

# Radioimmunoassay of unsulfated lithocholates

Alistair E. Cowen,<sup>1</sup> Melvyn G. Korman,<sup>2</sup> Alan F. Hofmann,<sup>3</sup> Jacqueline Turcotte,  
and Janet A. Carter

Gastroenterology Unit, Mayo Clinic and Mayo Foundation, Rochester, MN 55901

**Abstract** A simple, rapid, precise radioimmunoassay for total unsulfated species of lithocholate (lithocholyglycine, lithocholytaurine, and lithocholate) in serum is described. Antiserum was raised in rabbits by injection of lithocholyglycine coupled to bovine serum albumin (prepared by a carbodiimide method) and emulsified in complete Freund's adjuvant; antisera capable of measuring 40–120 pmol at 1:400 dilution were obtained. The tracer was [11,12-<sup>3</sup>H]lithocholyglycine. The radioimmunoassay featured a 2-hr binding step at 42°C and a 1-hr separation step using polyethylene glycol. The antibody had the following relative specificities: lithocholyglycine and lithocholytaurine, 1; lithocholate, 1.5; chenodeoxycholyglycine, 20; and deoxycholyglycine, 55. There was no binding of various other free or conjugated sulfated and unsulfated bile acids. The mean fasting-state level in 50 healthy subjects was 0.3  $\mu\text{mol/l}$  (0.14  $\mu\text{g/ml}$ ), but 11 of the 50 subjects had levels too low to measure by this technique.

**Supplementary key words** bile acids · lithocholic acid · serum bile acid levels

Lithocholic acid (lithocholate), which is formed in the distal intestine by bacterial 7 $\alpha$ -dehydroxylation of chenodeoxycholic acid (1), is a major fecal bile acid in man (2). Lithocholate is unequivocally hepatotoxic when fed to experimental animals (3), and this property has led to considerable speculation concerning its role in the initiation or perpetuation of various hepatic diseases (3, 4). These speculations have been re-emphasized recently because of the increasing use of chenodeoxycholic acid as a specific efficacious agent for the dissolution of cholesterol gallstones (5).

Several recent studies have shown that the metabolism of lithocholate is unique among the bile acids in man. 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 (6–8). Thus, conventional analyses of bile or serum may not provide accurate quantitation of the predominant chemical form of lithocholate, which is probably the sulfated conjugate (9, 10), in either of these fluids.

Lithocholate has been detected in serum of healthy

subjects and patients with liver disease (11–14). Despite this, the lack of sensitivity and the complexity of the methods have precluded systematic investigation of serum levels in man. Recently, Simmonds, et al. (15) developed a rapid, simple, valid radioimmunoassay for conjugates of cholic acid (choly conjugates). A subsequent study from this laboratory showed the apparent value of this technique for assessing disease activity in patients with chronic hepatitis (16), and for the development of a bile acid tolerance test (17, 18).

This paper describes a rapid, sensitive, simple radioimmunoassay developed for unsulfated species of lithocholate (unsulfated lithocholates) in serum. An accompanying paper describes a radioimmunoassay for sulfated lithocholates (19). Together, these two assays should enable measurement of total circulating lithocholate species in human serum.

## METHODS

### Tracer

[11,12-<sup>3</sup>H]Lithocholyglycine (glycolithocholic acid) was prepared by reductive tritiation of the glycine conjugate of the  $\Delta^{11-12}$ lithocholic acid, 3 $\alpha$ -hydroxy-chol-11,12-en-5 $\beta$ -oic acid (New England Nuclear, Boston, MA); specific activity was 0.5 Ci/mmol (20). Purity exceeded 98% when assessed by zonal scanning.

### Antisera

*Preparation of the immunogen, lithocholyglycine.* Lithocholic acid (Mann Research Laboratories, New York, NY) was recrystallized from methanol–water and conjugated with glycine methyl ester according to the

<sup>1</sup> Eli Lilly Fellow, Australia. Present address: Royal Brisbane Hospital, Brisbane, Australia.

<sup>2</sup> Eli Lilly Fellow, Australia. Present address: Prince Henry's Hospital, Department of Medicine, Monash University, Melbourne, Australia.

<sup>3</sup> To whom reprint requests should be addressed at Department of Medicine (8113), University Hospital, 225 West Dickinson Street, San Diego, CA 92103.

method of Norman (21). After adsorption chromatography on a column of silicic acid, the methyl ester was saponified to yield lithocholyglycine. The lithocholyglycine was dissolved in ethanol and converted to the tetrapropylammonium salt by alkanization with 1 N tetrapropylammonium hydroxide (Eastman Kodak, Rochester, NY).

*Coupling of lithocholyglycine to albumin.* The tetrapropylammonium salt of lithocholyglycine (10 mg) was dissolved in 1 ml of 0.1 N tetrapropylammonium phosphate buffer (pH 7.5) and mixed with 4 mg of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (Ott Chemical Company, Muskegon, MI) (22). Bovine serum albumin, 3 mg (Sigma Chemical Company, St. Louis, MO), was dissolved in 0.5 ml of 0.1 N tetrapropylammonium phosphate buffer, and this solution was added dropwise to the reaction mixture which was stirred magnetically. The pH was then adjusted to 7.5 and the mixture was stirred for 12 hr.

*Isolation of lithocholyglycine-albumin complex.* The lithocholyglycine-albumin complex was separated from unreacted lithocholyglycine by gel filtration on Bio-Gel P-10 (Bio-Rad, Richmond, CA). The complex was recovered in the void volume previously determined with blue dextran (Pharmacia Laboratories Inc., Piscataway, NJ). With a lithocholyglycine to albumin molar ratio of 25:1 to 30:1 in the reaction mixture, 10–12% of the bile acid was recovered as the lithocholyglycine-albumin complex. Radioactivity thus isolated could not be filtered through a membrane ultrafilter (Centriflo membrane ultrafilters, Amicon Corporation, Lexington, MA), nor did the addition of a 20-fold mass of lithocholyglycine prior to ultrafiltration displace the radioactivity from the lithocholyglycine-albumin complex.

### Immunization

Freshly prepared lithocholyglycine-albumin complex in 0.1 N tetrapropylammonium phosphate buffer was emulsified with an equal volume of Freund's complete adjuvant (Difco Laboratories, Detroit, MI); the final concentration was 100  $\mu$ g of lithocholyglycine and 0.4 mg of albumin per ml. One ml of this mixture was injected intracutaneously at multiple sites on the dorsal aspect of six New Zealand white rabbits. The antigen was injected at 2-week intervals for six doses and then at longer intervals according to the antibody titer.

Other rabbits were injected with the same complex with the addition of killed tubercle bacilli, together with additional administration of pertussis vaccine, with or without previous "priming" with 6-mercaptopurine (23). Assay for lithocholyglycine-binding anti-

body was begun after 3 months of immunization. Two rabbits (immunized by the initial method described above) gave serum of adequate titer (>40% binding of tracer at 1:400 dilution) for development and testing of a radioimmunoassay and continue to do so.

### Preparation of lithocholate-free serum

Lithocholates (both unsulfated and sulfated) were removed from serum by sequential extraction with charcoal and cholestyramine. Three hundred ml of serum was mixed with 6 g of wetted charcoal and incubated for 15 min; the larger particles were then sedimented by centrifugation and the serum was decanted. Cholestyramine (supplied by Dr. Herbert Sarett, Mead Johnson, Evansville, IN) was then mixed with the serum and shaken repeatedly over a 30-min interval. The serum was again centrifuged and then all traces of charcoal and cholestyramine were removed by Millipore filtration (Millex TN, Millipore, Bedford, MA). The method removed 97% or more of the [ $^3$ H]cholyglycine, [ $^3$ H]lithocholyglycine, and [ $^3$ H]sulfolithocholyglycine added in tracer amounts.

### Radioimmunoassay

*Stage 1. Binding of [ $^3$ H]lithocholyglycine to antibody.* Reaction mixtures were prepared containing the following: 0.1 ml of lithocholic acid-free human serum (diluted 1:2); 0.1 ml of [ $^3$ H]lithocholyglycine (approximately 1 pmol); 0.1 ml of unlabeled lithocholyglycine as standard or 0.1 ml of unknown serum for bile acid assay; 0.1 ml of antiserum (1:40 dilution); and buffer (potassium phosphate, pH 7.4) to a final volume of 1 ml. The components of the incubation mixture were diluted with or dissolved in the buffer. Incubation with antiserum was at 42°C for 2 hr; the tubes were then placed in a 4°C cold room for 30 min in preparation for the separation step.

*Stage 2. Separation of free and bound antigen.* Bound antigen was precipitated with polyethylene glycol (PEG; mol wt 6000) (24). To 1 ml of the reaction mixture, 0.5 ml of PEG solution (37.5 g/100 ml) was added to give a final PEG concentration of 12.5%. After the reaction mixture was allowed to stand for 15 min, the precipitated  $\gamma$ -globulin, including bound antigen, was separated by centrifugation at 1,200 g at 4°C for 30 min. The supernate, containing free [ $^3$ H]lithocholyglycine, was decanted into a scintillation vial and 14 ml of a toluene-detergent-scintillant solution (Ready-Solv, Beckman, Fullerton, CA) was added along with two drops of a 10% ascorbic acid solution. Radioactivity was measured in a Beck-

man LS-250 liquid scintillation counter; the statistical error of counting usually was  $\pm 3\%$ .

In the absence of antibody, 95% or more of the added [ $^3\text{H}$ ]lithocholyglycine was recovered in the supernate after addition of PEG. Thus, nonspecific binding and entrapment in the precipitate was small. They were not affected by doubling the concentration of bile acid-free human 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 6% or less.

### Calculation 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 lithocholyglycine was normalized to 100%.

Results for serum analysis are expressed as  $\mu\text{mol}$  of unsulfated lithocholates per liter of serum; unsulfated lithocholyglycine and unsulfated lithocholytaurine reacted equally with the antibody. Unfortunately, unconjugated (and unsulfated) lithocholate also reacted with the antibody, although its affinity was less than those of the lithocholy conjugates. Although it seems likely that the concentration of unconjugated (unsulfated) lithocholate in serum is quite low and that the values obtained with radioimmunoassay would be virtually identical to those obtained with an antibody that was completely specific for unsulfated conjugates of lithocholate, we have elected to term our values "unsulfated lithocholates". Obviously, the value measured by the radioimmunoassay will be a slight underestimate if the serum contains an appreciable concentration of unsulfated, unconjugated lithocholate.

## RESULTS

Antiserum from one rabbit (antiserum 4463) was used for characterizing the antibody and validating the final assay procedure. Antiserum from one other rabbit showed similar potency, specificity, and affinity (data not presented). Antibody appearance, titer, and specificity did not seem to be enhanced by the addition of killed tubercle bacilli, by simultaneous administration of pertussis vaccine, or by a 7-day treatment period with 6-mercaptopurine ceasing 10 days before the start of immunization.

### Binding of lithocholyglycine

Approximately 40% of tracer [ $^3\text{H}$ ]lithocholyglycine was bound by a 1:400 dilution of antiserum. Binding decreased linearly with dilution, on a logarithmic scale, from 1:400 to 1:1600. Percentage binding of tracer decreased linearly with a logarithmic increase in unlabeled lithocholyglycine concentration from 20 to 160 pmol (**Fig. 1**).

### Specificity

The relative binding affinities of lithocholate derivatives are shown in **Fig. 2**, and those of chenodeoxycholyglycine and deoxycholyglycine are shown in **Fig. 3**. The relative amounts of major bile acids and their conjugates required to displace 50% of the bound [ $^3\text{H}$ ]lithocholyglycine are shown in **Table 1**. Cortisol, estrogen, progesterone, testosterone, and cholesterol did not influence the binding of [ $^3\text{H}$ ]lithocholyglycine to antiserum. Serum from male and female germ-free rats and mice caused no significant displacement of tracer from antibody.

### Validation. Assay of human serum

Several dilutions of serum were assayed in 0.1 ml volumes. Displacement curves with serial dilutions of human sera (1  $\mu\text{M}$  and 50  $\mu\text{M}$ , respectively) were parallel with the lithocholyglycine displacement curve. Thus, the estimated bile acid value for whole serum was independent of its dilution in the assay system. The coefficient of variation was 3% within assays and 9% between assays.

Recovery experiments do not differ in principle from the standard curve (**Fig. 1**) which shows log-linear displacement of tracer between 40 and 120 pmol/assay tube.

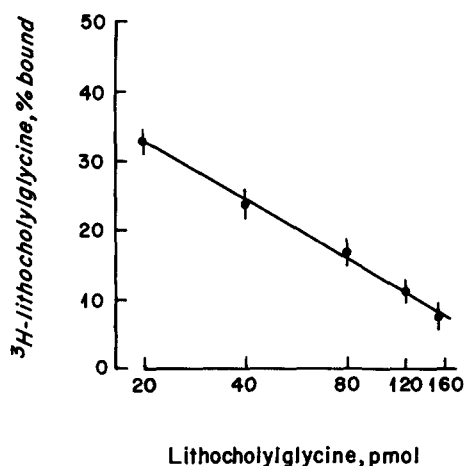
### Normal fasting-state values

Values for 50 normal subjects (40 male and 10 female) are summarized in **Fig. 4**. There was no obvious difference related to sex. The mean ( $\pm\text{SE}$ ) for this series was  $0.25 \pm 0.019 \mu\text{mol/l}$  of serum.

## DISCUSSION

### Sensitivity

The least amount of added lithocholyglycine required to cause a significant decrease in the binding of tracer to antiserum was 20 pmol. The use of 0.1 ml of serum in the assay system provides a sensitivity of 0.15–0.2  $\mu\text{mol}$ . Thus, the limit of sensitivity is not ideal because 11 of 50 normal subjects had values

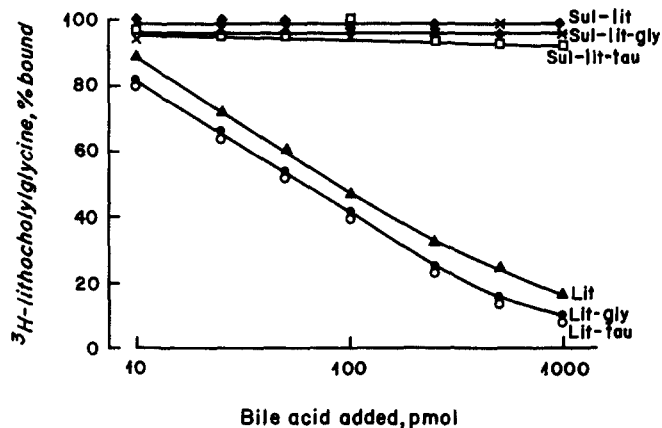


**Fig. 1.** Displacement of bound [<sup>3</sup>H]lithocholyglycine added mass of nonradioactive lithocholyglycine. Percent binding (mean  $\pm$  SE) is plotted against the mass of added lithocholyglycine on a logarithmic scale.

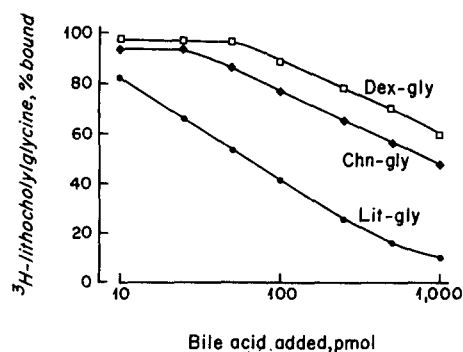
below this level; however, increased levels were clearly recognized.

### Validation

In an assay system developed primarily because of the difficulty and uncertainty of chemical methods of estimation, cross validation is difficult. It may be that we should term our measurements "immunoreactive". The measurement of serum bile acids by gas-liquid chromatography is complex, and the measurement of lithocholate is subject to a number of additional methodological problems because in bile and serum the lithocholate probably occurs predominantly as



**Fig. 2.** Specificity of antiserum used for radioimmunoassay of unsulfated lithocholates. Antibody binds lithocholyglycine (lit-gly) and lithocholytaurine (lit-tau) equally and also has some affinity for unsulfated unconjugated lithocholate (lit). Sulfated derivatives of lithocholate show no binding. Abbreviations: Sul-lit, sulfolithocholate; Sul-lit-gly, sulfolithocholyglycine; Sul-lit-tau, sulfolithocholytaurine.



**Fig. 3.** Specificity of antiserum used for radioimmunoassay of unsulfated lithocholates for glycine conjugates of dihydroxy bile acids. The antiserum has some affinity for chenodeoxycholyglycine (chn-gly) and deoxycholyglycine (dex-gly). See Table 1.

sulfated derivatives, and these are unlikely to be estimated validly without a solvolysis step. Thus, many of the reported methods for the estimation of serum bile acids are likely to have resulted in under-

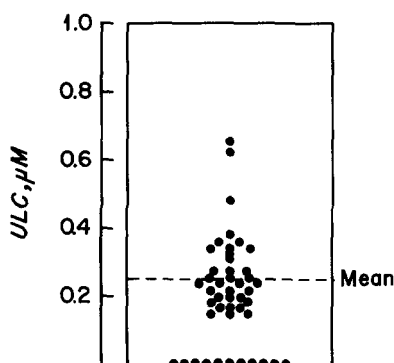
TABLE 1. Relative affinity of bile acids for antiserum to unsulfated lithocholyl conjugates

Bile Acid	Source	Relative amount required to displace 50% of bound tracer
Unsulfated monohydroxy bile acids		
Lithocholyglycine	a	1
Lithocholytaurine	a	1
Lithocholic acid	b	1.5
Isolithocholic acid	c	>10,000
5 $\alpha$ -Isolithocholic acid	c	>10,000
5 $\alpha$ -Lithocholic acid	b	>10,000
Sulfated monohydroxy bile acids		
Sulfolithocholyglycine	d	>10,000
Sulfolithocholytaurine	d	>10,000
Sulfolithocholic acid	d	>1,000
Unsulfated dihydroxy bile acids		
Chenodeoxycholyglycine	a	20
Chenodeoxycholytaurine	a	>10,000
Chenodeoxycholic acid	e	>10,000
Deoxycholyglycine	a	55
Deoxycholytaurine	f	>10,000
Deoxycholic acid	f	>10,000
Unsulfated trihydroxy bile acids		
Cholyglycine	a	>10,000
Cholytaurine	a	>10,000
Cholic acid	g	>10,000

The bile acids used in these experiments were all above 95% pure by thin-layer chromatography. The source of each is as follows:

- Synthesized by us using the mixed carboxylic carbonic anhydride method of Norman (21), with subsequent purification as described (29,30).
- Purchased from Calbiochem, La Jolla, California.
- Purchased from Steraloids, New Pawling, New York.
- Conjugated with glycine or taurine by the method of Norman (21) and then sulfated by the method of Palmer and Bolt (6).
- Donated by Diamalt GmbH, Redenfelden, Germany.
- Purchased from T. Schuchardt, Munich, Germany.
- Purchased from Aldrich Chemicals, Milwaukee, Wisconsin.





**Fig. 4.** Unsulfated lithocholate (ULC) concentrations in 50 healthy subjects. The mean includes all samples, even though the distribution appears to be bimodal in that 11 of the 50 healthy subjects had levels too low to displace significant tracer from the antibody.

estimation of lithocholate levels. The values obtained in our assay are higher than those obtained by some investigators using gas-liquid chromatography (10, 25, 26) but are comparable with those of others using apparently valid methods (12). The assay system behaves in a valid manner as determined by the usual criteria. In addition, levels of unsulfated lithocholates were too low to detect in serum of germ-free rats, providing additional evidence that the reactive substances measured in this assay were steroids produced by microbial action.

The only significant cross-reaction with bile acids normally present in serum was a 1:1.5 reaction with unsulfated, unconjugated lithocholate. The next closest cross-reaction was with chenodeoxycholyglycine which required 20  $\mu\text{mol}$  to register as 1  $\mu\text{mol}$  of lithocholate in the present assay. Thus, it is unlikely that other bile acids normally present in serum contributed to the levels of lithocholate found in healthy subjects. However, in severe liver disease, very high levels of chenodeoxycholate have been reported (13, 14) and, because this bile acid shows some affinity for the antibody, it could preclude accurate measurement of unsulfated lithocholates, especially since the concentrations of all secondary bile acids are often extremely low in patients with hepatobiliary disease.

We did not detect cross-reaction or nonspecific binding with other components of serum. The slope of displacement of [ $^3\text{H}$ ]lithocholyglycine by serial dilutions of human serum was identical to that for unlabeled lithocholyglycine. These findings indicate that the components of serum other than bile acids did not greatly influence this assay.

#### Methodological problems


**Solubility.** Sodium and potassium salts of lithocholyglycine have low solubilities in aqueous solu-

tions (27, 28). To maintain an aqueous system throughout the conjugation procedure, it was necessary to find a soluble salt. A systematic investigation of salts revealed that the quaternary ammonium salts were the most suitable (27) and that the tetrapropylammonium derivative was the shortest chain length providing adequate solubility for use in the assay procedure.

**Tracer.** The reduction of  $\Delta^{11-12}$ lithocholic acid, which is readily synthesized (20), provides a simple and inexpensive method of obtaining tracer of high specific activity.

**Bile acid-free serum.** The method of charcoal extraction previously described (15) did not remove all lithocholate derivatives from serum. However, the combination of charcoal extraction followed by cholestyramine extraction resulted in the removal of more than 96% of the lithocholate derivatives (including sulfates).

#### Future application

The methods described in this paper, together with a radioimmunoassay for sulfated lithocholates described in the accompanying paper (19) should now permit measurement of these two classes of bile acids in serum in health and in some instances of liver disease. The potential clinical application of these methods is discussed in the accompanying paper (19). 

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