Use of a Synthetic Soluble Bilirubin Derivative to Assess Interference in Creatinine Measurements

Carlo Franzini, Anna M. Morelli, and Gianpaolo Cattozzo

In assessing interference from bilirubin, the use of a synthetic soluble derivative (ditaurobilirubin, DTB) is recommended as a surrogate for the natural conjugates (Bc). We compared the interference effect of unconjugated bilirubin (Bu), Bc, and DTB, using six mechanized methods for serum creatinine measurement. No significant interference was noted in methods that include removal of proteins or in an enzymatic method involving NADH oxidation. Heavy (negative) interference was observed in an alkaline picate method, and in direct enzymatic methods based on hydrogen peroxide measurement: interference was always more pronounced in the presence of the two soluble derivatives (Bc and DTB), whose interference was of the same magnitude. These results point out the utility of testing for bilirubin interference by using soluble derivatives, in addition to Bu, and suggest the feasibility of using DTB as a surrogate for Bc for this purpose.

Additional Keyphrases: intermethod comparison; variation, source of

Because naturally occurring bilirubin derivatives (Bc) are not readily available, the use of a synthetic soluble derivative (ditaurobilirubin, DTB) has been suggested (1, 2) to assess bilirubin interference in analytical methods.1 DTB mimics many properties of Bc (3, 4), but we know of no reports about its interfering properties. Therefore, we investigated the interference from unconjugated bilirubin (Bu), DTB, and Bc in assays of creatinine, given that many methods for creatinine measurement are known to be highly sensitive to the presence of bilirubin (5).

Materials and Methods

Unconjugated bilirubin (Bu) was from Merck (Darmstadt, F.R.G.) and ditaurobilirubin (DTB) from Lee Scientific (St. Louis, MO). Conjugated bilirubin (Bc) was prepared from twofold-diluted (6) fresh human bile, following Lucassen’s procedure (7) but omitting the final washings with acetone and diethyl ether, and dissolving the precipitate in a small volume of water. After centrifuging, we immediately mixed 0.5 mL of the supernate with 30 mL of two serum pools (see below).

Two portions of a serum pool (total bilirubin 6.8 \text{\mu mol/L}; “direct” bilirubin 3.4 \text{\mu mol/L}) were enriched with creatinine, to final concentrations of about 180 (low pool) and 290 \text{\mu mol/L} (high pool). To 30 mL of each we added either Bc, as described above, or Bu (0.5 \text{\mu L} of Bu solution in sodium hydroxide, 0.1 \text{mL}, followed by 0.5 mL of hydrochloric acid, 0.1 \text{mL}), or DTB (0.5 \text{mL of 25.6 mmol/L, aqueous solution}). The supplemented and unsupplemented pools were then mixed in different proportions, to yield a total of 24 specimens, featuring two concentrations of creatinine and four concentrations of added Bu (from 0 to 525 \text{\mu mol/L}), DTB (from 0 to 420 \text{\mu mol/L}), and Bc (from 0 to 411 \text{\mu mol/L}). Total bilirubin concentration was measured with the proposed Reference Method (8).

Creatinine concentration was measured, in triplicate, by means of six mechanized procedures, with commercially available reagents, as follows. Method A: alkaline picate end-point reaction, with dialysis (performed with a SMA 8 analyzer; Technicon Instruments, Tarrytown, NY). Method B (9): glutamate dehydrogenase (EC 1.4.1.3) measurement of the ammonia generated by the

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1 Nonstandard abbreviations: Bu, unconjugated bilirubin; Bc, conjugated bilirubin; and DTB, ditaurobilirubin.

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action of creatinine iminohydrolase (EC 1.5.3.1) (performed with a Cobas-Fara instrument; Roche Diagnostics, Basel, Switzerland). Method C (performed with an Ektachem 700 analyzer; Eastman Kodak, Rochester, NY), and methods D and F (both performed with a Hitachi 705 analyzer; Boehringer Mannheim, Mannheim, F.R.G.) (10–14): enzymatic conversion of creatinine to creatine (creatinine amidohydrolase, EC 3.5.2.10) and then to sarcosine (creatinine amidohydrolase, EC 3.5.3.3), followed by peroxidase-coupled (EC 1.11.1.7) colorimetric measurement of the hydrogen peroxide resulting from the action of sarcosine oxidase (EC 1.5.3.1). Method E: kinetic picrate reaction (performed with a Chem 1 instrument; Technicon).

Results

For methods A–C, either no significant interference was observed, or the interference was small (less than ± 3.5%) when bilirubin concentration did not exceed 500 μmol/L. For methods D–F, measured creatinine values decreased with increasing bilirubin concentration. A second-degree polynomial regression gave the best fit to the experimental data (S^2 ≤ 1.6 μmol/L), and was applied to calculate, by mathematical interpolation, creatinine suppression-values at two bilirubin concentrations (50 and 400 μmol/L). Interferences from Bc and DTB did not significantly differ from each other (differences <3 S_{CP}), but were always significantly higher (differences in the range of 3.1–36 S_{CP}) than the interference from Bu (Table 1).

Discussion

In agreement with previous data (15), our results show that removal of proteins (by continuous dialysis or multilayer filtration) overcomes the interfering effect of bilirubin in bilirubin-sensitive reactions. Without removal of proteins, only one of the three analytical principles tested, enzymatic via NADH oxidation (but not alkaline picrate or enzymatic via hydrogen peroxide measurement), appears to be insensitive to interference from bilirubin.

Interference from soluble derivatives (Bc or DTB) always exceeds the interference from Bu, probably related to the solubility of the derivatives and to their possibly weaker linkage to protein. Whatever the mechanism, supplementing specimens with soluble bilirubin derivatives should always be included in experiments designed to assess bilirubin interference, as recommended (1, 2).

Our results definitively show that Bc and DTB cause equivalent (negative) interference. Therefore, DTB can be reliably used as a surrogate for Bc in testing for bilirubin interference; moreover, DTB is commercially available as an acceptably pure material (3).

An additional problem is the occurrence, in pathological sera, of a third molecular species of bilirubin, the so-called delta-bilirubin or biliprotein (16). Although delta-bilirubin reportedly shows a reactivity similar to Bc (16), its interfering behavior is unpredictable until sera are specifically tested for this species.

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References


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**Table 1. Change in Creatinine Concentration (μmol/L) in the Presence of Unconjugated Bilirubin and Ditaurobilirubin**

<table>
<thead>
<tr>
<th>Creat. concn*</th>
<th>Bu, 50</th>
<th>DTB, 50</th>
<th>Bu, 400</th>
<th>DTA, 400</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Method D</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>−5.3</td>
<td>−14°</td>
<td>−43</td>
<td>−72°</td>
</tr>
<tr>
<td>High</td>
<td>−8.8</td>
<td>−17°</td>
<td>−46</td>
<td>−104°</td>
</tr>
<tr>
<td><strong>Method E</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>−11</td>
<td>−21°</td>
<td>−85</td>
<td>−135°</td>
</tr>
<tr>
<td>High</td>
<td>−13</td>
<td>−26°</td>
<td>−100</td>
<td>−144°</td>
</tr>
<tr>
<td><strong>Method F</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>−11</td>
<td>−16°</td>
<td>−88</td>
<td>−110°</td>
</tr>
<tr>
<td>High</td>
<td>−16</td>
<td>−23°</td>
<td>−136</td>
<td>−163°</td>
</tr>
</tbody>
</table>

*Interpolated from the second-degree polynomial regression equation of measured creatinine values vs bilirubin concentration.

Low concentration of creatinine = 180 μmol/L; high concentration = 290 μmol/L. See text for description of methods.

(Decrease by DTB—decrease by Bu) > 3 S_{CP}.
Precipitation with Polyethylene Glycol and Density-Gradient Ultracentrifugation Compared for Determining High-Density Lipoprotein Subclasses HDL2 and HDL3

Kurt Widhalm and Renate Pakosta

The purpose of this study was to compare quantification of cholesterol in high-density lipoprotein subfractions HDL2 and HDL3 by precipitation with polyethylene glycol (PEG) with that by density-gradient ultracentrifugation. Fresh serum samples from 32 fasting, obese children were analyzed with precipitation reagent "Quantolip" (Immuno AG), and then fractionated with a Beckman TL 100 ultracentrifuge with a swinging-bucket rotor. After centrifugation we carefully removed the supernate with a syringe and measured the cholesterol from each fraction enzymatically with CHOD-PAP reagent (Boehringer Mannheim). The low-density lipoprotein (LDL), HDL2, and HDL3-cholesterol values measured by ultracentrifugation did not differ significantly from those obtained by precipitation; the correlation coefficients (r) between the two methods were 0.96 for LDL, 0.75 for HDL2, and 0.96 for HDL3. The relatively simple PEG precipitation method used in this study measures total HDL and its major subclasses HDL2 and HDL3 with accuracy and precision comparable with those of the well-established ultracentrifugation method.

The high-density lipoproteins (HDL) of human plasma are a heterogeneous group of particles of differing composition and size within the density range 1.063–1.21 kg/L.1 On the basis of flotation rate, two major populations of particles have been identified, HDL2 and HDL3 (1), corresponding to densities of 1.063–1.125 and 1.125–1.21 kg/L, respectively. The lower density of HDL2 reflects a greater lipid/protein ratio; other differences include a greater relative content of apoprotein C and a lower content of apoprotein A-II in HDL2 than in HDL3 (2).

It is well documented that the incidence of coronary heart disease in Western countries is negatively correlated with the plasma total HDL-cholesterol (HDL-C) concentration (3). This relationship also may reflect an underlying association between atherogenesis and the concentration of HDL2 especially (4).

The pathophysiological connection between a high risk for atherosclerotic diseases and low plasma HDL concentration is poorly understood. HDL has a close relationship to triglyceride metabolism, the HDL being created to a great extent during the enzymatic hydrolysis of triglyceride-rich lipoproteins, mainly very-low-density lipoprotein (VLDL), by lipoprotein lipase.

In addition, the hydrolysis of VLDL gives rise mainly to the HDL subfraction HDL2, a lipoprotein subclass considered to be potentially a better risk indicator for atherosclerosis than is total HDL. The well-established method for separating and quantifying HDL and its subclasses is ultracentrifugation (5–9), but this laborious and expensive technique is hardly suited for the routine clinical laboratory. Consequently, researchers have developed faster, less-expensive, and automated precipitation methods (10–13).

The purpose of this study was to compare the results of quantifying HDL2- and HDL3-C by precipitation with polyethylene glycol (PEG) with those by density-gradient ultracentrifugation.

Materials and Methods

Blood was sampled from 32 fasting, obese outpatients, ages 10.2–16.1 years. Their triglyceride concentrations ranged from 0.96 to 5.1 mmol/L and total cholesterol from 2.8 to 6.7 mmol/L. We determined LDL-C, HDL2-C, and HDL3-C in aliquots by precipitation with PEG. Samples were stored at −20 °C for no longer than six weeks before lipoprotein fractions were measured by ultracentrifugation with the Beckman TL 100 ultracentrifuge with a swinging-bucket rotor. The method is based on a difference in flotation rate of the HDL subclasses.

Preparation of Lipoprotein Fractionation by Ultracentrifugation

Measure 1-mL aliquots of serum into 2-mL (11 × 34 mm) polallomer centrifugation tubes. Layer 1 mL of 1.006 kg/L KBr solution over the surface. Centrifuge the tubes and their contents in the Beckman TL S55 swinging-bucket rotor for 18 h (10 000 × g, 10 °C). This is Spin I, during which the fraction defined as VLDL is floated to the top of the tube.

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1 Nonstandard abbreviations: HDL, LDL, VLDL, high-, low-, and very-low-density lipoprotein, respectively; PEG, polyethylene glycol; and C, cholesterol.

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Department of Pediatrics, Division Metabolic Diseases, University of Vienna, Währinger Gürtel 18–20, 1090 Vienna, Austria.