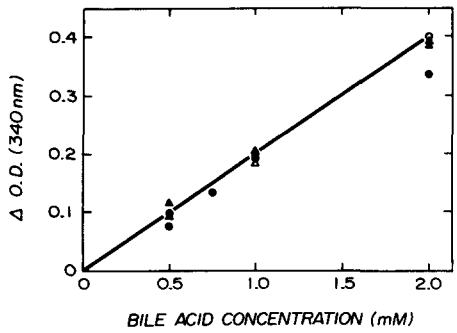

Fig. 1. The amount of activity after 30 min incubation of SDH with 3α-ol-7-one-5β-cholanoic acid (▲), 3α,12α-diol-7-one-5β-cholanoic acid (●), ursodeoxycholic acid (△), and taurocholic acid (○), the latter representing the commonly assayed bile acids. All bile acids were assayed in 0.1 ml of methanol. The incubation mixture contained 2 ml of glycine-hydrazine buffer, pH 9.4, 0.4 ml of NAD solution (2.3 μmol), 0.5 ml of SDH solution (0.1 mg), and 0.1 ml of bile acid at each concentration studied. After 30 min at 38°C the tubes were read at 340 nm. Control tubes lacking SDH were run simultaneously: ΔOD represents the difference between the test and control readings at 340 nm.

taken to determine if the routine enzyme assay used in our laboratory quantitatively measures these bile acids.

MATERIALS AND METHODS

Reagents

3α-hydroxysteroid dehydrogenase from *Pseudomonas testosteroni* (EC 1:1:1:50) (lot 48P286) was obtained from Worthington Biochemicals Corp., Freehold, NJ. The enzyme was dissolved daily in deionized-distilled water and kept on ice during the procedure. Each lot of enzyme was assayed to determine the concentration of the working solution. For four sequential lots, including lot 48P286 used in these studies, 0.2 mg/ml was optimal. NAD was obtained from Sigma Chemical Co., St. Louis, MO. A solution containing 62 μmol/10 ml in deionized-distilled water was prepared daily and stored in an ice bath. Glycine-hydrazine buffer was prepared from reagent grade chemicals as follows: 0.1 mol of glycine, 40 mmol of hydrazine sulfate, and 0.5 mmol of disodium ethylenediamine tetraacetate were dissolved, adjusted to pH 9.4 with sodium hydroxide, and brought to 100 ml.

Chromatographically pure conjugated bile acids were obtained from Calbiochem, San Diego, CA. Chromatographically pure cholic acid was obtained from Nu-Chek Prep., Elysian, MN. 3α-ol-7-one-5β-Cholanoic acid and 3α,7α-diol-12-one-5β cholanoic acid were purchased from Steraloids, Wilton, NH, and 3α,12α-diol-7-one-5β cholanoic acid was prepared from cholic acid by oxidation with *N*-bromosuccinimide (3). Thin-layer chromatography (TLC) with the solvent system isooctane–ethyl acetate–glacial acetic acid 5:5:1 was used to further purify this oxo bile acid (4). TLC plates 20 × 20 cm, 1.0 mm thick, were prepared using Silica Gel G (Brinkman Instruments, Westbury, NY). The plates were activated at 100°C for 2 hr before use.

Procedure for SDH Assay

Samples of bile acid in methanol (0.1 or 0.2 ml) were placed in two sets of tubes. To each tube 2 ml of glycine-hydrazine buffer and 0.4 ml of NAD solution were added. To one set of tubes (control), 0.5 ml of deionized-distilled water was added, while to the other set (test) 0.5 ml of enzyme solution was added. A blank with enzyme solution was prepared containing 0.1 or 0.2 ml of methanol. All tubes were incubated for 30 min at 38°C. The tubes were read against the blank at 340 nm; paired controls and tests were read alternately. Results are expressed as the difference in absorbance between the test and control tube readings (ΔOD). In our laboratory 0.1 ml samples of 1 mM solutions of the twelve free and conjugated forms of lithocholic acid, deoxycholic acid, chenodeoxycholic acid, and cholic acid gave a mean ΔOD of 0.187 ± 0.002 (n = 87). Taurocholic acid, with a mean ΔOD of 0.188 ± 0.004 (n = 13) was established as the daily working standard. Therefore, with each assay, standard tubes containing 0.1 ml of 1 mM taurocholic acid in methanol were simultaneously analyzed.

The 0.1 or 0.2 ml samples used in these studies contained *a*) different amounts of standard bile acids including 3α,12α-diol-7-one-5β-cholanoic acid, 3α-ol-7-one-5β-cholanoic acid, 3α,7α-diol-12-one-5β-cholanoic acid, and ursodeoxycholic acid; *b*) mixtures of cholic acid and oxo bile acids; or *c*) mixtures of oxo bile acids and normal or abnormal intestinal bile aspirates. Aspirates were diluted with an equal volume of methanol–water 1:30 and centrifuged before use.

RESULTS AND DISCUSSION

Free and conjugated forms of lithocholic acid, deoxycholic acid, chenodeoxycholic acid, and cholic acid have shown no significant difference in ΔOD after 30 min incubation with SDH under the assay conditions described. **Fig. 1** shows that the bile acids 3α-ol-7-one-5β-cholanoic acid, 3α,12α-diol-7-one-5β-cholanoic acid, and ursodeoxycholic acid give comparable activity at 30 min over a concentration range

of 0.5–2.0 mM. The 12-oxo bile acid, 3α,7α-diol-12-one-5β-cholanoic acid behaves in a like manner. The change in absorbance with incubation time from 7.5 min to 60 min was observed. At 7.5 and 15 min, 3α,12α-diol-7-one-5β-cholanoic acid showed less activity than taurocholic acid; the other three bile acids had slightly higher activity than taurocholic acid at these times. By 30 min, however, all the observed activities were similar.

To further substantiate that the assay system quantitatively measures the oxo bile acids, they were mixed in varying proportions with the more commonly occurring bile acids as well as with normal and abnormal human intestinal bile aspirates. In **Fig. 2,** the results are expressed as percentage of the theoretical total of the two components assayed independently. The assay system quantifies the two oxo bile acids under all conditions. Similarly, 3α,7α-diol-12-one-5β-cholanoic acid when added to aspirates gave quantitative results. These results extend the reported C-19, C-21 (5, 6), and C-24 (2, 6) compounds which serve as substrates for 3α-hydroxysteroid dehydrogenase.

Studies with ursodeoxycholic acid and the oxo bile acids demonstrate that the presence of 7β-hydroxyl, 7 oxo, 12 oxo, and 12α-hydroxyl groups does not affect the enzyme assay. 3α-SDH does not oxidize the 7β or 12α hydroxyls, further supporting the specificity of this enzyme. Beher and Lin (7), employing three crude extracts of SDH from *Pseudomonas testosteroni*, found no significant amount of 12α or 7α activity.

Turnberg and Anthony-Mote (8) studied the optimum conditions for the 3α-SDH assay of cholic acid, chenodeoxycholic acid, and deoxycholic acid as free and conjugated bile acids. They found that the best conditions for assaying mixtures of bile acids were: pH 10, a temperature of 26°C, and a reaction time of 40 min. At each temperature studied, however, deoxycholic acid was more sluggish than cholic acid. The authors suggested that the hydroxyl group at position 7 in the cholic acid might help bring the substrate into better apposition with the enzyme. In the studies reported here, 3α-ol-7-one-5β-cholanoic acid reacted more quickly than taurocholic acid, while 3α,12α-diol-7-one-5β-cholanoic acid reacted more slowly. The presence of an oxo group in the 7 position would seem, therefore, to have no effect on the substrate binding. It is also seen that a 7β hydroxyl (ursodeoxycholic acid) and a 7α hydroxyl group (taurocholic acid) had similar rates of reaction. Likewise substitution of a 12 oxo group (3α,7α-diol-12-one-5β-cholanoic acid) did not alter the rate of

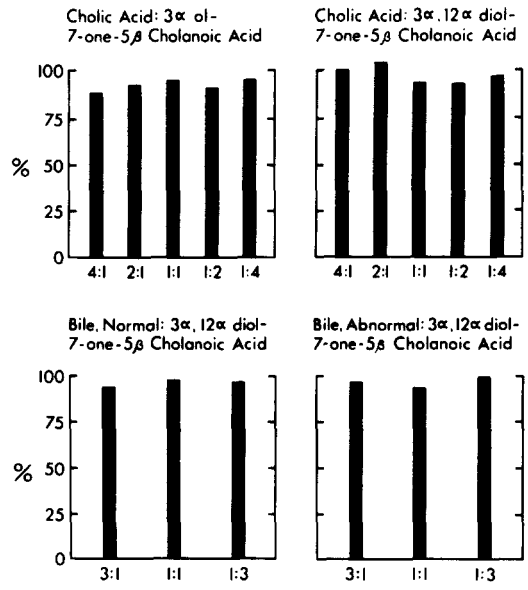

Fig. 2. SDH reactivity of mixtures containing keto bile acids. Upper left: ratios of cholic acid to 3α-ol-7-one-5β-cholanoic acid. Upper right: ratios of cholic acid to 3α,12α-diol-7-one-5β-cholanoic acid. Lower left: ratios of bile aspirates from normal subjects to 3α,12α-diol-7-one-5β-cholanoic acid. Lower right: ratios of bile aspirates from patients with abnormal bile acid distribution to 3α,12α-diol-7-one-5β-cholanoic acid. Results are expressed as the percentage of the sum of the two components measured separately. Duplicate experiments for each graphic reproduction were within 3%; total number of assays = 44.

reaction. Although free ursodeoxycholic acid and free oxo bile acids were employed for these studies, there is no reason to believe that conjugated forms would behave differently, as no difference was noted comparing free and conjugated cholic acid, deoxycholic acid, and chenodeoxycholic acid.

It is therefore our conclusion that the nature of the oxygen function at position 7 as well as the α or β orientation of the hydroxyl group has no dramatic effect on the rate of reaction of the SDH assay. It should be noted that there was no hydrazine present in Turnberg and Anthony-Mote's assay system to trap formed ketone products (8). At pH 10, a reverse enzyme reaction with accompanying formation of NAD from NADH would be unlikely; however, it is possible that this reaction could account for the apparent sluggish kinetics in the deoxycholic acid assay.

We conclude that discrepancies found in G/T ratios calculated from SDH and GLC data do not result from errors in the SDH assay caused by the presence of 3α,12α-diol-7-one-5β-cholanoic acid, 3α-ol-7-one-5β-cholanoic acid, or ursodeoxycholic acid in the samples.

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