Radioimmunoassay of sulfated lithocholates

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Abstract A sensitive, rapid radioimmunoassay for sulfated species of lithocholic acid (sulfolithocholyglycine, sulfolithocholyltaurine, and sulfolithocholic acid) was developed and used to measure the total concentration of sulfated lithocholates in serum from healthy human subjects. Sulfolithocholylglycine was conjugated to bovine serum albumin by a carbodiimide procedure, and the reaction product, emulsified in Freund's adjuvant, was injected into rabbits. The antiserum obtained was capable of binding 40% of [^{3}H]sulfolithocholylglycine at 1:1000 dilution. The assay featured a 2-hr binding step at 42°C followed by precipitation of bound tracer with polyethylene glycol at 4°C. The assay can be used with 0.1 ml of serum and was reproducible. The antibody had little affinity for the 3-sulfate of cholic or chenodeoxycholic acid, a number of steroid sulfates, or unsulfated bile acids. In 50 healthy subjects, mean (±SE) fasting-state serum levels of immunoreactive sulfated lithocholyl conjugates (+sulfated lithocholate) was 1.6 ± 0.1 μM; based on results with a separate radioimmunoassay for unsulfated lithocholyl conjugates, most of the lithocholate in serum in healthy man is present in sulfated form.

Supplementary Key words bile acids · lithocholic acid · solvolysis · serum bile acid levels.

Lithocholic acid, which is formed in the human intestine by bacterial 7α-dehydroxylation of chenodeoxycholic acid, is a major fecal bile acid in man (1). In man, lithocholic acid resembles other bile acids in being conjugated with glycine or taurine before biliary excretion, but, in contrast to other bile acids, lithocholic acid is also sulfated (2–4). As a consequence, lithocholic acid occurs in human bile predominantly as sulfated lithocholyl conjugates.

Sulfate esters of lithocholic acid were first recognized by Palmer (2, 5, 6), who noted that the usual preparation of bile acids for gas–liquid chromatography, which includes a vigorous alkaline saponification procedure, may result in extensive loss and degradation of sulfate esters. Because of this, older published data for the lithocholate content of bile may not be valid. In 1965, Sjövall and Vihko (7) showed that steroid sulfates were readily separated from nonsulfated steroids by nonaqueous gel permeation chromatography using Sephadex LH-20 columns. Recently, Stiehl (8) and Makino, Hashimoto, and Shinozaki (9) have reported serum values for sulfated lithocholates (sulfated lithocholyl conjugates + sulfated unconjugated lithocholate) determined by this technique.

Despite the excellent class separation afforded by nonaqueous gel permeation chromatography using Sephadex LH-20, quantitation of lithocholyl sulfates by subsequent gas–liquid chromatography is still complex, requiring multiple chemical steps (solvolysis and deconjugation, esterification with methanol, and derivatization). We have previously developed successful radioimmunoassays for conjugates of cholic acid (10) and chenodeoxycholic acid (11), and we reasoned that a similar radioimmunoassay for sulfated lithocholates might permit rapid and convenient measurement on small serum samples. The development of such a radioimmunoassay is reported here. The preceding paper (12) describes a similar radioimmunoassay for unsulfated lithocholates.

METHODS

Synthesis of tracer

[11,12-^{3}H]Lithocholylglycine (13) was sulfated by a modification of the method described by Palmer (6). Specific activity of the tracer used was 0.7 Ci/mmol. Purity exceeded 98% as determined by zonal scanning of thin-layer chromatograms.

Potassium phosphate buffer, pH 7.4, 0.01 M was used. Bile acid-free serum was prepared by combined charcoal–cholestyramine extraction (10, 11).

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Production of antiserum: immunogen

Lithocholylglycine (glycolithocholate) was synthesized from lithocholic acid (lithocholate) and glycine methyl ester as described (3). After purification by preparative thin-layer chromatography and saponification, the lithocholylglycine was sulfated by a modification of Palmer's method (2). Sulfolithocholylglycine was dissolved in 0.5 ml of 50% aqueous pyridine, and 4 mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (Ott Chemical Company, Muskegon, MI) was added while the mixture was stirred magnetically. Bovine serum albumin (Sigma Chemical Company, St. Louis, MO) 3 mg dissolved in 0.3 ml of saline, was added dropwise from a micropipet. The concentration of pyridine was then slowly increased to 80% over the next 4 hr by the dropwise addition of pyridine. The mixture was then stirred for another hour, diluted with 20 volumes of water, and lyophilized. To document covalent coupling, [^{3}H]sulfolithocholylglycine was used in the conjugation procedure. When the product was dialyzed against running tap water for 48 hr, 10–12% of the radioactivity did not pass through an ultrafilter (Centriflo membrane cones, Amicon filters, Amicon Corporation, Lexington, MA), despite the addition of a 20-fold excess of carrier sulfolithocholylglycine before filtration.

The crude reaction mixture was used for immunization. The sulfolithocholylglycine–albumin conjugate was not isolated from unreacted sulfolithocholylglycine by gel permeation chromatography, as has been customary for other bile acid radioimmunoassays (10, 11).

Immunization

The reaction mixture containing the sulfolithocholylglycine–albumin conjugate was dissolved in 3 ml of water and emulsified with an equal volume of Freund's complete adjuvant (Difco Laboratories, Detroit, MI) using a tissue homogenizer. The final concentration was 100 μg of sulfated lithocholylglycine and 0.4 mg of albumin per ml. This mixture was injected intracutaneously into multiple sites on the dorsal skin of six white New Zealand rabbits. The antigen was injected at 2-week intervals for 10 doses and then at longer intervals according to the antibody titer.

Assays for sulfated lithocholylglycine-binding antibody were begun after 3 months of immunization. Two sera showed adequate binding of [^{3}H]sulfolithocholylglycine (>40% of tracer bound at 1:1000 dilution) for development of a sensitive radioimmunoassay and continue to do so.

Radioimmunoassay

Stage 1. Binding of [^{3}H]sulfolithocholylglycine to antibody. The reaction mixture contained the following: 0.1 ml of bile acid-free serum diluted 1:2; 0.1 ml of [^{3}H]sulfolithocholylglycine ($\simeq$1.0 pmol); 0.1 ml of unlabeled sulfolithocholylglycine, as standard, or unknown serum for bile acid assay; 0.1 ml of antiserum (1:100 dilution); and buffer to a final volume of 1 ml. The components of the incubation mixture were diluted with or dissolved in 0.01 M potassium phosphate buffer, pH 7.4. Incubation was at 42°C for 2 hr. The tubes were then placed in a 4°C cold room for 30 min before the separation step.

Stage 2. Separation of free and bound antigen. Bound antigen was precipitated by polyethylene glycol (PEG; mol wt 6000). To 1 ml of Stage 1 reaction mixture was added 0.5 ml of 37.5% PEG yielding a concentration of 12.5% (wt/vol) (14). After the mixture stood for 15 min, the precipitated γ-globulin, including the bound antigen, was sedimented by centrifugation at 1200 *g* at 2400 rpm 4°C for 30 min. The supernate, containing free [^{3}H]sulfolithocholylglycine, was decanted into scintillation vials, and 14 ml of a toluene–detergent–scintillant solution (Ready-Solv VI, Beckman, Fullerton, CA) was added. Radioactivity was measured in a Beckman LS-250 liquid scintillation counter; the statistical error of counting usually was ±3%.

The separation of free antigen from bound antigen, based on radioactivity counts, was highly reproducible. In the absence of antibody, 95% or more of the [^{3}H]sulfolithocholylglycine added to the reaction mixture was recovered in the supernate after antibody precipitation with PEG. Thus, nonspecific binding and entrapment in the precipitate were small; further, they were not affected by doubling the concentration of bile acid-free serum in the reaction mixture. The mean value for three replicates decanted as supernate in the absence of antibody was taken as the "100% free" value. Replicates in one assay and means for successive assays usually differed by 3% or less.

Calculation and expression of results. Bound radioactivity was calculated by subtracting the radioactivity in the supernate in the presence of antibody from the "100% free" value for the supernate in the absence of antibody. Bound radioactivity in the absence of added unlabeled sulfolithocholylglycine was normalized to 100%. Results for serum analyses are expressed as molar concentrations (μmol of sulfated lithocholates/l of serum) since our antibody reacted not only with sulfated lithocholyl conjugates, but also with sulfated (unconjugated) lithocholic acid.

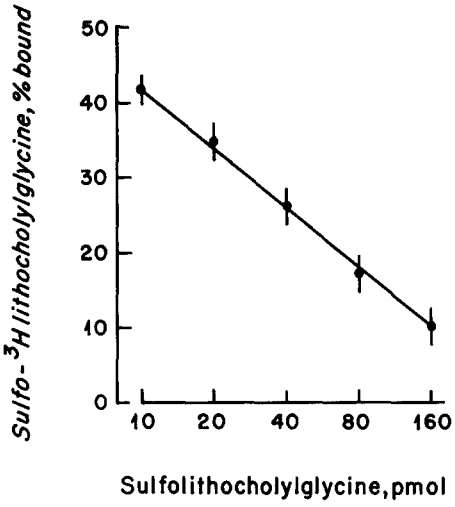

Fig. 1. Displacement of bound [^{3}H]sulfolithocholylglycine by added nonradioactive sulfolithocholylglycine. The amount of added sulfolithocholylglycine is plotted logarithmically on the abscissa; the percent binding (M ± SE) of the tracer is shown on the ordinate.

RESULTS

Sensitivity and specificity of antibody

Antiserum from one rabbit (antiserum 4468) was used for characterizing the antibody and validating the final assay procedure.

Approximately 50% of the tracer dose of [^{3}H]-sulfolithocholylglycine was bound by a 1:400 dilution of antiserum. Binding decreased linearly with dilution, on a logarithmic scale, from 1:400 to 1:3200. Percentage binding of tracer decreased linearly with a logarithmic increase in the unlabeled sulfolithocholylglycine concentration from 10 to 160 pmol (**Fig. 1**).

Specificity

The relative amounts of lithocholate derivatives and major bile acids required to displace 50% of bound [^{3}H]sulfolithocholylglycine are summarized in **Table 1**, and specificity of the antibody for the six lithocholate species anticipated to be in serum is shown in **Fig. 2**. The following sulfated steroid compounds did not cause 50% displacement of tracer when added in quantities of 10,000 pmol: 3-sulfocholate, 3-sulfo-chenodeoxycholate, cholesterol sulfate, estrone sulfate, estriol sulfate, dehydroisoandrosterone sulfate, 5α-androstan-3α-ol-17-one-3-sulfate, 5-androstan-3β-ol-17-one-3-sulfate, ethiocholanolone sulfate, and 11-ketoandrosterone sulfate. Serum samples from germ-free male and female rats and mice (Sprague-Dawley, Madison, WI) were assayed and caused no displacement of tracer from antibody, i.e., had levels of sulfolithocholate too low to measure.

Assay of human serum

Dilutions of human serum were assayed using 0.1 ml volumes. Displacement curves with serial dilutions of human sera (1 μM, 50 μM) were parallel with the sulfolithocholylglycine displacement curve (**Fig. 3**). Thus, the estimated bile acid value for whole serum was independent of its dilution in the assay system.

Precision, sensitivity, and recovery experiments

Using a serum sample with a level of 2 μM, the coefficient of variation was 2% within assays and 8% between assays. The assay could measure levels as low as 0.1 μM. In recovery experiments, sulfolithocholylglycine was added to serum samples from patients containing concentrations of 0.2, 0.5, and 2.1 μM of sulfated lithocholates to give final concentrations of 0.2, 0.4, 0.8, and 1.6 μM. When recovery (observed value minus initial value) was plotted against amount added, there was excellent agreement (slope = 1.0; r = 0.9).

Normal values

Values for 50 normal subjects (40 males and 10 females) are summarized in **Fig. 4**. There was no difference related to sex. The mean (±SE) for this series was 1.56 ± 0.11 μM. In these 50 subjects, most of the immunoreactive lithocholate was sulfated.

DISCUSSION

Methodological problems

Synthesis of immunogen. In contrast to our findings with other bile acids (10, 11), coupling of sulfolitho-

TABLE 1. Relative binding of lithocholyl derivatives in radioimmunoassay

Bile Acid	Relative amount required to displace 50% of bound tracer[a]
Sulfolithocholylglycine	1
Sulfolithocholyltaurine	1
Sulfolithocholate	1
Lithocholate	>200
Lithocholylglycine	>200
Lithocholyltaurine	>1000

[a] The following bile acids required 1000 pmol to cause 50% displacement of bound tracer: cholate, cholylglycine, cholyltaurine, chenodeoxycholate, chenodeoxycholylglycine, chenodeoxycholyltaurine, deoxycholate, deoxycholylglycine, deoxycholyltaurine, and 3-sulfocholate. The bile acids used were of high purity; their source is reported in the accompanying paper (12).

cholylglycine by the carbodiimide method did not occur under the usual reaction conditions. We reasoned that the sulfate group might cause electrostatic repulsion and that this could be diminished, or the protein conformation could be altered, by using a nonaqueous solvent. Coupling was not achieved with a pyridine concentration of less than 75%. When the pyridine concentration exceeded 80%, irreversible protein precipitation occurred.

Production and properties of antiserum

Sensitivity and specificity. The least amount of added lithocholylglycine required to cause a significant decrease in the binding of tracer to antiserum was 10 pmol. The use of 0.1 ml of serum in the assay system provided a sensitivity of less than 0.1 μM, sufficient for accurate determinations of levels found in all 50 healthy subjects examined.

Not only the sulfated lithocholylglycine and sulfated lithocholyltaurine, but also the sulfate of unconjugated lithocholate, had equal affinities for the antiserum. Thus, the antibody measures sulfated lithocholate and sulfated lithocholyl conjugates. However, we think it likely that the concentration of sulfolithocholate (lithocholate sulfate or sulfated lithocholic acid) is negligible in plasma since *a*) all injected lithocholate is completely sulfated before excretion into bile (3, 4) and *b*) sulfated bile acids are reabsorbed poorly from the small intestine (4, 15, 16). Further, we found that sulfolithocholate, in contrast to other bile acids, was not fully conjugated during hepatic passage (3). If sulfolithocholate were absorbed from the intestine, it should appear as such in bile. Yet, if labeled lithocholate is given to healthy volunteers and the chemical form of radioactivity excreted in bile is assayed for several days, no sulfolithocholate radioactivity is observed (3, 4). Therefore, our assay probably measures sulfated lithocholyl conjugates and is therefore analagous to our other assays for cholyl conjugates (10) and chenodeoxycholyl conjugates (11). This speculation can be tested if antibodies are developed that are specific for sulfolithocholate or for sulfolithocholyl conjugates; for example, our antibody to cholyl conjugates showed much less affinity for unconjugated cholate (10).

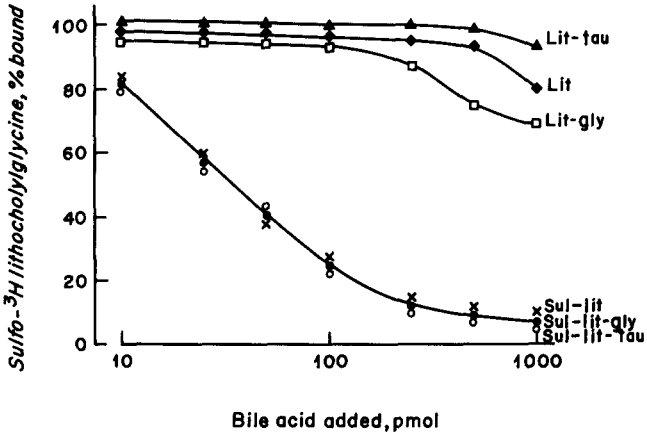

Fig. 2. Specificity of antiserum used for radioimmunoassay of sulfated lithocholates. Abbreviations: Lit-tau, lithocholyltaurine; lit, lithocholate; lit-gly, lithocholylglycine; sul-lit, sulfolithocholate; sul-lit-gly, sulfolithocholylglycine; sul-lit-tau, sulfolithocholyltaurine.

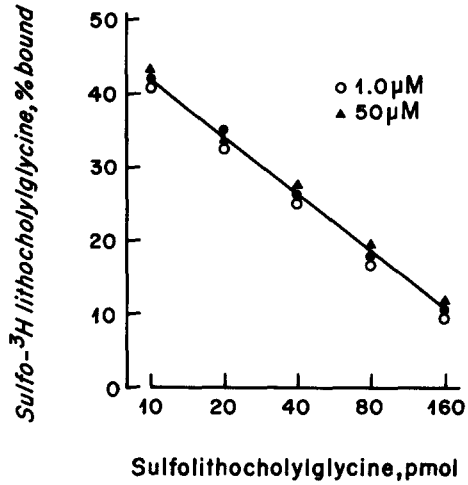

Fig. 3. Effect of dilution of two serum samples on binding of [^{3}H]-sulfolithocholylglycine. The serum sample with the original level of 50 μM was from a deeply jaundiced individual.

Accuracy. The absence of detectable levels in germ-free animals suggests that other steroidal compounds did not react with the antiserum. We also found no binding of other sulfated steroids which can occur in high concentrations under certain circumstances, such as pregnancy. Despite the above, the values obtained using this antibody are higher than those previously reported using gas–liquid chromatography, either not preceded by (17) or preceded by (18) a solvolysis step.

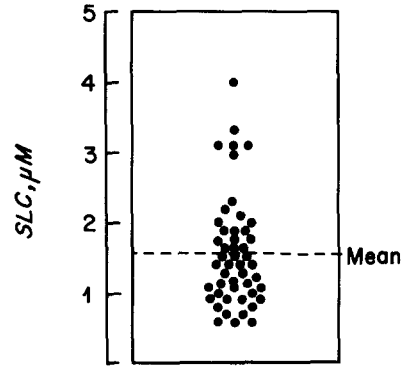

Fig. 4. Levels of sulfated lithocholates in 50 healthy fasting adult men and women, as determined by our radioimmunoassay.

After this work had been completed, a radioimmunoassay for sulfolithocholylglycine was reported by Demers and Hepner (19). The specificity of antiserum raised by these workers cannot be compared with ours, since they give no values for cross reactivity with sulfolithocholate or sulfolithocholyltaurine. With their radioimmunoassay, they found the average serum level of sulfated lithocholates to be 0.06 μM, which is an order of magnitude lower than that observed by us. We are unable to account for this discrepancy, but note that their, and not our, results are similar to those reported by other workers using gas–liquid chromatography (17, 18).

Radioimmunoassay vs gas–liquid chromatography

The present method can be carried out with 0.1 ml of serum, and one technician can assay 30 samples in 24 hr. Thus, the method is far more sensitive and far more rapid than gas–liquid chromatographic analysis. The method is subject to interference by any sulfated steroid that reacts with the antibody and, conversely, the assay will not detect sulfates of monohydroxy bile acids other than lithocholate that do not react with the antibody. Determination of sulfated lithocholates using inverse isotope dilution by coupled gas–liquid chromatography–mass spectrometry, with selective ion monitoring, should offer both specificity and sensitivity; but this technique is available in only a few laboratories and, as yet, the preparation of sulfated lithocholates containing stable isotopes has not been reported.

Potential clinical application

Recent studies have shown that about one-fifth of the lithocholate formed in the distal small intestine is absorbed (4), conjugated and sulfated during hepatic passage (3), and excreted in stool without extensive enterohepatic cycling (16). However, during intestinal transit, some lithocholate is liberated by bacterial deconjugation and desulfation, and this returns to the liver to again be conjugated and sulfated (16). Any defect in conjugation or sulfation could cause increased serum concentrations of lithocholate, which might initiate or perpetuate hepatic damage.

The most likely clinical application would be for the detection of impaired or absent sulfation of lithocholate, such as occurs in the rhesus monkey (20) and could occur as an inborn error of metabolism in man, since the concentration of secondary bile acids is extremely low in patients with cirrhosis (8) or cholestatic liver diseases (9, 21, 22). We think it unlikely that the assay will have clinical utility in such situations. Further, as noted, the high ratio of primary to secondary bile acids, together with the lack of ideal specificity for the antibody, raises the possibility that the radioimmunoassay might not give valid results. We have used the present radioimmunoassay to show that sulfation is unimpaired in patients receiving chenodeoxycholic acid for gallstone dissolution (23), despite such patients having a several-fold increase in lithocholate absorption (4).

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