Determination of the Sum of Bilirubin Sugar Conjugates in Plasma by Bilirubin Oxidase

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Background: A reliable indicator of cholestasis is the presence of abnormal concentrations of bilirubin mono- and diglucuronide [conjugated bilirubin (CB)] in blood. A routine assay of CB is available only to those who possess a certain type of clinical analyzer. We describe a two-point manual method for CB that could be adapted as a rate assay to automated clinical analyzers.

Methods: The measurement of CB is based on its oxidation to biliverdin by bilirubin oxidase. The resulting decrease in absorbance at 460 nm is proportional to the CB concentration. The assay is calibrated with solutions of ditaurobilirubin in human serum.

Results: Under the conditions of the assay (0.1 mol/L glycine buffer, pH 10.0; reaction time, 2 min), only 5% of unconjugated bilirubin is oxidized and δ-bilirubin is not oxidized at all. Results obtained with the bilirubin oxidase method agreed well with those obtained by HPLC. The long-term CVs at CB concentrations of 6 and 63.4 mg/L were 20% and 2.6%, respectively. The reference values, established by analyzing 51 plasma specimens from healthy adults, were 0.0–1.2 mg/L, with a mean value of 0.2 mg/L.

Conclusions: The proposed method for CB has good analytical specificity and obviates the requirement for HPLC or a dry chemistry analyzer. The measurement of CB in blood is superior to the measurement of direct bilirubin because an abnormal concentration of direct bilirubin does not necessarily indicate the presence of cholestasis.

The presence of cholestasis in liver disease is indicated by a rise in conjugated bilirubin (CB)3 in blood. The most common test for detecting cholestasis is the measurement of direct-reacting bilirubin (DB), consisting of the two glucuronides, δ-bilirubin, and depending on the specificity of the method, variable amounts of unconjugated bilirubin (UB) (1). However, high concentrations of DB in plasma do not necessarily indicate cholestasis because δ-bilirubin persists in plasma long after cholestasis is relieved. A better indicator of hepatobiliary excretion is the presence of abnormal concentrations of CBs in blood (2).

In 1987, Kosaka et al. (3) published a method for measuring total serum bilirubin and its fractions by use of bilirubin oxidase (BO) and the diazo reaction. Among those fractions was the sum of mono- and diglucuronide (CB). To demonstrate the specificity of the assay for CB, Kosaka et al. compared the CB values in sera obtained with HPLC with those of the enzymatic method, using correlation/regression statistics. The slope of the regression equation indicated that the CB values obtained with the enzymatic method were 13.3% higher than those obtained with HPLC, but it is impossible to tell whether this difference is attributable to the oxidation of UB or δ-bilirubin or to different methodological principles.

Our study complements the work of Kosaka et al. (3). Specifically, we provide quantitative data regarding the extent of oxidation of UB. Using pure δ-bilirubin (isolated from human serum), we demonstrated that under the assay conditions this fraction was not oxidized at all. We evaluated the rates of oxidation of ditaurobilirubin (DTB) in human serum, human serum albumin (HSA), and bovine serum albumin (BSA), and demonstrated that DTB in BSA is not suitable as a calibrator for this assay. We determined the optimum activity of BO in the reaction mixture so that the oxidation of UB is kept at no more
than 5%, and by adding mannitol (a free-radical scavenger) to the reaction mixture, we reduced the spontaneous oxidation of UB or DTB (a blank reaction) ostensibly by free radicals.

A routine assay for CB is available only to those who have certain types of clinical analyzers. The more generally available measurement is that of DB. The availability of a practical CB method adaptable to a variety of clinical analyzers might increase recognition of the clinical value of routine CB testing. We describe here a two-point manual procedure for CB that could be easily adapted as a rate procedure to automated clinical analyzers.

**Materials and Methods**

**Bilirubin standard solutions.** We prepared bilirubin standard solutions by adding UB Standard Reference Material 916a (National Institute of Standards and Technology, Gaithersburg, MD) to pooled human sera as described elsewhere (4). These solutions were stored at −70 °C until used.

**DTB calibrator solutions.** DTB calibrator solutions were prepared by dissolving DTB (Porphyrin Products) in pooled human sera. The concentration of bilirubin, expressed as the UB equivalent, was determined by the reference method for total bilirubin (4). The DTB calibrator solutions were stored at −70 °C until used.

**Bile isolate.** We prepared solutions of bile isolate, a mixture of bilirubin mono- and diglucuronide prepared according to the method of Lucassen (5), in pooled human sera. The percentage of composition of the bilirubin fractions in the enriched pool, established by HPLC was as follows: δ-bilirubin, 6%; bilirubin monoglucuronide (CBm), 34%; bilirubin diglucuronide (CBD), 57%; UB, 3%.

δ-Bilirubin. δ-Bilirubin was isolated from human serum, as described previously (6).

**BO.** The lyophilized enzyme (Genencor) was supplied in vials containing (according to the supplier) 200 U of BO. We dissolved the contents of the vial in 5 mL of distilled water, measured the BO activity (see “Measurement of BO activity”), dispensed 1-mL aliquots of the solution into small plastic tubes, and stored the tubes at −70 °C until used.

**Tris buffer.** The composition of the Tris buffer was as follows: 0.1 mol/L Tris, pH 8.5, containing 40 mmol/L sodium cholate, 15 mmol/L sodium dodecyl sulfate, and 50 mmol/L mannitol.

**Glycine buffer.** The composition of the glycine buffer was as follows: 0.1 mol/L glycine, pH 10.0, containing 50 mmol/L mannitol.

**HPLC.** We used a Hewlett-Packard Model 1090 chromatograph equipped with a Micronex RP-30 column (Sekisui Chemical Co.). The preparation of the specimen and the procedure for separation of the bilirubin fractions was performed as described by Adachi et al. (7) except that plasma was diluted twofold with saline instead of 0.1 mol/L acetic acid. The bilirubin concentrations in the fractions were calculated by multiplying the peak area of each fraction (as ratio of the total area) by the total bilirubin concentration in the specimen.

**Enzymatic procedure for bilirubin conjugates**

**Principle.** At pH 10.0, BO selectively oxidizes bilirubin conjugates to their corresponding biliverdins. The decrease in absorbance at 460 nm is proportional to the concentration of bilirubin conjugates.

We used a Gilford Stasar III spectrophotometer for absorbance measurements.

**Test.** Under subdued light, we added to a test tube 1.0 mL of glycine buffer, pH 10.0, and 50 µL of either heparin-treated plasma or serum. After a 5-min incubation at 37 °C, we added 10 µL of BO solution containing 0.6 U (measured with UB as the substrate in Tris buffer, pH 8.5; see below). We mixed the solution, started a stopwatch, and aspirated the mixture into the spectrophotometer cuvette, which was thermostated at 37 °C. The absorbance was recorded automatically by the instrument every 10 s for 2 min. We analyzed controls and calibrators (DTB) in the same way. (Note: in this procedure, the volume of the BO solution may vary, e.g., from 10–50 µL, which is acceptable as long as the activity of the enzyme per test remains between 0.5 and 0.8 U.)

**Blank.** To measure the blank, we used the same procedure as for the test but substituted glycine buffer for BO.

**Calculations.** The concentration of CB in the specimens was calculated by comparing the difference in absorbance (A) between the blank and the test at 2 min (ΔA = Ablank − Atest) to that of the calibrator, or it was obtained from a calibration curve constructed by analyzing multiple calibrators; for this study we included four calibrators (25, 50, 100, and 200 mg/L DTB) in every analytical run.

**Measurement of BO activity**

There was a wide variation in the activity of BO (units in vial) purchased from various sources and between BO lots from the same source. For example, we assayed vials containing 200 U of BO according to the manufacturer and found activities from 80 to 650 U. This may be related to the lack of a uniform procedure for establishing BO activity.

Because UB is oxidized to some extent in this CB method and because the extent of oxidation depends on the activity of BO in the reaction mixture, it was necessary
to assay BO preparations so that the activity of enzyme used in the CB method was between 0.5 and 0.8 U per test. We used the following procedure for measuring the activity of BO: We dissolved the contents of the vial in 5 mL of distilled water and used a small aliquot to make a series of dilutions in water. Each dilution was assayed for BO activity by use of a 200 mg/L UB solution in pooled human sera as described under "Enzymatic procedure for bilirubin conjugates", that is, using 1.0 mL of buffer, 50 μL of UB solution, and 10 μL of BO. The enzyme dilution that provided a linear decrease in absorbance between 10 s and 1 min was used to calculate the activity of BO in U/L from the ΔA/min and the molar absorptivity of UB in Tris buffer, which is 54 700 L · mol⁻¹ · cm⁻¹. The BO activity in the vial was calculated by multiplying the measured activity by the dilution factor and by the volume (in liters) used to reconstitute the BO in the vial.

We want to point out that the activity of the BO in the oxidation of CB (DTB as the substrate in glycine buffer, pH 10.0) is 34-fold lower than the activity in the oxidation of UB (UB as the substrate in Tris buffer, pH 8.5).

KINETICS OF BO
The kinetics of BO were determined with DTB as substrate by varying the DTB concentration while keeping the BO activity and the initial oxygen concentration constant. All reagents were equilibrated with air. The assay solution consisted of 1.0 mL of glycine buffer, 50 μL of DTB in pooled human sera, and 10 μL of BO solution (0.6 U). The reaction was initiated by adding BO and followed by monitoring the decrease in absorbance at 460 nm. The maximum reaction rates (ΔA/min) were calculated from the initial linear region of the reaction curves (<60 s) and corrected for the spontaneous oxidation of DTB, if there was any. The Kₘ and Vₘₐₓ were estimated from the double reciprocal (Lineweaver-Burk) plot.

RESULTS

SPECIFICITY OF THE ENZYMATIC METHOD
A serum specimen from a jaundiced patient was analyzed by HPLC before and after incubation with BO for 30 min (Fig. 1). The CBd was almost completely oxidized, and the CBm was oxidized to a great extent. δ-Bilirubin was slightly oxidized, whereas the increase in the peak height of UB could be attributed to partial hydrolysis of CBm or CBd. When UB solutions in serum were analyzed by the proposed method for CB, only 5% of UB was oxidized under the conditions of the assay (Fig. 2). The absorbance of pure δ-bilirubin, isolated from human serum (6), remained unchanged even when the reaction time was extended to 10 min.

REACTION RATES
Shown in Fig. 3 are the reaction rates for DTB in human serum, bile isolate, δ-bilirubin, and a typical patient’s plasma with a high concentration of CB. The reaction rates
for DTB in three protein matrices are depicted in Fig. 4. DTB in BSA was oxidized much slower than DTB in human serum or HSA.

**Dependence of CB Values on the Oxidation (Reaction) Time**

Patient specimens with high CB concentrations and bile isolate in human serum were analyzed by the proposed method except that the absorbance of the reaction mixtures was followed for up to 10 min. CB concentrations were calculated for various reaction times by use of the corresponding $D_A$ values of DTB calibrator solutions. The results in Table 1 show a dependence of CB values on the reaction time. The 10-min CB value of each specimen was assumed to represent complete oxidation of CB and was used to calculate the percentage of CB oxidized at other times.

**Linearity, Precision, Interference by Hemoglobin**

The linearity of the proposed method was evaluated by analyzing dilutions of a patient’s plasma specimen with high CB concentration (232 mg/L) and dilutions of a DTB solution (196 mg/L) in human serum. Under the conditions of the assay linearity (milligrams of CB per liter of reaction mixture) extended to at least 9.4 mg/L, corresponding to 200 mg/L CB in plasma or DTB in human serum (Fig. 5).

The long-term precision of the assay was established by analyzing over a period of 6 months two controls prepared from sera with high concentrations of CB; aliquots of the controls were kept at −70 °C until used. Shown in Table 2 are precision data for the controls and the reproducibility of the $\Delta A_{460}$ nm of a DTB solution in human serum.

The interference of hemoglobin was evaluated by adding various amounts of a hemolysate to aliquots of a jaundiced plasma specimen. At a hemoglobin concentration of 3 g/L (the highest concentrations tested), the CB concentration of the specimen increased from 73.0 to 75.2 mg/L, a negligible change.

**Comparison of CB Values by Various Methods**

Plasma specimens with increased CB values were analyzed by the enzymatic method. The mean CB value was 0.2 mg/L, with a range of 0.0–1.2 mg/L. The total bilirubin values of the 51 specimens were 3–11 mg/L (range), with a mean of 6 mg/L.

**Formation of Biliprotein**

Solutions of DTB (220 mg/L) in HSA (40 g/L) and BSA (40 g/L) were allowed to stand at room temperature (in the dark) for up to 60 h. The DTB solution in HSA was analyzed by HPLC 1 and 24 h after it was prepared, and the DTB solution in BSA was analyzed 1 and 60 h after preparation. The chromatograms showed a bilirubin fraction that eluted at the same time (14.6 min) as the naturally occurring biliprotein in human serum. Furthermore, the absorption peak at 450 nm was superimposed on the peak at 280 nm. The amounts of biliprotein formed for DTB in HSA were 3.4 and 10 mg/L at 1 and 24 h, respectively; for DTB in BSA, the amounts of biliprotein formed were 4.8 and 13.8 mg/L at 1 and 60 h, respectively. Because the absorptivity at 450 nm of the biliprotein formed in human blood from CB and serum albumin is much higher than that of UB (6), the amounts of the DTB-biliprotein calculated from the HPLC tracings are most likely overestimated.

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**Table 1. Dependency of CB values (mg/L) on oxidation time.**

<table>
<thead>
<tr>
<th>Time, min</th>
<th>Bile Isolate</th>
<th>Patient 1</th>
<th>Patient 2</th>
<th>Patient 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg/L</td>
<td>%</td>
<td>mg/L</td>
<td>%</td>
</tr>
<tr>
<td>1</td>
<td>67</td>
<td>54</td>
<td>82</td>
<td>65</td>
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<td>10</td>
<td>125</td>
<td>100</td>
<td>126</td>
<td>100</td>
</tr>
</tbody>
</table>

* CB values were calculated using the $\Delta A$ of DTB at the same times.

* Percentage of the 10-min value, which was assumed to be 100% complete.
The BO kinetics experiment was conducted with three DTB solutions having concentrations from 49.2 to 147.6 mg/L. A double reciprocal (Lineweaver-Burk) plot provided a straight line (Fig. 7) described by the equation: 

\[ y = 1.79x + 0.0105; r^2 = 0.999. \]

The \( K_m \) and \( V_{max} \) values calculated from this equation were 172 \( \mu \)mol/L and 95 \( \mu \)mol/min, respectively.

**Discussion**

We developed an enzymatic method for measuring the concentration of the sum of the two bilirubin glucuronides in serum/plasma that could be adapted to a variety of clinical analyzers. The measurement of CB is preferable to that of DB (by the various versions of the diazo method) because it permits distinguishing between the presence and absence of cholestasis.

The procedure may be calibrated with DTB calibrators in human serum or in HSA; DTB in BSA is oxidized much slower than in human serum or HSA (Fig. 4) and, therefore, is not a suitable calibrator. HPLC analysis of DTB solutions in human serum, HSA, or BSA revealed that DTB reacts spontaneously with albumin to yield small amounts of a biliprotein analogous to that formed in the reaction between albumin and bilirubin glucuronides. Presumably, DTB is linked to albumin through the sulfonic acid group and a free amino group on the albumin molecule. The DTB-albumin complex starts forming upon the addition of DTB to the protein solution and increases with time when the solution is allowed to stand at room temperature. The reaction stops upon freezing. This biliprotein is likely present in calibrators and controls that contain DTB. At this time, we do not know whether this biliprotein is oxidized by BO in the proposed assay.

Our findings do not support the conclusion of Franzini and Cattozo (8) that “After prolonged incubation at 37 °C (up to 24 h) of DTB solutions in different protein matrices (HSA, HSR and BSA) no evidence for biliprotein formation has been obtained” (8). It appears that these authors

### Table 2. Long-term precision of the enzymatic assay for CB.

<table>
<thead>
<tr>
<th>DTB calibrator (54 mg/L), ( \Delta A_{460 \text{ nm}} )</th>
<th>CB in control, mg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Level 1</td>
</tr>
<tr>
<td>Mean</td>
<td>0.183</td>
</tr>
<tr>
<td>SD</td>
<td>0.003</td>
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<tr>
<td>CV, %</td>
<td>1.8</td>
</tr>
<tr>
<td>( n )</td>
<td>11</td>
</tr>
</tbody>
</table>

Fig. 6. Comparison of CB values in patient plasma specimens obtained with the BO and HPLC methods.

*Line A, CB <100 mg/L: \( y = 0.64 (\pm 0.04)x + 3.0 (\pm 1.6); r^2 = 0.86; \bar{x} = 26.6 \) mg/L; \( \bar{y} = 20.1 \) mg/L; \( n = 34. \) Line B, CB >100 mg/L: \( y = 1.21 (\pm 0.13)x - 32.5 (\pm 20.1); r^2 = 0.91; \bar{x} = 145.4 \) mg/L; \( \bar{y} = 144.1 \) mg/L; \( n = 10. \)*
considered the small amounts of biliprotein (~5 mg/L) shown in Table 2 of Ref. (8) to be artifacts. It should be pointed out that Franzini and Cattozo used gel filtration to isolate the biliprotein, a technique that may be less sensitive and specific than HPLC.

The specificity of the CB measurement is very good because δ-bilirubin is not oxidized and only ~5% of the UB is oxidized regardless of its concentration; this small interference for UB is considered tolerable because it will not lead to erroneous diagnoses. The interference by hemoglobin is negligible.

The 2-min reaction time was chosen to keep the oxidation of UB at a minimum. We believe that an essential requirement for a reliable procedure for measuring CB is specificity, that is, measuring as little UB and δ-bilirubin as possible. Because we made these measurements with a manual spectrophotometer, it would not have been practical to use a shorter reaction time. If this method were to be adapted to automated clinical analyzers, the reaction time would most likely be reduced to 30 s or less.

The precision of the method is comparable to that obtained by the diazo DB methods and adequate for clinical purposes. Claims about the accuracy of the method must be qualified. Because of its specificity, the method can be considered as being accurate, although it is not certain that it measures the exact concentration of the two bilirubin glucuronides because there are many potential sources of error. The molar absorbivities of DTB, CBm, and CBD may not be identical, and the reaction rate of CB in serum appears to be slower than that of the DTB calibrator (Table 1). In addition, the oxidation rate of CBm may not be the same as that for CBD. Despite these uncertainties the measurement of CB is still valid because absolute accuracy is not that relevant because once the diagnosis of hepatobiliary dysfunction is established, the exact concentration of CB in serum is of little help to the clinician. It is the disappearance of CB from blood that signals that the hepatobiliary obstruction has been relieved.

Because the concentration of CB in the serum of healthy adults is too low to be detected by routine laboratory methods (9), the near zero CB values obtained by this method in healthy subjects are not unexpected.

Comparison of values obtained with the BO method and those obtained with HPLC is somewhat puzzling. It appears that samples with a CB concentration <100 mg/L have a slope different from those of samples with a CB concentration >100 mg/L. We have no explanation for this inconsistency.

Under the conditions of the assay, bilirubin is oxidized to biliverdin even in the absence of BO. This spontaneous oxidation has been observed previously (3) and is enhanced by the presence of transition metal ions, which are ubiquitous in plasma. By adding 50 mmol/L mannitol to the glycine buffer (10), we were able to prevent the spontaneous oxidation, which could introduce an inaccuracy in the CB measurement. In the absence of mannitol, we have observed a mean decrease of 1.4 mA/min, which was reduced to 0.15 mA/min when mannitol was added to the buffer.

References


Fig. 7. Kinetics of BO with DTB as substrate at pH 10.0.
Lineweaver-Burk plot: \( y = 1.79x + 0.0105; r^2 = 0.999 \). [S], concentration of substrate.