Development, Validation, and Application of a Single-Tube Radioimmunoassay for Cholic and Chenodeoxycholic Conjugated Bile Acids in Human Serum

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A single-tube radioimmunoassay for both cholic acid and chenodeoxycholic acid conjugates in serum is based on the use of [1-14C]glycocholic acid and [U-3H]glycochenodeoxycholic acid as tracers. The assay was shown to be specific, sensitive, accurate, and precise (CV = 13% at low concentrations, 5.5% at higher concentrations) when used for serum samples from subjects and patients with increased bile acid in the serum because of liver disease, and results correlate well with those by gas chromatography (r = .99).

Additional Keyphrases: diagnosis of hepatobiliary disease · gas chromatography compared · liver function · relation to bilirubin concentration and γ-glutamyltransferase activity · normal values

Several techniques have been developed for measuring bile acids in serum (1-4), the most recent and sensitive being radioimmunoassay (RIA), originally developed by Simmonds et al. (5) and later by others (6-10). RIA is considerably more sensitive than gas-liquid chromatography or enzymatic determination, and is much simpler than most gas-chromatographic techniques. However, radioimmunoassay only provides information on the bile acid (or class of bile acids) detected by the particular antiserum used. In the diagnosis of hepatobiliary disease, it might be useful to be able to measure the concentrations of the two principal bile acid conjugates simultaneously.

These are the predominant bile acids present in serum in normal persons and in cases of liver disease, and the ratio of the two classes of bile acid conjugates has been considered by some authors to have diagnostic (11-13) or prognostic (14, 15) significance.

We have developed highly specific antisera by immunizing rabbits with cholyglycine and chenodyoxycholyglycine covalently coupled to bovine serum albumin. The acquisition of these antibodies and the commercial availability of a 14C tracer for cholic acid conjugates enabled us to develop a single-tube RIA for these classes of conjugated bile acids.

In this paper we report the development, validation, and application of this method to healthy subjects and to patients with liver disease. A correlation with the conventional liver-function tests is then reported.

Materials and Methods

Reagents

All reagents were of analytical grade, obtained from usual commercial sources.

Potassium phosphate buffer (pH = 7.4 at 22 °C) concentration was 10 mmol/liter.

Serum free of bile acids was prepared according to the method of Simmonds et al. (5), which provided a serum 99% free from bile acids. [1-14C]Glycocholic acid and [U-3H]glycochenodeoxycholic acid were used as markers of their complete extraction.

(NH4)2SO4; saturated solution.


Preparation of Antigen and Antibody

Four New Zealand rabbits were immunized with chenodeoxycholyglycine and four with cholyglycine during five months. The carbodiimide method was applied in order to couple bile acids with proteins (5). The molar ratio albumin/bile acid was 1/10 to 1/15 for both of the bile acids.

The removal of free bile acids has proved to be useless. Each antigen was emulsified with Freund’s complete adjuvant (1/1 by vol) and injected subcutaneously in five areas of the animals’ backs, according to Simmonds et al. (5). Subsequent injections of the same dose were repeated weekly.

Twice a month, 5 ml of blood was drawn by cardiac...
puncture. The serum thus obtained was sterilized by passage through a 0.22-μm (av pore diameter) filter (Millipore Corp, Bedford, Mass. 01730), lyophilized, and stored at 4 °C. Under such conditions, antisera were stable for at least a year; reconstitution with phosphate buffer did not significantly alter the titer.

Antisera were used without any further purification.

Antisera for cholyglycine and chenodeoxycholylglycine provided about 40% bound (B/T) when respectively diluted 300- and 500-fold with buffer.

Other Procedures

Tracers: [1-14C]Glycocholic acid (51.7 Ci/mol) (The Radiochemical Centre, Amersham, England, HP79LL) and [U-3H]glycochenodeoxycholic acid (5.0 kCi/mol) (New England Nuclear, Worcester, Mass. 01608) were proved to be 97–99% pure by thin-layer chromatographic zonal scanning (16).

For the simultaneous determination of the two bile acids, about 10,000 dpm for [3H]chenodeoxycholylglycine (~600 pg/tube) and 1000 dpm for [1-14C]cholylglycine (~6000 pg/tube) were used in each tube, diluted in phosphate buffer.

Standards. Stock solutions, 0.2 mmol/liter in phosphate buffer, were stored at ~20 °C. Properly diluted with bile acid-free serum (10-fold diluted in phosphate buffer), they provided a series of solutions from 5 to 160 pmol/tube. Concentrated solutions were measured by enzymatic determination, with use of 3α-hydroxysteroid dehydrogenase (EC 1.1.1.50) (18), and proved to be stable if stored at ~20 °C.

Procedure for double RIA. The serum samples for analysis were diluted five- and 10-fold when obtained from normal subjects, and 50- to 200-fold when obtained from patients with liver disease.

Bile-acid-free serum (100 μl, diluted threefold with phosphate buffer) and 200 μl of each tracer were added to each tube, followed by 100 μl of each standard solution (or sample). Finally, 100 μl of each antisera, properly diluted, and 100 μl of phosphate buffer were added.

Blanks consisted of the following:

B0: 100 μl of bile acid-free serum (diluted threefold with phosphate buffer), 200 μl of each tracer, 100 μl of bile acid-free serum (diluted 10-fold with phosphate buffer), and 400 μl of phosphate buffer.

B1: 100 μl of bile-acid-free serum (diluted threefold with phosphate buffer), 200 μl of each tracer, 100 μl of each antisera, 100 μl of bile-acid-free serum diluted 10-fold, and 200 μl of phosphate buffer. Each tube was vortex-mixed strongly, and 1 ml of saturated (NH4)2SO4 was added to all samples after 3 h at room temperature (further incubation did not result in a higher fraction of tracer bound, Figure 1). The contents of the tubes were stirred again and centrifuged at 3500 rpm for 25 min at room temperature.

The two bile acids were separately determined as described by Simmonds et al. (5).

Liquid scintillation counting. The counting was performed on the unbound tracer. The supernatant fluid was removed into a counting vial and 15 ml of Unisolve was added.

Samples were counted with an Analytic Isocap 300 Liquid Scintillation Counter (Searle Analytic Inc., Des Plaines, Ill. 60018). Both isotopes were simultaneously counted, during a single counting period, in two analyzer channels (A3 for the low-energy isotope, B3 for the high-energy isotope).

All the samples, counted for 10 min, gave a mean efficiency of 36% for 3H and 46% for 14C.

Only three efficiency calibration curves were required because of the minimal and uniform quenching in all samples: the % efficiency vs. ESR curve for low-energy isotope, as counted in channel A3; the % efficiency vs. ESR curve for the high-energy isotope, as counted in channel A3; and the % efficiency vs. ESR curve for the high-energy isotope as counted in channel B3.

When: h1 = % efficiency/100, the fractional efficiency for the low-energy isotope in channel A3, C1 = % efficiency/100, the fractional efficiency for the high-energy isotope in channel A3, C2 = % efficiency/100, the fractional efficiency for the high-energy isotope in channel B3, 3H-cpmA = count/min, as counted in channel A3, for H3, and 14C-cpmB = count/min, as counted in channel B3, for 14C, then disintegrations per minute for 14C (Dc) and for 3H (DH) are determined as follows:

\[
D_c = \frac{14C - cpm_B}{C_2}
\]

\[
D_H = \frac{3H-cpm_A - (D_c \cdot C_1)}{h_1}
\]

Calculation. The antibody-bound fraction was expressed as a percentage of B/B0 vs. log dose. The dose-response curve has been described by a four-parameter logit function (17):

\[
Y = \frac{a - d}{1 + \left(\frac{x}{c}\right)^b} + d
\]

where: Y = B/B0; a = response for zero-dose, d = response for infinite-dose, c = "midrange" (effective dose

Fig. 1. Relationship between per cent bound, normalized, of conjugated cholic acid (CCA) and conjugated chenodeoxycholic acid (CDDCA) vs. duration of incubation.
Table 1. Cross Reactivity of Various Bile Acids with Antisera Specific for Chenodeoxycholic Acid Conjugates (A) and for Cholic Acid Conjugates (B) *

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>Chn-gly</th>
<th>Chn-tau</th>
<th>Chi-gly</th>
<th>Chl-tau</th>
<th>Dex-gly</th>
<th>Dex-tau</th>
<th>Chn</th>
<th>Lit</th>
<th>Chl</th>
<th>Dex</th>
<th>Lit-gly</th>
<th>Lit-tau</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>100</td>
<td>100</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>4</td>
<td>1</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>B</td>
<td>—</td>
<td>100</td>
<td>100</td>
<td>1</td>
<td>—</td>
<td>2</td>
<td>6</td>
<td>—</td>
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<td>—</td>
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<td>—</td>
</tr>
</tbody>
</table>

* Abbreviations are: Chn-gly, chenodeoxycholyglycine; Chn-tau, chenodeoxycholyltaurine; Chi-gly, cholyglycine; Chl-tau, cholytaurine; Dex-gly, deoxycholyglycine; Dex-tau, deoxycholyltaurine; Chn, chenodeoxycholic acid; Chl, cholic acid; Lit, lithocholic acid; Dex, deoxycholic acid; Lit-gly, lithochocholyglycine; and Lit-tau, lithochocholyltaurine.

Table 2. Logit Parameters of the Dose–Response Curves (Mean Values ± SD) of 20 Determinations *

<table>
<thead>
<tr>
<th>Tracer</th>
<th>Simultaneous determination</th>
<th>Separate determination</th>
</tr>
</thead>
<tbody>
<tr>
<td>[U-3H]Glycochenodeoxy</td>
<td>a</td>
<td>b</td>
</tr>
<tr>
<td>cholic acid</td>
<td>88.7 ± 3.9</td>
<td>1.60 ± 0.47</td>
</tr>
<tr>
<td>[U-3H]Glycocholic</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>[14C]Cholyl-glycine</td>
<td>91.2 ± 4.5</td>
<td>1.3 ± 0.2</td>
</tr>
</tbody>
</table>

* For symbols a, b, c, d, see text.

for 50% dose-response) (pmol/tube), and b = slope factor.

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 suitable computer program.

Subjects

Concentrations of the two conjugated bile acids (µmol/liter) were determined for 20 control subjects, 20 patients with liver cirrhosis, and 20 patients with obstructive jaundice. Liver disease had been previously assessed by liver biopsy and conventional liver-function tests. Diagnosis of obstructive jaundice had been confirmed surgically.

The sera were separated and stored at –20 °C in glass vials until analyzed.

Results

Specificity. Simultaneous determination of the two bile acids was possible because of the high specificity of the two antisera.

Table 1 shows the cross reaction of the two antisera with the other bile acids normally found in human serum. In each tube, the concentration of the bile acids added ranged from 40 to 640 pmol/tube. No cross reaction was found between the two antisera and the other bile acids; they cross reacted with their own respective unconjugated antigen only to a very limited extent (Table 1). The antisera reacted equally to glycine and taurine conjugates.

Standard curve. Figure 2 shows the dose–response curves for the two classes of bile acids in single and simultaneous determinations. Each point represents the mean value of 10 determinations ± 1 SD. The midrange (Table 2) of the [U-3H]glycocholic acid (c = 21.05 ± 6.3) dose–response curves did not significantly differ when single determinations were carried out. For the former dose–response curve, simultaneous determination did not appear to affect the midrange (c = 20.4 ± 6.8). When [1-14C]glycocholic acid was used as tracer; however, the dose–response curve midrange was higher than for [U-3H]glycocholic, both when single (c = 41.4 ± 9.5) and simultaneous (c = 57.9 ± 9.0) determinations were done. Simultaneous determination also did not appear to affect the value of the dose–response curve midrange when a 14C-labeled tracer was used (Table 2).

The slopes of the curves appeared to be greater for the [U-3H]glycochenodeoxycholic acid than for [1-14C]-glycocholic acid. All the points of the composite standard curve differed significantly from one another, and the coefficients of variation were 8% or less. The smallest amount of unlabeled antigen that could be distinguished from no antigen was 5 pmol/tube for conjugated chenodeoxycholic acid (CCDCA) assay and 10 pmol/tube for conjugated cholic acid (CCA) assay.

No interference of 3H with 14C counting was found, while 14C interference on 3H counting was minimized.
by the low degree of quenching. This allowed direct calculation (without correction for the low-energy isotope's count) of the high-energy isotope's disintegration rate, while the low-energy isotope's disintegration rate required a correction for the high-energy isotope's count.

The simultaneous determination of the two standard curves, where the proportion of the two antigens were inverted, so that CCA/CCDCA ratio varied within a wider range than in human normal serum, confirmed no interference of the two isotopes on each other when the low-energy isotope dpm was properly corrected for the high-energy one (Figure 3).

**Bile acids in tested serum.** Simultaneous and single determination of each sample gave identical values, as also was true for those sera with inverted CCA/CCDCA ratio (Figure 4).

Serum conjugated cholic acid values were the same whether a $^{14}$C-labeled tracer or a $^3$H-labeled tracer was used (Figure 5).

Concentrations of each of the two conjugated bile acids, assessed by simultaneous determination, appear in Figure 6, and values for them in serum as well as other liver-function tests are in Table 3; in pathological conditions, serum bile acids were increased in cases where results for the other liver-function tests were normal (Figures 7 and 8, Table 3).

In obstructive jaundice both conjugated cholic acid ($P < 0.001$) and conjugated chenodeoxycholic acid ($P < 0.01$) were related with bilirubin values, as well as with $\gamma$-glutamyltransferase (EC 2.3.2.2) values ($P < 0.01$ and $P < 0.1$, respectively).
In cirrhotic patients a high correlation was found between conjugated chenodeoxycholic acid and bilirubin ($P < 0.01$) and an even better correlation between conjugated chenodeoxycholic acid and $\gamma$-glutamyltransferase ($P < 0.001$).

A good correlation was found when conjugated cholic acid values were plotted vs. bilirubin ($P < 0.1$) and $\gamma$-glutamyltransferase ($P < 0.05$).

No significant relationship was found with the other liver-function tests.

**Parallelism.** Samples with high bile-acid concentrations (cirrhosis) were diluted 20-, 40-, 80-, 120-, and 160-fold, to study the behavior of the antibody in comparison to a standard curve for both. The plot of standard dilution vs. tracer binding (Figure 9) paralleled the standard curves, which indicated that the antibody was reacting only with the bile acid that represented its own antigen.

**Accuracy and precision.** Serum bile acids from 15 unknown samples were determined both by radioimmunoassay and by gas chromatography (17). Bile acids were extracted from the sera with Amberlite XAD$_2$ (20). After saponification of the extract, bile acid methyl acetyl derivatives were detected by use of a 2 m $\times$ 0.4 cm glass column packed with 3% AN 600 on Anakrom Q (110–120 mesh), with nordeoxycholic acid as internal standard (21). Figure 10 shows the analysis of regression; it reveals a close agreement of values at the highest concentrations, while at the lowest concentrations, the gas-chromatographic method is at the limit of sensitivity.

The intra-assay precision, verified in 20 determinations of the samples in the same assay, was satisfactory; the coefficient of variation was 8% or less at the lowest concentration of bile acids. The intra-assay precision was estimated in the samples from a normal subject and a patient with cirrhosis, measured in 10 different assays. The coefficient of variation was 13% at the lowest concentration and 5.5% at the highest concentration of bile acids.

**Analytical recovery.** In 10 samples from control subjects, to which an amount of cholyglycine and chenodeoxycholylglycine equal to 0.2 $\mu$mol/liter was added, recovery ranged from 96 to 107%.

**Discussion**

Results show the validity of the proposed method. Simultaneous determination of the two bile acids was possible because of the high specificity of the two antisera.

This method has been proved to be both sensitive, specific, and easily done. In fact, no preliminary pro-

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**Table 3. Laboratory Findings in the Groups Considered**

<table>
<thead>
<tr>
<th>Test</th>
<th>Normal value</th>
<th>Cirrhosis $n = 20$</th>
<th>% Abnormal</th>
<th>Obstructive jaundice $n = 20$</th>
<th>% Abnormal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean $\pm$ SD</td>
<td></td>
<td></td>
<td>Mean $\pm$ SD</td>
<td></td>
</tr>
<tr>
<td>Bilirubin, mg/liter</td>
<td>&lt;1.0</td>
<td>1.87 $\pm$ 0.79</td>
<td>80</td>
<td>6.8 $\pm$ 5.1</td>
<td>100</td>
</tr>
<tr>
<td>Alk. phosphatase, U/liter</td>
<td>&lt;200</td>
<td>276 $\pm$ 85</td>
<td>60</td>
<td>444 $\pm$ 389</td>
<td>80</td>
</tr>
<tr>
<td>$\gamma$-GT, <strong>U/liter</strong></td>
<td>&lt;28</td>
<td>47.1 $\pm$ 19</td>
<td>80</td>
<td>162.7 $\pm$ 149</td>
<td>95</td>
</tr>
<tr>
<td>AST, $^b$ U/liter</td>
<td>&lt;20</td>
<td>46.7 $\pm$ 24</td>
<td>60</td>
<td>54.9 $\pm$ 23</td>
<td>90</td>
</tr>
<tr>
<td>ALT, $^c$ U/liter</td>
<td>&lt;30</td>
<td>43.3 $\pm$ 24.2</td>
<td>60</td>
<td>49.4 $\pm$ 18.6</td>
<td>80</td>
</tr>
<tr>
<td>CCA, $^d$ $\mu$mol/liter</td>
<td>&lt;1</td>
<td>15.4 $\pm$ 9.5</td>
<td>100</td>
<td>21.3 $\pm$ 10.3</td>
<td>100</td>
</tr>
<tr>
<td>CCDDA, $^e$ $\mu$mol/liter</td>
<td>&lt;1.5</td>
<td>36.2 $\pm$ 20.3</td>
<td>100</td>
<td>13.5 $\pm$ 8.3</td>
<td>100</td>
</tr>
</tbody>
</table>

* $^{a}$ $\gamma$-Glutamyltransferase, $^{b}$ aspartate aminotransferase, $^{c}$ alanine aminotransferase, $^{d}$ cholic acid-conjugated bile acid, $^{e}$ chenodeoxycholic-conjugated bile acid.
procedure is required. The tracers, as well as the other reagents, can be obtained from the usual commercial sources; and only a short analysis time and small serum samples are required. Simultaneous determination of the two classes of bile acids did not seem to affect the sensitivity of the assay; when a proper \(^{14}\text{C}/\text{H}^3\) ratio was used, good results were achieved.

Use of a \(^{14}\text{C}\)-labeled tracer resulted in a less-sensitive dose–response curve, because of the lower specific activity of a \(^{14}\text{C}\)-tracer; but use of such tracer does not compromise the sensitivity of the assay to such an extent as to cause it to operate at the lower limit of sensitivity.

In normal as well as in certain pathological conditions, such as cirrhosis and chronic active liver disease, the \(\text{CCA/CCDCA}\) ratio is less than unity, because chenodeoxycholic acid is the major bile acid in serum; cholic acid predominates in jaundice. By simultaneous determination of the two primary bile acids, it is possible to learn their ratio in serum by a single assay.

It is not the aim of the present paper to assess the validity of measuring serum conjugated cholic acid and conjugated chenodeoxycholic acid as an index of liver function. Nevertheless, in all the patients with cirrhosis of the liver or obstructive jaundice that we have examined, serum conjugated cholic acid and conjugated chenodeoxycholic acid values are increased, while other usual liver-function tests give an abnormal result in a smaller percentage of cases (Table 3), confirming that increased serum conjugated cholic acid and conjugated chenodeoxycholic acid is the most sensitive liver-function test, and that an inverted conjugated cholic acid/conjugated chenodeoxycholic acid ratio (i.e., \(>1\)) is a marker of jaundice.

Our results appear in good agreement with those (4–7, 19, 20) of many authors (Figure 11), which confirms the validity of the present method as well as its applicability in diagnosis of hepatobiliary disease.

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References