notes on methodology

Lyophilized 7α-hydroxysteroid dehydrogenase: a stable enzyme preparation for routine bile acid analysis

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Summary Preparations of 7α-hydroxysteroid dehydrogenase (7α-HSDH) from *Escherichia coli* strain 23 can be frozen and thawed without significant loss of activity. 7α-HSDH may then be lyophilized into powder form, which is stable for more than 6 months (3% loss of activity). The lyophilized 7α-HSDH preparation has the additional advantage over previously described preparations of a low and stable fluorescence background when applied to the fluorometric estimation of bile acids, especially in combination with thin-layer chromatography. Analysis of duodenal aspirates from 18 normal subjects gave bile acid ratios identical with those reported earlier and obtained by using gas–liquid chromatography. A significant difference in the glycine: taurine ratio between males and females was observed.

Supplementary key words bile acid fluorometric assay · thin-layer chromatography of bile acids

A 7α-hydroxysteroid dehydrogenase, totally devoid of other hydroxysteroid dehydrogenases, can be obtained from a high percentage of *Escherichia coli* strains (1, 2). This enzyme has been demonstrated to be particularly useful for the fluorometric estimation of components of mixtures of dihydroxy bile acid conjugates inseparable by thin-layer chromatography (2, 3). Major problems in the practical application of this enzyme have been the relative instability of previously described preparations (a life-span of only 10–20 days) (2, 3) and the rather variable increase in nonspecific fluorescence background on aging of the aqueous preparation.[1] The apparent instability of the aqueous *E. coli* B preparation on freezing (1) has led us to investigate the effect of freezing–thawing and lyophilization of an *E. coli* strain of higher enzyme content, with the aim of obtaining a vastly enhanced stability in the preparation. Lyophilization has proved useful in stabilization of *Pseudomonas testosteroni* 3α-HSDH (4) and for other microbial enzyme preparations (5) and was therefore considered to be of potential usefulness in preparing 7α-HSDH.

All bile acids were obtained from Calbiochem, Los Angeles, Calif. Nicotinamide adenine dinucleotide (NAD) was from Sigma Chemical Co., St. Louis, Mo. Todd Hewitt broth was purchased from BioQuest (Becton, Dickinson and Co.), Cockeysville, Md. Glycine, sodium hydroxide, potassium and sodium phosphates, methanol, 95% ethanol, toluene, and acetic acid were from Fisher Scientific Co., Montreal, P.Q. Molybdatophosphoric acid was purchased from E. Merck, Darmstadt, West Germany. Partially purified 3α-HSDH was a product of Worthington Biochemical Corp., Freehold, N.J.

7α-HSDH from E. coli *23.* 7α-HSDH was prepared from a 500-ml stationary phase culture of *E. coli,* strain 23 (serological type 0127:B8), as previously described (2). After ultracentrifugation of the enzyme, the preparation (approx. 15 ml) was rapidly frozen over the maximal surface area of a 50-ml flask by liquid nitrogen and lyophilized for 48 hr in a VirTis lyophilizer. The powdered enzyme was distributed into 4–6 vials and stored at −20°C. Storage conditions at room temperature and 4°C were also tested. Lyophilized 7α-HSDH (6.5 mg/1.5 ml) was reconstituted with ice-cold distilled water prior to use and was used within 4 days.

Assay for 7α-HSDH activity. Reaction mixtures consisted of NAD (1.7×10^{-3} M), glycine–NaOH buffer (0.05 M, pH 9.5), glycochenodeoxycholic acid (10^{-3} M), and 100 μl of reconstituted lyophilized 7α-HSDH (total reaction volume 3.0 ml, pH 9.2). The reaction was initiated with enzyme, and the formation of NADH was followed spectrophotometrically by the increase in absorbance at 340 nm as previously described (1). 1 unit of 7α-HSDH was equivalent to the formation of 1 mole of NADH/min at 25°C.

Estimation of individual bile acids in bile-rich duodenal aspirates. Bile-rich duodenal aspirates were extracted according to Folch, Lees, and Sloane Stanley (6). 20 μl of a 1.0-ml reconstituted extract in methanol–3% hydrogen peroxide 4:1 (v/v) was applied to 20 × 20 cm thin-layer chromatography plates, coated with silica gel to a thickness of 0.5 mm, that had been predeveloped with chloroform–methanol–water 85:35:5 (v/v/v). The bile acid mixture was chromatographed in a preequilibrated tank (3 hr) containing toluene–acetic acid–water 10:10:1. Marker columns of the plates (standards and samples) were treated with 3.5% molybdatophosphoric acid spray reagent, and the plates were heated to 140°C for 10 min (7). Unsprayed columns of the plate were divided to cor-

Abbreviations: C, cholic acid; CDC, chenodeoxycholic acid; DC, deoxycholic acid; 3α-HSDH, 3α-hydroxysteroid dehydrogenase; 7α-HSDH, 7α-hydroxysteroid dehydrogenase.

[1] Macdonald, I. A., C. N. Williams, and D. E. Mahony. Unpublished results.

TABLE 1. Stability of lyophilized 7α-HSDH in sodium- or potassium-containing preparations

Storage Conditions	Cation in Buffer System	Activity[a]	%
Freshly reconstituted	Na^+	1.35 ± 0.06	100
3 months at room temperature (22°C)	Na^+	0.35 ± 0.02	30
3 months at 4°C	Na^+	1.00 ± 0.04	74
6 months at −20°C	Na^+	1.31 ± 0.05	97
Freshly reconstituted	K^+	0 ± 0.00	0

[a] Units/mg of lyophilized powder; duplicate determinations.

TABLE 2. Bile acid ratios in bile-rich duodenal aspirates from healthy volunteers[a]

Sex	Number	Glycine:Taurine[b]	Cholate:Chenodeoxycholate:Deoxycholate[b]
M	10	3.63 ± 0.45	45.22 ± 2.53:32.33 ± 2.85:22.89 ± 1.84
F	8	2.07 ± 0.33	45.62 ± 1.76:36.00 ± 2.82:18.37 ± 1.66

[a] Duplicate fluorometric analyses were performed on aspirates obtained after intravenous cholecystokinin (40 units) on two to four separate occasions.
[b] Bile acid ratios ± 1 SEM.

respond to four readily visualizable bands: (*a*) taurocholic acid, (*b*) taurochenodeoxycholic and taurodeoxycholic acids, (*c*) glycocholic acid, and (*d*) glycochenodeoxycholic and glycodeoxycholic acids as identified by standards. Areas corresponding to *a, b, c,* and *d* were eluted by two 5-ml portions of methanol and the extracts were dried under nitrogen; the residues were then dissolved in a total volume of 200 μl of methanol. Reconstituted eluates of the four areas were subjected to 3α-HSDH estimation (20 μl of a 1 mg/ml solution) (2, 8) while the "dihydroxy" areas *b* and *d* were subjected to 7α-OH quantification by 20 μl of lyophilized 7α-HSDH solution in the fluorometric assay system (2). The conjugated deoxycholate content of bands *b* and *d* was estimated by subtraction of the chenodeoxycholate estimation (7α-HSDH) from the total (3α-HSDH) as before (2, 3).

Background fluorescence estimations. Fluorescence values (excitation of 365 nm and emission at 472 nm) were measured before the addition of enzyme in blank, sample, and standard glycochenodeoxycholic acid cuvettes and at the completion of the reaction (usually 20 min). Fluorescence increments due to enzyme, as well as nonspecific background, were recorded, and the former were correlated with the age of the enzyme preparation. The statistical analysis of the bile acid ratios for significance was performed by the group *t* test (9).

Lyophilized 7α-HSDH in 0.1 M sodium buffer, pH 7.0, may be stored at −20°C for a period of 6 months with about 3% loss of activity (**Table 1**). Storage under warmer conditions was unsatisfactory because it resulted in loss of enzyme activity. However, no measurable loss of activity was incurred on reconstituting lyophilized powder that had been repeatedly subjected to warming to room temperature and promptly refrozen. Reconstructed lyophilized powder (6.5 mg of powder/1.5 ml of water) could be stored at 4°C unfrozen, or it could be rapidly frozen and stored at −20°C for several days with very little loss of enzyme activity.

In contrast to the earlier observations on *E. coli* B 7α-HSDH (1), the corresponding enzyme of *E. coli* strain 23 (2) was stable to either slow or rapid freezing and rethawing (95% activity retained). However, when strain 23 7α-HSDH was diluted in the sodium buffer and slowly frozen, inactivation was demonstrated in more dilute preparations. This inactivation of dilute enzyme could be largely prevented by rapid freezing. Protection against inactivation could also be effected by increased enzyme concentrations (undiluted *E. coli* 23 7α-HSDH) as well as by rapid freezing. 7α-HSDH, even in concentrated solution, was shown to be unstable at pH values less than 5; 80% inactivation occurred in concentrated enzyme solutions in 24 hr at pH 5.0. Curiously, a complete loss of enzyme activity occurred when the enzyme in 0.1 M potassium buffer, pH 7.0, was lyophilized (Table 1), even though there was no loss of activity in the fresh enzyme after either rapid or slow freezing in this buffer system.

Duplicate fluorometric estimations of individual bile acids with 3α- and 7α-HSDH were reproducible within ±3%. Average ratios for the glycine conjugates:taurine conjugates and for cholate:chenodeoxycholate:deoxycholate (C:CDC:DC) derived from duplicate fluorometric estimations for 18 normal subjects are presented in **Table 2.** The values for these ratios were generally reproducible within ±5% of the mean for samples taken on two consecutive days from one individual. The glycine:taurine ratio was shown to be significantly higher in males than in females (3.63 compared with 2.07, $P < 0.02$), but there was no significant sex difference between the C:CDC:DC ratios.

In the 7α-HSDH assay, fluorescent background associated with the enzyme was found to increase with aging of the enzyme preparation (fresh or reconstituted lyophilized powder) at 4°C. A similar increase in background was obtained when the enzyme solution was passed through a 0.22-μm Millipore filter on day 1 (**Table 3**). An augmented nonspecific background increase in the assay was also observed on storage of the lyophilized powder at room temperature or at 4°C for a prolonged period. No measurable increase in background was observed on storage of powder for 6 months at −20°C. (Individual bile acids could be quantified fluorometrically in 20 min with 6-month-old enzyme, as with the fresh preparation.) In addition to "enzyme background," a rather variable nonspecific background was measurable and directly additive

TABLE 3. Effect of aging of reconstituted lyophilized 7α-HSDH preparations on background fluorescence contribution[a]

Age of Preparation[b]	Background Fluorescence Range due to Enzyme		Nonspecific Background Fluorescence Range	Total Background Fluorescence Range[c]
	Unfiltered	Filtered		
1	5–6	4–6	15–25	20–31
4	9–10	8–10	15–25	24–35
7	14–15	14–15	15–25	29–40
21	35–40	36–42	15–25	50–67

[a] Data from duplicate estimations of eight eluates of the "dihydroxy-bands" on thin-layer chromatography of bile-rich duodenal aspirates. One enzyme preparation was unfiltered and a second was passed through a Millipore filter on day 1.

[b] Stored sealed at 4°C.

[c] Total background due to both the enzyme (either filtered or unfiltered) and the nonspecific background.

with the enzyme background (Table 3). The total background fluorescence of 21-day-old aqueous enzyme (approx. 60) represents about 25% of the fluorescence due to the oxidation of 7α-OH groups for a typical thin-layer chromatographic eluate (approx. 200–250). The nonspecific background for the glycochenodeoxycholic acid standard (which showed only one spot on thin-layer chromatography) was identical with that of a blank reaction mixture without substrate or enzyme (approx. 9).

Lyophilized 7α-HSDH offers several advantages over the aqueous preparation reported on earlier. It is stable over long storage periods at −20°C. It can be reconstituted in a convenient volume and concentration and is usable over a 4-day period. The high values of nonspecific fluorometric background in aged aqueous preparations can be avoided. The use of lyophilized enzyme improves the analysis by stabilizing the background readings and avoids the obvious inconvenience of continuous production of enzyme and interbatch variations.

The average C:CDC:DC ratios for both sexes did not vary significantly from each other or from recently reported values obtained by gas–liquid chromatography (10, 11), and the values for glycine:taurine fall well into the normal range previously reported (12). We confirmed the previously reported sex difference for the glycine:taurine ratio (13). We were not able to confirm sex differences in the C:CDC:DC ratio previously reported (13).

Significant alterations in fluorescent background due to "aging" of the aqueous or reconstituted preparation occur long before enzyme deactivation. These background increases were attributed to irreversible changes in some of the preparation components rather than to secondary bacterial growth. This effect is considerably reduced on purification of the enzyme[1] and can largely be avoided by the use of reconstituted 7α-HSDH preparations less than 4 days old.

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