Results with Six "Kit" Radioimmunoassays for Primary Bile Acids in Human Serum Intercompared

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We examined six radioimmunoassay procedures for measuring primary bile acids in human serum (two 3H-labeled and four 125I-labeled). A significant (p < 0.01) correlation was observed between measurements in the assay both for cholic acid and chenodeoxycholic acid, at low and high concentrations of serum bile acids. All kits were acceptable with respect to accuracy, precision, stability, and analytical recovery. All six procedures gave similar results for chenodeoxycholic and cholic acid in sera of 80 healthy subjects; the agreement was also close when the two primary bile acids were compared with their sum in serum. Normal values ranged from 0.4 to 2.5 μmol/L for conjugated chenodeoxycholic acid and from 0.3 to 1.5 μmol/L for conjugated cholic acid. The 125I assays do not require liquid-scintillation equipment but 125I induces a decrease in the affinity constant of antibody. The sensitivity of the assays was still adequate for measuring bile acids in the serum of healthy fasting persons and liver-disease patients.

Additional Keyphrases: cholic acid • chenodeoxycholic acid • reference intervals

Extensive studies on bile acid concentrations in serum are not yet available, because the usually used technique—gas chromatography (1-3)—is laborious. At present, the diagnostic significance of information on bile acid concentration in serum is still uncertain (4, 5), but many authors have proposed that such information is an index to liver function (1-10).

The recent development of radioimmunoassays (RIA)2 for bile acids in serum (11-19) allows their more convenient determination; however, RIA techniques require standardization and validation before values from different sources can be compared. Tritiated bile acids were first used as tracers (11), but recently 125I has been introduced (18), to avoid liquid scintillation counting and to improve the specific activity of the tracer. Those authors found that the antibody titer increased 50- to 500-fold over that used in the tritium assay but sensitivity was maintained (18).

Methods proposed by different authors differ with respect to labeled antigen, antibody specificity, whether or not bile acids are extracted from the serum sample, and the separation of free and antibody-bound antigen.

Here we compare two tritiated RIAs, specific for cholic (CCA) and chenodeoxycholic (CCDCA) acid conjugates, developed in our laboratory and validated by us (15), in terms of precision, sensitivity, specificity, accuracy, and analytical recovery, with four commercially available 125I radioimmunoassays: two for CCA, one for CCDCA, and one for primary bile acids (cholic and chenodeoxycholic acid).

We also discuss the application of such techniques to a selected population of healthy adult subjects.

Materials and Methods

125I Procedure

The following bile acid RIA kits were either purchased or obtained by donation:
1. Cholylglycine (RIA) (polyethylene glycol separation) Diagnostic Kit (Abbott Labs., North Chicago, IL 60064).
2. Glycocholic acid RIA-Kit (Nordic Lab, OY SF 90100 Oulu, Finland).
3. Glycochenodeoxycholic acid RIA-Kit (Nordic Lab).
4. Conjugated Bile Acid Solid-Phase Radioimmunoassay Kit 125I (Becton Dickinson, Orangeburg, NY 10962).

These kits were used according to the manufacturers' instructions.

3H Procedure

CCA and CCDCA were determined as we reported earlier (15). Briefly: [3H]glycocholic acid (spec. acty. = 5 kCi/mol) and [3H]glycochenodeoxycholic acid (spec. acty. = 5 kCi/mol), both from New England Nuclear Corp., Boston, MA 01608, were used as tracers. The unlabeled glyco-conjugated bile acids (Calbiochem, La Jolla, CA 92037) were coupled to bovine serum albumin by the carbodiimide reaction (11).

Antisera produced in New Zealand White rabbits were diluted 1000- and 20000-fold in the final dilution for CCA and CCDCA assays, both giving 50% binding when 0.5 pmol of labeled antigen was used in each tube. Bound antigen was precipitated with a saturated solution of ammonium sulfate. Each antiserum was found to be highly specific for each glycine- and taurine-conjugated bile acid. Studies on cross-reaction, parallelism, and recovery, to access reproducibility, precision, and accuracy, have been reported (15). The RIA procedure was the same as in that paper, except that a final volume of 0.4 mL/tube was used instead of 1.0 mL, to make our assay conditions as comparable as possible to the commercial ones.

Table 1 summarizes the above procedures.

Sample Preparation

The assay developed in our laboratory was performed directly on serum samples, without previous extraction of bile acids; results of studies performed on the same sera with and without extraction of bile acids with ethanol (0.1 mL of serum plus 0.9 mL of ethanol) were closely related (unpublished data). In addition, a comparison of CCA and CCDCA values determined with gas-liquid chromatography (20) validated the direct RIA assay, showing that an extraction step was not required. This may be due both to the high affinity constant of our antisera (Table 2) and to the weak binding of bile acids with proteins (11).

The Nordic Lab methods include a preliminary extraction of bile acid from the serum. The Abbott method is direct but includes the use of anilinonaphthalene sulfonic acid to break
Table 1. Characteristics of the Six Kits

<table>
<thead>
<tr>
<th>Kit</th>
<th>Acts directly on serum</th>
<th>Sample vol, μL</th>
<th>No. of pipetting steps</th>
<th>Sepn. of bound/free</th>
</tr>
</thead>
<tbody>
<tr>
<td>3H-RIA (CCA, CCDC)</td>
<td>Yes</td>
<td>10</td>
<td>4</td>
<td>(NH₄)₂SO₄</td>
</tr>
<tr>
<td>¹²⁵I, Nordic (CCA, CCDC)</td>
<td>No</td>
<td>20</td>
<td>6</td>
<td>PEG</td>
</tr>
<tr>
<td>¹²⁵I, Becton-Dickinson</td>
<td>Yes</td>
<td>25</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>¹²⁵I, Abbott</td>
<td>Yes</td>
<td>25</td>
<td>4</td>
<td>PEG</td>
</tr>
</tbody>
</table>

* Solid phase, separation not required. PEG, polyethylene glycol.

the bile acid/protein binding. The Becton Dickinson method is direct (antibodies coated on the tubes).

**Instruments**

For the ³H-RIA, the radioactivity of the free count (supernate) was counted with a liquid scintillation spectrometer (Analytic Isocap 300; Searle Analytic Inc., now Tracer Analytic, Elk Village, IL 60007) after the addition of 15 mL of "Unisolve" (Koch-Light Lab., Ltd., England) as a scintillation cocktail.

The ¹²⁵I bound count was counted with a gamma spectrometer (Packard Instrument Co., Downers Grove, IL 60515).

**Calculation**

Antibody binding was expressed as a logit of B/B₀ (the amount relative to the amount bound for zero dose) vs log dose. The dose-response curve has been described by Rodbard as a four-parameter logit function (21). The best sigmoidal curve was computed by the least-squares method, and the amount of each bile acid was interpolated automatically by use of a computer program.

Final results were expressed in micromoles per liter. From the standard curve data, the affinity constant and the concentration of the antisera were calculated by plotting the bound/free ratio (B/F) vs the concentration of free antigen (F), a Scatchard plot.

**Subjects**

Using both the ³H and ¹²⁵I procedure, we studied 25 overnight-fasting subjects, including both normal subjects and hepatobiliary disease patients, to have a wide range of serum bile acid concentration.

In addition, better to define the normal serum primary bile acid values, we evaluated cholic and chenodeoxycholic acid concentrations in sera from 80 healthy fasting adults (40 men and 40 women), in whom any liver or intestinal disease had been previously excluded by means of conventional biochemical and roentgenographic examinations.

**Results**

**Standard Curves**

Figures 1–3 show the six standard curves for cholic acid and chenodeoxycholic acid assays. The percent bound normalized at zero dose (logit scale) is plotted vs the different standard antigens, expressed as picomoles per tube, to compare the different techniques. Each point represents the mean and SD of five determinations.

Table 2 shows logit parameters (a = % response for zero dose; b = slope factor; c = midrange, i.e., the effective dose for 50% dose-response, in picomoles per tube; and d = % response extrapolated to infinite dose) for each of the six assays. The affinity constant for, and concentration of, each antibody are reported in the same table.

**Analytical Variables**

**Precision.** Values for three serum pools, with high, medium,
and low bile acid concentrations, were established by gas chromatography (20). Ten aliquots of each pool sera were diluted in an assay and these data were used to assess intra-assay variance (Table 3). These pools were used as quality controls for subsequent routine assays. The inter-assay variance found for the pools is also shown in Table 3.

Analytical recovery for 10 samples of normal serum, to which known amounts of cholyglycine and chenodeoxycholyglycine were added, ranged from 90 to 109% for all the methods assessed; parallelism was assessed by use of a sample with high bile acid concentration, diluted several fold. The

![Fig. 2](image-url) Standard radioimmunoassay curves for two chenodeoxycholic acid methods compared
Each point represents the mean (2 SD shown) of five determinations. A. $^3$H-RIA; B, $^{125}$I-RIA, Nordic Lab Kit

![Fig. 3](image-url) Standard radioimmunoassay curve for Becton Dickinson primary bile acid method
Each point represents the mean (2 SD shown) of five determinations

![Fig. 4](image-url) Relationship between chenodeoxycholic acid concentrations in serum as measured by the $^3$H-RIA method and $^{125}$I Nordic Lab methods
Dotted line, theoretical line of identity

plot of standard dilution vs tracer binding paralleled the standard curves for all the assays used.

Specificity. Table 4 illustrates the specificity of the various antisera as reported by the different manufacturers; the cross reactivity values are greatly heterogeneous. The $^3$H-RIA methods we developed are extremely specific for their corresponding conjugated bile acid; they give the same response for the glyco- and tauro-conjugated forms, whereas the cross reactivity with the corresponding free bile acid is very low.

The $^{125}$I Nordic Lab kit for cholic acid, on the contrary, measures both free and glyco-tauro conjugated bile acids; the $^{125}$I Nordic Lab kit for chenodeoxycholic acid measures free and conjugated bile acids, but shows a relatively low cross reactivity with taurochenodeoxycholic acid (50% less). The $^{125}$I CG Abbott kit measures glycocholic acid, but with a consistent cross reactivity for taurocholic acid (16.5%) and glycochenodeoxycholic acid (14.5%). Finally, the $^{125}$I Becton Dickinson kit for the four primary bile acids presents an enormous difference between the glyco- and tauro-conjugated forms; the cross reaction for glycocholic acid is 50% less with respect to the corresponding taurocholic acid (Table 4).

### Table 3. Precision of Replicate and Sample-to-Sample Assays of the Six Procedures Compared

<table>
<thead>
<tr>
<th>Method</th>
<th>Low concn. pool</th>
<th>Medium concn. pool</th>
<th>High concn. pool</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Inter-batch</td>
<td>Intra-batch</td>
<td>Inter-batch</td>
</tr>
<tr>
<td>$^3$H-CCA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$^3$H-CCDA</td>
<td>0.45 7.2</td>
<td>0.48 7.6</td>
<td>1.5 7.4</td>
</tr>
<tr>
<td>$^{125}$I-Abbott cholyglycine</td>
<td>0.40 7.8</td>
<td>0.44 8.1</td>
<td>1.4 7.8</td>
</tr>
<tr>
<td>$^{125}$I-Nordic Lab cholic acid</td>
<td>0.38 8.5</td>
<td>0.42 9.0</td>
<td>1.2 8.5</td>
</tr>
<tr>
<td>$^3$H-CCDCA</td>
<td>0.68 7.0</td>
<td>0.72 7.8</td>
<td>2.4 9.0</td>
</tr>
<tr>
<td>$^{125}$I-Nordic Lab chenodeoxycholic acid</td>
<td>0.55 8.1</td>
<td>0.60 9.2</td>
<td>2.1 10.0</td>
</tr>
<tr>
<td>$^{125}$I-Becton Dickinson</td>
<td>1.25 10.1</td>
<td>1.45 16.5</td>
<td>4.2 7.5</td>
</tr>
</tbody>
</table>

**Intermethod Comparison**
Serum samples from 25 fasting subjects were assayed for
Table 4. Relative Cross Reactivity of Different Bile Acids with the Six Antisera

<table>
<thead>
<tr>
<th>Bile acid</th>
<th>% cross reactivity with antisera of method</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Chenodeoxycholylglycine</td>
<td>0.1</td>
</tr>
<tr>
<td>Chenodeoxycholyltaurine</td>
<td>—</td>
</tr>
<tr>
<td>Cholic acid</td>
<td>100</td>
</tr>
<tr>
<td>Cholyltaurine</td>
<td>100</td>
</tr>
<tr>
<td>Deoxycholylglycine</td>
<td>1</td>
</tr>
<tr>
<td>Deoxycholyltaurine</td>
<td>—</td>
</tr>
<tr>
<td>Lithocholylglycine</td>
<td>0.1</td>
</tr>
<tr>
<td>Lithocholyltaurine</td>
<td>—</td>
</tr>
<tr>
<td>Ursodeoxycholylglycine</td>
<td>—</td>
</tr>
<tr>
<td>Ursodeoxycholyltaurine</td>
<td>—</td>
</tr>
<tr>
<td>Chenodeoxycholic acid</td>
<td>2</td>
</tr>
<tr>
<td>Cholic acid</td>
<td>6</td>
</tr>
<tr>
<td>Deoxycholic acid</td>
<td>0.5</td>
</tr>
<tr>
<td>Lithocholic acid</td>
<td>—</td>
</tr>
<tr>
<td>Ursodeoxycholic acid</td>
<td>—</td>
</tr>
<tr>
<td>Sulfated bile acids</td>
<td>—</td>
</tr>
</tbody>
</table>

1. $^3$H CCA; 2. $^{125}$I Abbott cholylglycine; 3. $^{125}$I Nordic Lab cholic acid; 4. $^3$H CCDCA; 5. $^{125}$I Nordic Lab chenodeoxycholic acid; 6. $^{125}$I Becton Dickinson bile acids.

CCA and CCDCA by both the $^3$H-RIA and the two $^{125}$I-RIA procedures. As indicated in Figures 4–6, CCA and CCDCA determined by $^3$H-RIA procedures was highly correlated with the corresponding concentrations in serum by the $^{125}$I methods ($r > 0.9$).

The slope and the coefficient of the regression line, reported in the same figures, indicate close agreement of the four methods; any difference may be ascribed to the different specificity of the antisera. The correlation between $^3$H-RIA and $^{125}$I (Becton Dickinson) RIA was obtained by plotting the sum of CCDCA and CCA, measured independently with the $^3$H-RIA; this correlation was not as close as those reported above.

Purity. The reagents in the Nordic Lab kits are in a lyophilized form; those in the other kits are in solution, ready for use. We assessed the purity of the tracer by measuring the radiochemical purity by scanning zones of thin-layer chromatograms on silica gel plates developed with benzene/ethanol/acetic acid (60/30/1 by vol), both on a newly acquired kit and on one at its expiration date. All the tracers were more than 95% pure.

Bile acids in the serum of healthy subjects. Table 5 shows values for primary bile acids in sera from a normal adult population. These data show no significant sex-related differences. Moreover, the values given by the different methods are in close agreement with the mean normal values; the $^{125}$I Nordic Lab chenodeoxycholic acid kit, however, gives a small underestimation. Other authors (25) have reported mean normal chenodeoxycholic acid values quite similar to those we obtained with our $^3$HCCDCA method, even though they used independent methods.

On the other hand, the Becton Dickinson method seems to overestimate, when the data on combined primary bile acids in serum are compared with the separate determination of the two.

Discussion

Our aim was to compare results obtained with some different commercially available kits for serum bile acid determination, to facilitate standardization of normal values and experimental procedures. Although serum bile acid determinations have been proposed as a sensitive index of liver function, there are still discrepancies among results by the different techniques used, in terms of the serum bile acid values in physiological and pathological conditions.

The Abbott, Nordic Lab, and Becton Dickinson procedures are quite similar, differing only in the separation step. The sensitivity of these three methods (evaluated by using the midrange of the standard curves) is about 20 pmol/tube. The Nordic Lab kits have the enormous disadvantage that there must be a preliminary extraction of bile acids with ethanol; this extraction is important, as reported by the authors (22), particularly for sera with relatively high bile acid concentrations, for which the serum sample must be more diluted. The

Table 5. Comparison Between the Mean Values by the Different Assays of Serum Primary Bile Acids

<table>
<thead>
<tr>
<th>Method</th>
<th>Women (n = 40)</th>
<th>Men (n = 40)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>$^3$H CCA</td>
<td>0.55</td>
<td>0.25</td>
</tr>
<tr>
<td>$^{125}$I-Abbott cholylglycine</td>
<td>0.68</td>
<td>0.40</td>
</tr>
<tr>
<td>$^{125}$I-Nordic Lab cholic acid</td>
<td>0.70</td>
<td>0.30</td>
</tr>
<tr>
<td>$^3$H CCDCA</td>
<td>1.10</td>
<td>0.80</td>
</tr>
<tr>
<td>$^{125}$I-Nordic Lab chenodeoxycholic acid</td>
<td>0.88</td>
<td>0.65</td>
</tr>
<tr>
<td>$^{125}$I-Becton Dickinson</td>
<td>2.40</td>
<td>2.00</td>
</tr>
</tbody>
</table>
Becton Dickinson kit is faster, because it does not require bound/free separation (antibody is coated on the wall of the tube); the Abbott kit is direct but includes a separation of bound and free antigen with polyethylene glycol.

The $^3\text{H}$-RIA method, even though involving a labeled antigen with a lower specific activity than in the commercial kits, is more sensitive (2–5 pmol/tube). This can be explained both by a higher affinity constant of the antibody used (Table 3) and by the loss of immunoreactivity of the histamine bile acid derivatives used in the $^{125}\text{I}$-conjugation. On the other hand, the use of $^{125}\text{I}$-labeled antigen provides the advantage that antibody consumption is reduced and no liquid-scintillation equipment is required, so that the assay cost is less. The precision of the methods and the linearity and analytical recovery are maintained within acceptable limits.

The specificity of the kits we compared is extremely variable. The Abbott kit should measure only cholyglycine, but there is a substantial cross reactivity with glycochenodeoxycholic (14.5%) and taurocholic (16.5%) acids. This gives a 20–30% overestimate for a serum sample from a normal subject with a glycochenodeoxycholic and taurocholic acid content of 1 and 0.2 µmol/L, respectively. The $^{125}\text{I}$ Nordic Lab kit for chenodeoxycholic acid underestimates taurochenodeoxycholic acid by 50%, whereas the Becton Dickinson kit for primary bile acids underestimates cholyglycine by 59% with respect to the other primary bile acids.

One must also consider the different response between the glyco- and tauro-conjugated forms. In childhood there may be a preferential conjugation with taurine over glycine (23). Furthermore, in patients with bile acid malabsorption, the serum becomes enriched during the day with the glyco-conjugated forms (24). Despite the heterogeneity of the antibodies compared, we noted a good correlation for both high and low values as measured by the different kits. To improve the quality of the assay, it will be necessary to develop new antigens in which the conjugating protein does not mask the specific bile acid function, such as the amino acid side chain and the 3,7,12-hydroxy groups; only in this way it will be possible to produce specific antibodies for an individual bile acid.

We emphasize that the Nordic Lab and Becton Dickinson kits do not include information regarding the cross reactivity with sulfated bile acids, which can be as much as 15% of the total bile acids in normal subjects and 70% in patients with cholestasis (7).

All the kits seem to provide reliable results; the insignificant differences in the mean values are probably accounted for by the different specificity of the antisera used.

Therefore, values ranging from 0.4 to 2.5 µmol/L for conjugated chenodeoxycholic acid and from 0.3 to 1.5 µmol/L for conjugated cholic acid can be considered normal.

In conclusion, all these commercially available methods are suitable for measuring serum bile acids. Use of $^{125}\text{I}$ tracer has remarkably simplified the method and decreased costs without, however, increasing sensitivity with respect to that of the corresponding $^3\text{H}$ methods.

The purity of the $^{125}\text{I}$-labeled antigens is satisfactory, as is also their specific activity as indirectly measured by reaction with our antisera previously titrated with a $^3\text{H}$-labeled bile acid for which the specific activity is well known (5 kCi/mol).
For all 125I tracers assessed, the antibody titer increased by about 30- to 50-fold (unpublished data).

With respect to which bile acid is most useful in liver disease diagnosis—i.e., only one primary bile acid, the sum of them, or other bile acids—this problem is beyond the scope of this paper, but it will be ascertained from extensive application of these methods to hepatobiliary disease.

References