Determination of Conjugated and Total Bilirubin in Serum of Neonates, with Use of Bilirubin Oxidase

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A new, simple, and rapid assay for conjugated bilirubin that does not require serum-matrix standards was developed by using the enzyme bilirubin oxidase (EC 1.3.3.5). This procedure can also be modified to measure total bilirubin. Measurements from these assays were compared with results obtained with the Sigma 605 (Jendrassik–Gröf method), Sigma 550/551 (Walters–Gerarde method), and Kodak Ektachem (BuBc) bilirubin assays. The one-year study involved serum specimens from 283 infants younger than 30 days. Linear-regression analysis of data for conjugated bilirubin collected by this assay and by the Kodak Ektachem assay yielded a slope of 0.975, an intercept of 0.088, an $S_x$ of 1.47 mg/L, and a correlation coefficient of 0.94 for a total of 49 specimens. Correlation was also good ($r = 0.95$) between results for total bilirubin by this assay and both the Sigma 605 and the Kodak Ektachem methods.

**Additional Keyphrases:** enzyme methods · multilayer filter analysis compared

Many common in vitro assay methods for bilirubin involve use of Ehrlich’s diazotized sulfanilic acid (1). The Jendrassik–Gröf procedure, in which the diazo reagent is used, is recommended for total bilirubin determination (1, 2). In contrast to total bilirubin measurement, no recommended method exists for conjugated bilirubin determination. Recently, an Ektachem neonatal bilirubin slide and a "Dri-stat" enzymatic bilirubin assay have been developed by Kodak and Beckman Instruments, respectively. The Kodak Ektachem (BuBc) method is based on the binding of bilirubin species to a cationic polymeric mordant, which allows measurement of both unconjugated and conjugated bilirubin (3, 4). The "Dri-stat" bilirubin reagent uses the enzyme bilirubin oxidase (EC 1.3.3.5) for determination of total bilirubin (5). Currently, the only approach suggested for determining conjugated bilirubin by using bilirubin oxidase is in a patent (6).

We propose here a novel assay for conjugated bilirubin by using bilirubin oxidase; in contrast to other procedures, this assay requires no serum-based standards and also permits measurement of total bilirubin. Bilirubin oxidase, derived from the fungus *Myrothecium verrucaria*, catalyzes the oxidation of bilirubin to biliverdin and the oxidation of biliverdin to a pale purple pigment without catalyzing the oxidation of hemoglobin (7, 8). This degradation causes the characteristic yellow absorption band for bilirubin (400–470 nm) to disappear. Because the products of the enzymatic reaction do not absorb in the yellow band (7, 8), we have exploited this loss in absorbance to estimate the concentrations of total and direct bilirubin. In the former assay, we use sodium cholate to dissociate unconjugated bilirubin from serum albumin. In the latter assay, we use acidic conditions, under which bilirubin oxidase catalyzes only conjugated bilirubin oxidation in serum (6). We also compared results by three commercially available methods for total and conjugated bilirubin: diagnostic kits and reagents 550/551 and 605 from Sigma Co., St. Louis, MO, and the Ektachem slide assay from Eastman Kodak, Rochester, NY. In the Sigma methods, diazotized sulfanilic acid is used. Sigma diagnostic kit 605 is based on the Jendrassik-Gröf procedure (9), the recommended procedure for estimation of total bilirubin (1, 2).

**Materials and Methods**

Chemicals, Standards, Preparation Procedures

All reagents were analytical grade. Phosphate buffer (50 mmol/L, pH 7.45) was prepared from mono- and di-basic sodium phosphate salts. Sodium cholate solution (~40 mmol/L) was prepared by dissolving 1.72 g of cholic acid sodium salt (Sigma) in 100 mL of phosphate buffer. Citric acid buffer (0.1 mol/L, pH 4.5) was prepared by dissolving citric acid monohydrate and adjusting the pH with a 1.0 mol/L NaOH solution.

Bilirubin ditaurine conjugate, disodium salt (Porphyrin Products, Logan, UT), was used without further purification. Its absorptivity (e) in citric acid buffer, calculated according to Caraway (10), was 54 L·g⁻¹·cm⁻¹ at 460 nm.

Lyophilized bilirubin oxidase was from Amano International Enzyme, Troy, VA; its vendor-specified activity was 2.27 kU/g. We first dissolved the enzyme in NaHCO₃ (20 mmol/L, pH 8.3) buffer, then desalted it by passing the enzyme solution over a 25-mL G-25 Sephadex column pre-equilibrated with the buffer. Desalted enzyme, examined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis as described by Yang et al. (11), showed a single protein band indicating high enzyme purity. The enzyme protein concentration was estimated by the assay of Lowry et al. (12). Protein concentrations of stock bilirubin oxidase solutions were 13 g/L and 70 mg/L.

Bilirubin diagnostic kits and reagents, no. 550/551, based on the method of Walters and Gerarde (13), and no. 605, based on the method of Jendrassik and Grof (9), were from Sigma.

Lyophilized bilirubin standards—total bilirubin concentrations of 20, 50, 100, and 150 mg/L in a buffered human serum albumin solution—were from Sigma. To reconstitute the standards, we dissolved the contents of each vial in 3.0

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mL of distilled water. After reconstitution, we prepared 75 and 125 mg/L bilirubin solutions by mixing equal volumes of the 50 and 100 mg/L bilirubin standards and of the 100 and 150 mg/L bilirubin standards, respectively. All bilirubin standard solutions were kept in the dark at 4 °C for a week or frozen at −20 °C if stored for longer than that.

All specimens were blood-serum samples from newborn infants, as provided by the Chemistry Laboratory of the Brigham and Women’s Hospital, Boston, MA. Because of the lability of bilirubin to light, the specimens were kept in the dark until analysis. If not analyzed within 2 h after specimen collection, the serum samples were stored at −20 °C.

Apparatus

Bilirubin absorbance and initial enzymatic rates of bilirubin oxidation were measured spectrophotometrically at 460 nm with a Perkin-Elmer 553 Fast Scan UV/Vis spectrophotometer and R 100 recorder (Perkin-Elmer Corp., Analytical Instruments, Norwalk, CT). The measurement cuvette was a 1.4-mL, 1-cm-lightpath, quartz, standard ultraviolet-transmitting cell with a fitted Teflon stopper (Spectrocell Inc., Orlando, PA). We also used a Kodak Ekcthem analyzer equipped with a data logger and both a 400- to 420-nm and a 460-nm wavelength filter.

Conjugated Bilirubin Assay Procedure

Add 10 μL of bilirubin oxidase solution (13 g/L) to a cuvette containing 0.94 mL of citric acid buffer; use this to adjust the spectrophotometer to zero. Then add 50 μL of serum specimen, mixing quickly by inverting the cuvette, and record the absorbance for 3 min (the absorbance usually plateaus within 1 to 2 min). Estimate the conjugated bilirubin concentration from the decrease in absorbance (10), using the absorptivity of ditaurate bilirubin conjugate, a surrogate for conjugated bilirubin (the chromatographic and chemical properties of commercially available ditaurate-conjugated bilirubin are similar to those of human-conjugated bilirubin (15), and its absorptivity is also used in the conjugated Kodak Ekcthem assay):

$$\text{conc, g/L} = \frac{\Delta A}{\epsilon \times l \times d}$$

where $\Delta A$ = absorbance change

$\epsilon$ = absorptivity

(L·g$^{-1}$·cm$^{-1}$)

$l$ = cuvette light path

(cm)

d = specimen dilution

(= 20)

Total Bilirubin Oxidase Assay Procedure

After adjusting the spectrophotometer to zero absorbance with 0.95 mL of sodium cholate phosphate buffer, add 50 μL of serum or bilirubin standard (in serum albumin) to the cuvette, quickly invert to mix, and record the absorbance at 460 nm. Then add 10 μL of bilirubin oxidase (original concentration, before addition, 70 mg/L), quickly mix, and record the absorbance change for 3 min. Construct a standard curve by plotting the absorbance of the total bilirubin standards after 3 min vs their initial concentrations. Estimate the total bilirubin concentration of the test specimens by comparing their absorbance after 3 min with the reference curve.

Data Analysis

All the serum specimens were from infants younger than 30 days. Because of the small amount of serum collected from each blood sampling, not all bilirubin assays were carried out for each serum specimen. All data were analyzed by least-squares linear regression analysis (15, 16).

Results

Conjugated Bilirubin Oxidase Assay

Figure 1 shows a typical example of the decrease in absorbance and the absorbance plateau after mixing 50 μL of serum (with 63 mg/L total bilirubin and about 18 mg/L conjugated bilirubin) with 0.95 mL of citric acid buffer containing bilirubin oxidase. Table 1 summarizes the comparison studies and shows good correlation between the conjugated enzymatic assay and the Kodak Ektachem assay for 49 specimens. The conjugated bilirubin concentrations of the specimens were 2 to 18 mg/L, a typical range during the neonatal period, with concentrations exceeding 14 mg/L considered indicative of liver disease (17). The Sigma 605 and 551 assays, which are based on the reaction of diazotized sulfanilic acid reagent and conjugated bilirubin to form azobilirubin in the absence of unconjugated bilirubin displacers, were unreliable in our hands. The absorbance for azobilirubin kept increasing; the failure to reach an eventual plateau in absorbance precluded attempts to estimate the conjugated bilirubin concentration. After about 10 min, depending on the sample, the specimen’s azobilirubin absorbance eventually reached a value reflecting the total bilirubin concentration of the specimen, which suggested that unconjugated bilirubin was slowly dissociated from serum albumin and interfering with the conjugated bilirubin concentration measurement.

Total Bilirubin Oxidase Assay

Table 1 shows good agreement between the bilirubin oxidase, the Sigma 605, and the Kodak Ektachem assays of total bilirubin. The range of the total bilirubin concentrations in the specimens was about 30 to 200 mg/L, which is typical of total bilirubin concentrations in neonates under-
Table 1. Linear Regression Equations and Parameters of Conjugated and Total Bilirubin Assays

<table>
<thead>
<tr>
<th>y</th>
<th>x</th>
<th>Regression equation</th>
<th>$S_{yr}$</th>
<th>$r$</th>
<th>n</th>
<th>Range, mg/L</th>
<th>$S_{x}$</th>
<th>$S_{y}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Box.Bc</td>
<td>Kod.Bc</td>
<td>$y = 0.068 + 0.975x$</td>
<td>1.47</td>
<td>0.94</td>
<td>49</td>
<td>2–18</td>
<td>0.057</td>
<td>0.34</td>
</tr>
<tr>
<td>Box.Bt</td>
<td>605.Bt</td>
<td>$y = 2.57 + 0.934x$</td>
<td>12.3</td>
<td>0.95</td>
<td>50</td>
<td>40–200</td>
<td>0.045</td>
<td>5.8</td>
</tr>
<tr>
<td>Box.Bt</td>
<td>Kod.Bt</td>
<td>$y = -6.56 + 1.024x$</td>
<td>12.4</td>
<td>0.95</td>
<td>75</td>
<td>30–200</td>
<td>0.040</td>
<td>4.8</td>
</tr>
<tr>
<td>Kod.Bt</td>
<td>605.Bt</td>
<td>$y = 13.60 + 0.827x$</td>
<td>9.6</td>
<td>0.96</td>
<td>66</td>
<td>30–200</td>
<td>0.028</td>
<td>3.2</td>
</tr>
<tr>
<td>550.Bt</td>
<td>Kod.Bt</td>
<td>$y = -23.93 + 1.229x$</td>
<td>31.0</td>
<td>0.84</td>
<td>43</td>
<td>40–210</td>
<td>0.126</td>
<td>15.4</td>
</tr>
</tbody>
</table>

$S_{yr}$ = standard error estimate; $S_{x}$ = standard deviation of slope; $S_{y}$ = standard deviation of intercept. n = no. of serum specimens.

Discussion

Conjugated Bilirubin Assays

The goal behind the development of a conjugated bilirubin assay is to separate conjugated bilirubin and unconjugated bilirubin. The immediate problem in dealing with bilirubin oxidase as a means of determining the amount of conjugated bilirubin in any given blood plasma sample is that the enzyme reacts favorably with both the conjugated and the unconjugated species. Because of this, the focus of development of the conjugated assay must lie with the determination and the creation of a suitable environment in which the conjugated species of bilirubin reacts with the enzyme but the unconjugated species does not. Takayama et al. (6) observed that, in the pH range 3.5–4.5, bilirubin oxidase catalyzes only the oxidation of conjugated bilirubin in icteric plasma. In the present assay we used a buffer of pH 4.5. As Table 1 shows, there was very good agreement between the conjugated enzymatic assay and the conjugated Kodak Ektachem assay, the latter having been judged (4, 18) the best of several commercially available methods for estimating the conjugated bilirubin species—as compared with liquid chromatography, a very specific method.

Total Bilirubin Assay

Unconjugated bilirubin is poorly soluble; it circulates in the blood bound to serum albumin (19). This binding to serum albumin protects it from oxidation by bilirubin oxidase (20). To speed the assay, sodium cholate is used to disrupt this protective binding and therefore increase the rate of reaction.

Results by the bilirubin oxidase, the Sigma 605, and the Kodak Ektachem total bilirubin assays correlated well (Table 1). The major difference between these assays may be their sensitivity to δ-bilirubin. This form of bilirubin, which reacts with diazotized sulfanilic acid reagent, is included in the total bilirubin concentration value measured by the Jendrassik–Grôf assay (Sigma kit 605) but is not measured by the Kodak Ektachem assay (3). Bilirubin oxidase probably does not catalyze the degradation of the protein-bound fraction, δ-bilirubin, because unconjugated bilirubin bound to serum albumin is protected from degradation (20).

In our study, however, the effect of the difference in sensitivity for δ-bilirubin between the Jendrassik–Grôf, Kodak Ektachem, and enzymatic methods should be minimal because in the plasma of infants younger than 28 days, δ-bilirubin is <2% of total bilirubin (21).

In conclusion, the use of bilirubin oxidase for measurement of total and conjugated bilirubin concentrations provides a simple and rapid micromethod. In considering a procedure for the measurement of conjugated bilirubin, the bilirubin oxidase and Kodak Ektachem methods should be further investigated, being potentially free of interference from δ-bilirubin and hemoglobin as compared with the diazotized sulfanilic acid reagent.

References


