Bioluminescence measurement of primary bile acids using immobilized 7α-hydroxysteroid dehydrogenase: application to serum bile acids

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Abstract A simple, rapid, and sensitive bioluminescence method for measuring primary bile acids has been developed and validated. The method is based on enzymatic dehydrogenation of bile acids using a bacterial 7α-hydroxysteroid dehydrogenase that is co-immobilized on Sepharose 4B beads with NADH:FMN oxidoreductase and a bacterial luciferase. The assay is specific for 7α-hydroxy bile acids and has a detection limit of 0.5 pmol/tube, with a linear range of 0.5–50 pmol/tube. The assay shows good precision (6–8% intra-assay; 8–10% inter-assay). The values obtained with the bioluminescence assay showed good agreement with those obtained by gas–liquid chromatography, radioimmunoassay, or end-point enzymatic assays. When applied to the measurement of serum bile acids, there was no interference from serum albumin, and the effect of other dehydrogenase activity in serum could be eliminated by heating the sample prior to assay. Since the method is rapid (1 minute), extremely sensitive (requires only 10 μl of serum), and specific, it appears to be the best method currently available for the measurement of serum primary bile acids.—**Roda, A., L. J. Kricka, M. DeLuca, and A. F. Hofmann.** Bioluminescence measurement of primary bile acids using immobilized 7α-hydroxysteroid dehydrogenase: application to serum bile acids. *J. Lipid Res.* 1982. **23:** 1354–1361.

Supplementary key words bacterial luciferase • NADH:FMN oxidoreductase • radioimmunoassay • enzymatic method

Bile acids are a group of acidic steroids formed in the liver from cholesterol. After conjugation with glycine or taurine, bile acids are secreted into bile and undergo an enterohepatic circulation, since they are actively reabsorbed by the terminal ileum (1). In man, five bile acids constitute most of the circulating bile acids. Of these five, three predominate: the primary bile acids, cholic acid and chenodeoxycholic acid, and the secondary bile acid, deoxycholic acid. The other two bile acids, ursodeoxycholic acid and lithocholic acid, are present in much smaller proportions, usually less than 5%. Chenodeoxycholic acid and cholic acid are formed directly from cholesterol and are termed primary bile acids; the remaining bile acids, deoxycholic acid, lithocholic acid, and ursodeoxycholic acid, are formed from the primary bile acids by dehydroxylation or epimerization of the 7-hydroxy group. Since intestinal bacteria are involved partially or completely in these biotransformations, these bile acids are termed secondary bile acids.

Serum bile acids originate from the intestinal absorption of bile acids during enterohepatic cycling and their levels increase postprandially. Both the fasting state and postprandial levels of serum bile acids are determined by the momentary balance between intestinal absorption and hepatic uptake (2). An increased serum bile acid level in the fasting state or postprandial is considered to be a specific indicator of liver disease, reflecting decreased hepatic uptake or systemic shunting of portal venous blood (3). A decreased level, especially postprandially, is an indicator of bile acid malabsorption that usually reflects ileal dysfunction (4–6).

Three methods have been used to measure serum bile acids. Gas–liquid chromatography determines individual steroid moieties, but the method is rather insensitive and sample preparation is laborious, requiring deconjugation and derivatization steps (7–10). Enzymatic methods based on group-specific steroid dehydrogenases isolated from bacteria (11, 12) have been used successfully to measure total 3α-hydroxylated (13) and 7α-hydroxylated (14) serum bile acids. Such meth-

Abbreviations: NAD, nicotine adenine dinucleotide; BSA, bovine serum albumin; FMN, flavin mononucleotide; DTT, dithiothreitol; HSD, hydroxysteroid dehydrogenase; ox-red, oxido-reductase; LDH, lactate dehydrogenase. In this paper, bile acids will be referred to by trivial names only. The suffix "yl" is used to denote the bile acid moiety of the glycine and taurine conjugates, which are N-acyl amidates.

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ods, as originally reported, were insensitive, but sensitivity can be increased modestly by detection of NADH fluorescence (15, 16). Recently, still more sensitive enzymatic techniques have been reported in which the dehydrogenation of the bile acid is signaled by reduction of a fluorescent (17) or colored (18) redox indicator. An enzyme-cycling technique has also been reported (19), but this requires a lengthy sample incubation.

Radioimmunoassay or similar competitive binding techniques are the only conventional analytical methods that exhibit sufficient sensitivity to detect the diurnal variation in serum bile acid levels in healthy individuals (4) or the impaired postprandial elevation in patients with ileal dysfunction (4–6). Antibodies are available that are specific for an individual amidated bile acid, i.e., a single glycine or taurine conjugate, such as cholylglycine (20); or for both conjugates of a single steroid moiety, i.e., cholyl conjugates (21) or for chenodeoxycholyl conjugates (22); or for most primary bile acids (23). Although radioimmunoassay exhibits adequate sensitivity, the specificity of most reported radioimmunoassays is not entirely satisfactory: unconjugated bile acids, which may constitute a considerable fraction of the serum bile acids (24), are not determined, as the antibodies react only with conjugated bile acids in most assays reported to date (25).

Recently, bioluminescence assays have been described for testosterone and androsterone using either 3α- or 3β-hydroxysteroid dehydrogenase co-immobilized with NADH:FMN oxidoreductase and bacterial luciferase (26). These assays exploit the tremendous sensitivity of the bioluminescence detection method for NADH; the detection limits for testosterone and androsterone were 0.8 pmole.

Both 3α- and 7α-hydroxysteroid dehydrogenases of acceptable purity for use in enzymatic bile acid analysis are now currently available from several commercial suppliers. The 7α-hydroxysteroid dehydrogenase may be considered to be specific for primary bile acids, as the major secondary bile acids do not possess a 7α-hydroxy group.

We report here the development, validation, and application of a bioluminescence assay of 7α-hydroxy bile acids based on 7α-hydroxysteroid dehydrogenase that has been co-immobilized with NADH:FMN oxidoreductase and a bacterial luciferase.

MATERIALS AND METHODS

Reagents

NAD and BSA were purchased from Calbiochem-Behring Corporation (La Jolla, CA); FMN, decanal, and dithiothreitol (DTT) were obtained from Sigma Chemical Company (St. Louis, MO); cyanogen bromide was supplied by the Eastman Chemical Company (Rochester, NY); and Sepharose 4B was purchased from Pharmacia Fine Chemicals (Piscataway, NJ).

Bile acids were obtained from a variety of commercial sources and were purified by crystallization or chromatography before use or further modification. Cholic and lithocholic acids were generous gifts of Canada Packers, Ltd., Toronto, Canada; and chenodeoxycholic and ursodeoxycholic acids were gifts of Diamalt, Pharmazell, Redenfelden, West Germany. 7-Keto derivatives were prepared by selective oxidation (27). Glycine and taurine conjugates were synthesized (28). All bile acids used had a purity of >98% by thin-layer chromatography, showing only one major spot when a 200-μg sample was chromatographed.

7α-Hydroxysteroid dehydrogenase (6.2 U/mg solid, Lot No. 116C-6840-1) from *Escherichia coli* was purchased from Sigma and used without further purification. It was found to be free of aldehyde dehydrogenase activity; i.e., there was no reaction with decanal present in the luminescent assay buffer. The enzyme was also free of 3α-hydroxysteroid dehydrogenase and lactate dehydrogenase activity. There was, however, an insignificant contamination with malate dehydrogenase. Luciferase was isolated from a frozen cell paste of *Beneckea harveyi*, strain B-392 (29, 30). NADH:FMN oxidoreductase was purified according to the method of Jablonski and DeLuca (31). Both enzymes, whose final purification step was gel permeation chromatography on a Sephadex G-100 column, were stored in 0.1 M phosphate buffer, pH 7.0, containing DTT (2.0 mM) at −70°C.

Immobilization procedure

Luciferase, NADH:FMN oxidoreductase, and 7α-hydroxysteroid dehydrogenase were co-immobilized on cyanogen bromide-activated Sepharose according to the method of Ford and DeLuca (26). A minor modification to the method was made by dialyzing the enzyme mixture, prior to immobilization, against sodium pyrophosphate (0.1 M, pH 8.0) rather than against sodium bicarbonate (0.1 M, pH 8.0).

The coupling mixture was composed of 1 ml luciferase (13 mg/ml, 6×10^6 relative light units/ml), 0.2 ml NADH:FMN oxidoreductase (6.8 mg/ml, 10.45 U/ml), and 3.5 mg 7α-hydroxysteroid dehydrogenase (6.2 U/mg solid). The volume of the coupling mixture was adjusted to 2.5 ml by the addition of sodium pyrophosphate (0.1 M, pH 8.0). A 2-ml portion of the coupling mixture was reacted with 1 g of activated Sepharose 4B.

To assess the efficiency of the immobilization procedure, enzyme activities in the coupling mixture were determined before and after addition of the activated Sepharose, permitting the activity of the enzymes (both adsorbed and covalently linked) to be determined by difference. Soluble and Sepharose-bound luciferase and oxidoreductase activities were measured as described previously (26). These measurements were made after the Sepharose-bound enzymes had been stored for several days in phosphate buffer (0.1 M, pH 7.0) containing BSA (2 g/l), DTT (0.1 mM), and sodium azide (0.2 g/l), since post-immobilization rises in enzyme activity had been previously observed for Sepharose-bound enzymes. 7α-Hydroxysteroid dehydrogenase activity was assayed by adding either 10 μl of the soluble enzymes or 50 μl of Sepharose-bound enzyme to 1 ml of sodium pyrophosphate (0.1 M, pH 8.5), containing 50 μl aqueous NAD (0.02 M) and 50 μl chenodeoxycholylglycine (100 μM) and measuring the increase in absorbance at 340 nm. Protein concentration was calculated from the absorbance at 280 nm.

Bioluminescence assay of bile acids using 7α-hydroxysteroid dehydrogenase

Principle. 7α-Hydroxysteroid dehydrogenase catalyzes the conversion of the bile acid 7α-hydroxyl group to a keto group. NADH is produced in the reaction, and in the presence of NADH:FMN oxidoreductase it converts FMN to its reduced form. This, in the presence of bacterial luciferase, reacts with decanal and oxygen to produce FMN, decanoic acid, and light. The peak intensity of the emitted light is proportional to the concentration of bile acid in the initial reaction.

$$7\alpha\text{OH bile acid} + \text{NAD} \overset{7\alpha\text{-HSD}}{\rightleftharpoons} \text{7-oxo bile acid} + \text{NADH}$$

$$\text{H}^+ + \text{NADH} + \text{FMN} \overset{\text{NADH-FMN ox-red}}{\rightleftharpoons} \text{NAD}^+ + \text{FMNH}_2$$

$$\text{FMNH}_2 + \text{decanal} + \text{O}_2 \overset{\text{luciferase}}{\longrightarrow} \text{FMN} + \text{decanoic acid} + \text{H}_2\text{O} + \text{light}.$$

Assay Buffer. An emulsion of decanal in water, prepared by shaking decanal (5 μl) with 10 ml of water, was made fresh each day and stored at 4°C. A decanal emulsion (100 μl) was added to 200 μl of aqueous FMN (73 μM), 1.0 ml aqueous NAD (0.02 M), and 10.0 ml of phosphate buffer (0.1 M, pH 7.0); after mixing, the solution was stored at room temperature in the absence of light. This solution was stable for 3–4 hr.

Bioluminescence measurements. Light emission was measured using an Aminco Chem-Glow photometer. Since there is no accepted light standard, all luminescence measurements are relative.

Enzyme-bound Sepharose (170 g/L) was diluted 1:9, v/v, with phosphate buffer (0.1 M, pH 7.0) containing BSA (2 g/l), DTT (0.1 mM), and sodium azide (0.2 g/l).

Procedure. Fifty μl of the Sepharose suspension was added to the assay tubes (6 × 50 mm, Kimble, Division of Owens-Illinois, Toledo, OH), followed by 500 μl of the assay buffer. Serum, usually a 10-μl sample, was diluted 1:4 with 0.1 M sodium phosphate buffer, pH 7, and then heated for 15 min at 70°C. For a sample with a concentration > 50 μM, a second dilution, usually 1:10, v/v, with phosphate buffer was required.

For the assay, a 0.1-ml sample of the appropriately diluted serum or a 0.1-ml sample of standard was added. The tube was then placed into the luminometer and the light emission was recorded continuously, using an Aminco recorder, until peak light emission was obtained; this occurred in 1–2 min after the start of the reaction. A standard curve of the concentration of bile acid versus peak light emission was plotted and values of the unknown were read directly from the curve. The assay was carried out at ambient temperatures (18–20°C).

Standards and standard curves. Standard curves were constructed using dilutions (1–100 μM) of the various free and conjugated 7α-hydroxy bile acids in phosphate buffer (0.1 M, pH 7.0). Each standard was assayed in duplicate. To test the linearity of the assay, standard curves were examined for cholate, cholylglycine, cholyltaurine, chenodeoxycholate, chenodeoxycholylglycine, and chenodeoxycholyltaurine.

Recovery. Aliquots of a solution of chenodeoxycholyltaurine (50 μM) were added to a serum sample with a low level of bile acid (1.60 μM) or to a sample with a high bile acid level (21.3 μM). Samples were assayed for bile acids before and after the addition of the chenodeoxycholyltaurine to permit calculation of percentage recovery.

The effect of dilution was assessed using a serum sample with an elevated bile acid concentration. The sample was diluted (2-fold, 5-fold, 10-fold, and 20-fold) with 0.1 M phosphate buffer, pH 7.0, and then assayed using the bioluminescent assay.

Specificity. The specificity of the assay was assessed by measuring the enzymatic reaction with 200 μM solutions of other bile acids and steroids. Bile acids that were tested lacked a 7α-hydroxy group (either having no substituent at the 7 position, or a 7-keto group) or a 7β-hydroxy group; these included deoxycholic, deoxycholylglycine, deoxycholyltaurine, lithocholic, lithocholylglycine, lithocholyltaurine, ursodeoxycholic, ursocholic acid, and the 7-keto derivatives of chenodeoxycholic and cholic acids. Other steroids that might react with the enzymes were also tested. These included testoster-

one, androsterone, progesterone, estradiol, and cholesterol.

Interferences. Intrinsic serum dehydrogenase activity was considered a possible source of interference, since it could contribute NADH to the oxidoreductase and would thus be assayed as bile acid. To quantitate the possible interference from serum dehydrogenase activity, two groups of studies were conducted. First, increasing amounts of a solution of lactate dehydrogenase (Calbiochem-Behring Corporation, La Jolla, CA; 6260 IU/ml) were added to a serum sample to give final lactate dehydrogenase activities of 40, 400, and 4000 IU/ml. These LDH-spiked serum specimens were then assayed for bile acids. The specimens were also assayed for NADH, using a Sepharose-bound NADH:FMN oxidoreductase-luciferase preparation containing no 7α-hydroxysteroid dehydrogenase. Second, a serum sample with an elevated lactate dehydrogenase level was assayed for bile acids and NADH before and after heating at 70°C for 15 min.

Protein effects. Primary bile acids are known to be tightly bound to albumin in human serum (32). The effect of human serum albumin concentration on the assay was assessed by comparing results obtained for chenodeoxycholylglycine or cholylglycine prepared either in phosphate buffer or phosphate buffer containing various concentrations of human serum albumin.

Precision. Inter-assay precision was assessed by ten replicate measurements during 1 day of two serum samples, one with a low and one with a high concentration of bile acids. Intra-assay precision was determined from daily (duplicate) determinations of serum bile acid concentrations in the same specimens used in the inter-assay precision study.

Validation. The bioluminescence assay was validated on serum bile acid samples by comparing results with those obtained by gas–liquid chromatography and with those obtained by radioimmunoassays for cholyl conjugates and chenodeoxycholyl conjugates.

Gas–liquid chromatography

Serum samples from patients with various types of liver disease (7, 25) were generously supplied by Dr. Gerard van Berge Henegouwen, Municipal Hospital, Arnhem, The Netherlands. Individual bile acids had been measured in these samples by gas–liquid chromatography, as reported by his laboratory (7).

Radioimmunoassay

Radioimmunoassay for serum bile acids was carried out as described previously (21, 22) on 50 samples. The antisera used were specific for cholyl conjugates or chenodeoxycholyl conjugates and did not react with unconjugated bile acids.

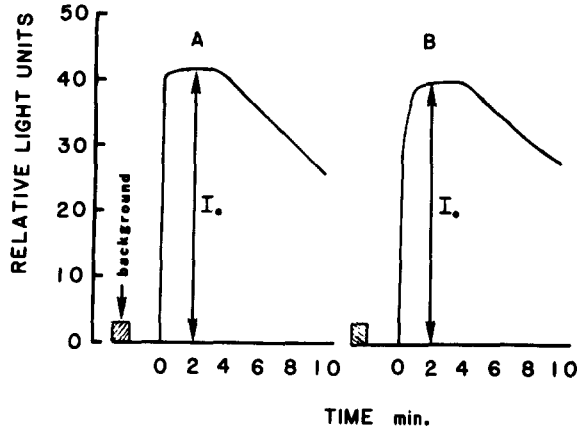

Fig. 1. Time course of light emission for A) a 5 μM chenodeoxycholyltaurine standard (left) and B) a serum sample (right). The relative net light units are obtained by subtracting the background intensity value from the peak of the light emission plateau.

Stability of immobilized enzyme preparation

To define the stability of the immobilized enzymes when dispersed in buffer, a series of assay tubes containing 50-μl aliquots of a 10-fold dilution of the stock enzyme-bound Sepharose in phosphate buffer were stored at room temperature. The stability of the enzyme-bound Sepharose was assessed by carrying out duplicate assays of a standard solution of chenodeoxycholylglycine (25 μM) over a period of several months.

RESULTS

Enzyme immobilization

More than 90% of total enzymatic protein and more than 90% of the activity of each enzyme were removed from solution by the coupling procedure. However, enzymatic activity of the immobilized enzymes differed. The immobilized oxidoreductase activity was identical to that in the reaction mixture, whereas only 12% of luciferase and 20% of the 7α-hydroxysteroid dehydrogenase activity was immobilized. The figures for immobilization efficiency are only approximate, since the enzyme assays on homogeneous and heterogeneous systems may not be strictly comparable.

Kinetics of the assay

Light was produced rapidly and reached a plateau at 1 min (**Fig. 1**). The time course of light emission was similar for both standards (Fig. 1a) and serum samples (Fig. 1b). In initial studies, we found that the reaction rates of the two primary bile acids were not identical, and were dependent on the relative concentration of the 7α-hydroxysteroid dehydrogenase. If beads were prepared with too little enzyme, cholate reacted more

TABLE 1. Recovery of different amounts of chenodeoxycholyltaurine added to two sera at low and high bile acid concentrations

Initial Bile Acid Concentration in Sample	Chenodeoxycholyltaurine Added (A)	Bile Acid Measured (M)	Differences (M − A)	Recovery
	μmol/l			%
1.60	0	1.60	1.60	100
1.60	1.60	3.10	1.50	94
1.60	3.20	4.85	1.65	103
21.30	0	21.2	21.3	100
21.30	21.30	42.0	20.7	97
21.30	42.60	62.2	19.6	92

slowly than chenodeoxycholate. However, the preparation of beads containing a high concentration of enzyme resulted in comparable rates of dehydrogenation for each of the primary bile acids.

Standard curve

A linear response in the range 1.5 to 50 μM was obtained for all nine bile acids present in the enterohepatic circulation of man, i.e., the glycine and taurine conjugates of 7α-hydroxy cholic and chenodeoxycholic acids, as well as the unconjugated acids. No differences were observed between the unconjugated or glycine- or taurine-conjugated forms of either primary bile acid. The detection limit (i.e., the concentration giving twice the background signal) was 0.5 μmol/l (5 pmol/tube).

Recovery

Recovery of added chenodeoxycholyltaurine to serum samples was excellent (**Table 1**).

Specificity

At concentrations of 200 μM, none of the bile acids lacking a 7α-hydroxy group reacted with the enzyme. Other physiological steroids did not react with the enzyme, even if present in high concentrations (data not presented).

Precision

Inter-batch precision of the assay at two different bile acid concentrations was satisfactory, the coefficient of variation for ten assays being zero. Results are summarized in **Table 2**.

TABLE 2. Inter-assay and intra-assay precision of bioluminescence assay

	Inter-assay			Intra-assay		
	Mean	SD	CV%	Mean	SD	CV%
Low concentration pool (μmol/l)	1.63 ± 0.16		9.8	1.60 ± 0.10		6.2
High concentration pool (μmol/l))	21.10 ± 1.80		8.5	21.30 ± 1.60		7.5

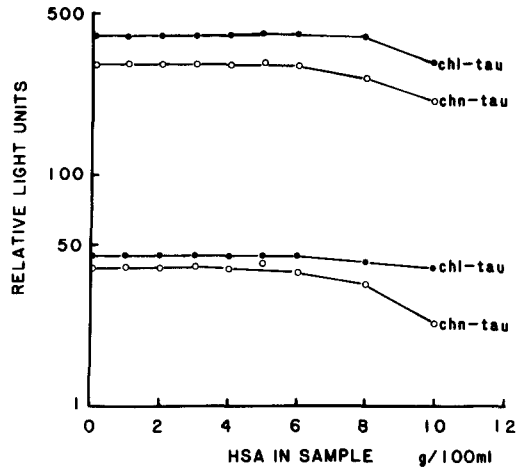

Fig. 2. Influence of addition of human serum albumin (HSA) on light emission of standards containing low or high bile acid concentrations. The concentration of HSA in the standard is shown. chl-tau, cholyltaurine; chn-tau, chenodeoxycholyltaurine.

Interferences

When lactate dehydrogenase was added to sera, light production increased in proportion to the amount added even with Sepharose beads prepared without the 7α-hydroxysteroid dehydrogenase. This nonspecific light production could be abolished by heating samples at 70°C for 15 min.

The presence of human serum albumin (at a physiological concentration of 3 g/dl) in the bile acid standards did not affect the dose-response relationship (**Fig. 2**).

Validation

Fig. 3 shows results obtained using the bioluminescence assay compared with those using gas–liquid chromatography. **Fig. 4** compares results obtained with the bioluminescent technique with those obtained by radioimmunoassay. The agreement between results obtained by the bioluminescent technique was excellent with those obtained by gas–liquid chromatography or radioimmunoassay, despite the fact that radioimmunoassay does not determine unconjugated bile acids, which may be present in plasma in low concentrations (24).

Stability studies

Storage at room temperature for 2 days produced a 30% loss in activity of the Sepharose-bound enzymes; this increased to 50% after 4 days and complete loss of activity after 1 week. In contrast, there was no loss of activity of the Sepharose beads when stored at 4°C in buffer containing azide, BSA, and DTT.

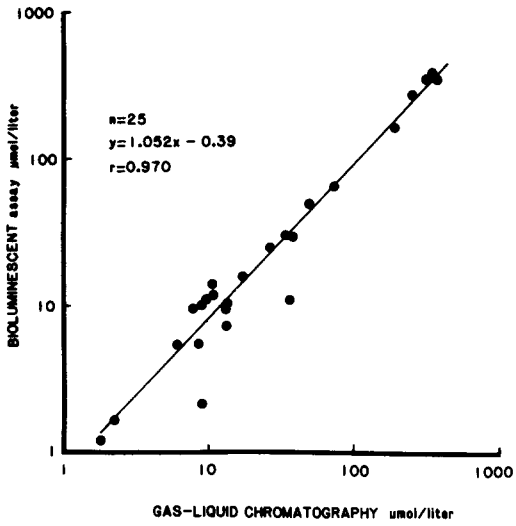

Fig. 3. Results obtained by the bioluminescence assay (ordinate) compared with those obtained by gas–liquid chromatography (abscissa) on serum samples from patients. The elevated values (>2 μM) indicate the samples from patients with liver disease. Samples were supplied and had been analyzed by Dr. Gerard van Berge Henegouwen (7).

DISCUSSION

Method ideality

These results indicate that the bioluminescence technique described here appears to be an ideal method for measuring primary bile acids, i.e., 7α-hydroxy bile acids, in biological fluids. The sensitivity of the bioluminescence assay is comparable to that of published competitive binding assays and superior to all endpoint enzymatic methods reported to date. The method is rapid (1 min) and is potentially less expensive than competitive binding techniques. The specificity is excellent, as the only 7α-hydroxysteroids present in serum are bile acids.

The use of a Sepharose-immobilized enzyme system offers a number of advantages over homogeneous, endpoint, or kinetic enzymatic methods. These include increased catalytic activity per unit enzyme, a potentially reusable form of the enzymes, and a stable analytical reagent. During 6 months, our enzyme preparation remained completely stable when stored at 4°C in phosphate buffer containing BSA, azide, and DTT.

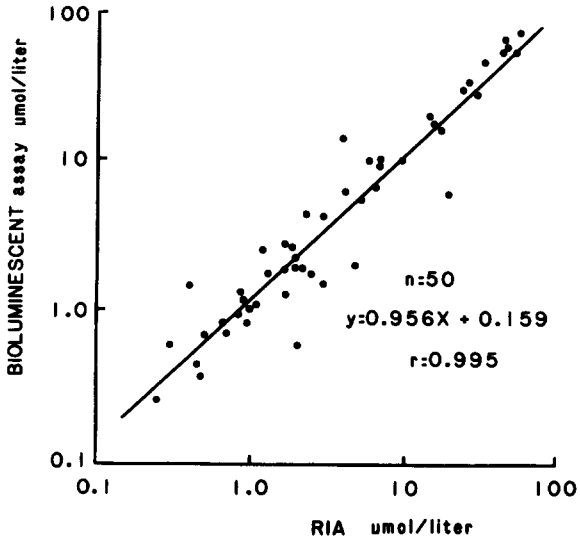

Fig. 4. Results obtained by the bioluminescence assay (ordinate) compared with those obtained by radioimmunoassay (abscissa). The positive intercept may reflect the presence of unconjugated bile acids in serum that are measured by the bioluminescence technique, but not by radioimmunoassay.

Validity

The output of luminescence was linear and recovery of added standards was excellent. Results with the bioluminescence assay agreed well with those obtained by gas–liquid chromatography. Agreement between the bioluminescence assay and gas–liquid chromatography should be excellent, as both methods determine unconjugated bile acids, glycine conjugates, and taurine conjugates. Bile acids are sulfated in cholestasis (33) at both the 3 or 7 positions (34), but sulfated bile acids are not present in serum in cholestasis in appreciable amounts (33), and a solvolysis step was not included prior to the gas–liquid chromatographic analysis for these samples.

The agreement with radioimmunoassay was good, although the correlation showed a positive intercept. This may reflect the presence of unconjugated bile acids that are not determined by radioimmunoassay. The presence of unconjugated bile acids in serum has recently been established by gas–liquid chromatography (24) as predicted from pharmacokinetic modeling of the enterohepatic circulation of bile acids (35).

Application to serum

The bioluminescence assay described here appears to work well with human serum. The intrinsic dehydrogenase activity was eliminated satisfactorily by heating at 70°C for 15 min. Serum albumin did not influence the assay because, as a result of the dilutions, the total albumin concentration in the assay tube was less than 1 g/l. The method could be used to measure the concentration of the other major serum bile acids, deoxycholic and cholic, and their conjugates by performing additional assays with an immobilized12α-hydroxysteroid dehydrogenase and an immobilized 3α-hydroxysteroid dehydrogenase (36).

Clinical utility

The bioluminescence assay described here appears to be the method of choice if a nonautomated method

is to be used for the measurement of serum bile acids. In animals, serum bile acid levels appear to be an excellent test to detect liver injury (37). The cost–benefit aspects and clinical utility of serum bile acid measurements continues to be widely debated (38).

Recently, these assays have been performed using the immobilized enzymes in a flow cell, and this has demonstrated the reusability of these reagents.

The low cost, simplicity, and commercial availability of the procedure described here should facilitate prospective studies designed to assess clinical utility of serum bile acid levels.

We acknowledge the skillful technical assistance of Diane Stone, advice from Dr. Ian Macdonald, donations of pure bile acid standards by Alan T. Johnson and Eva Ljungwe, secretarial assistance of Joy Sato, and editorial assistance of Vicky Huebner. The Medical Research Council (London) is gratefully acknowledged for a Travelling Fellowship (to L.J.K.). Supported in part by NIH Grant AM 21506, a grant-in-aid from the Stokely Van Camp Foundation, the Biliary Disease Research Fund, as well as a grant from the National Science Foundation.

Manuscript received 11 March 1982 and in revised form 9 July 1982.

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