Measurement of Direct Bilirubin by Use of Bilirubin Oxidase

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We developed an enzymatic method for measuring direct-reacting bilirubin (DBIL) in serum. At pH 4.5, bilirubin oxidase (BOX) oxidizes mono-conjugated bilirubin, di-conjugated bilirubin, and most of the δ-bilirubin to biliverdin. The resulting decrease in absorbance at 460 nm is linearly related to the concentration of DBIL in serum. Mean DBIL values in the 51 patients' sera examined by the BOX method and a diazo procedure (Clin Chem 1982;28:2305) were 45.4 and 42.8 mg/L, respectively. For the same samples, mean values for DBIL and conjugated bilirubin by the Kodak "Ektachem" methods were 50.2 and 24.8 mg/L, respectively. Hemoglobin, up to 1.5 g/L, does not interfere. Unconjugated bilirubin reacts negligibly. Day-to-day CVs were 2.2% and 2.4% at DBIL concentrations of 37 and 74 mg/L, respectively.

Methods commonly used to measure direct-reacting bilirubin (DBIL) are based on either the diazo reaction or direct spectrophotometry. DBIL values obtained by diazo procedures vary with the concentration and type of the diazo reagent (1), the pH of the reaction mixture (2), and the duration of the reaction (3). These factors are mainly responsible for the large variability in DBIL values obtained for specimens analyzed by different methods (4). The non-quantitative reaction of bilirubin diglucuronide (dB) (5), δ-bilirubin (Bδ) (1), and pure ditaurobilirubin (DTB) (6) in the direct diazo assay has been amply documented.1 Direct spectrophotometry is not affected by the variables associated with diazo assays, but it is available only to those who possess a certain type of instrumentation (7, 8).

Bilirubin oxidase (EC 1.3.3.5; BOX) from Myrothecium verrucaria MT-1 (9, 10) catalyzes the oxidation of bilirubin to biliverdin. Perry et al. (11) used BOX, at pH 8.2, for measuring total bilirubin in serum. Here we describe the use of the same principle for the determination of DBIL in serum. At pH 4.5, BOX catalyzes the oxidation of bilirubin monoglucuronide (mB), dB, and most of the Bδ. Unconjugated bilirubin (B0) is not oxidized at this pH. The proposed method is calibrated with solutions of ditaurobilirubin in human serum. We evaluated the precision, linearity, and specificity of the method and compared the results for patients' sera with those obtained by a diazo procedure (12) and by a film-based spectrophotometric method (8).

Materials and Methods

Stock ditaurobilirubin standard, 200 mg/L. We used commercial grade DTB (Porphyrin Products, Logan, UT 84321)

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1 Nonstandard abbreviations: HPLC, "high-performance" liquid chromatography; TBIL, total bilirubin; B0, unconjugated bilirubin; DBIL, direct-reacting bilirubin; mB, bilirubin monoglucuronide; dB, bilirubin diglucuronide; Bδ, bilirubin conjugates (with glucuronic acid); B0, δ-bilirubin; DTB, ditaurobilirubin; BOX, bilirubin oxidase.

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and a purified preparation, lot 56S, donated by Dr. Howard A. Lee (Lee Scientific, Inc., St. Louis, MO 63144). We prepared solutions by dissolving about 30 mg of DTB in 2 mL of water and diluting to 100 mL (volumetric flask) in pooled human sera with negligible bilirubin concentration.

Standard blank. Place 4 mL of water in a 200-mL volumetric flask and dilute to volume with the pooled sera used to prepare the stock standard solution.

Working DTB standards. Dilute the stock DTB standard with the standard blank to give DTB concentrations of 10, 20, 50, 100, and 200 mg/L. Dispense these solutions into polypropylene tubes and store at −70°C. These standards are stable for at least six months.

The concentration of DTB (expressed as B0) in the stock standard was established by the method of Doumas et al. (13). Because it has been shown previously that the DTB preparations used in this study were free of Bδ (6), the DBIL concentration in the standard is equal to the value obtained from the analysis for total bilirubin.

Bilirubin conjugates (Bδ). We prepared solutions of Bδ, isolated from human bile (14), by dissolving the isolate in human serum, dispensing aliquots in vials, and lyophilizing them. The percentage composition of the enriched serum pool, which was established with the use of HPLC (15) by Robert Ferris (Eastman Kodak Company), was: Bδ, 9%; bilirubin monoglucuronide, 42%; bilirubin diglucuronide, 44%; B0, 5%.

Bδ standard solutions. These were prepared by adding Bδ (Standard Reference Material no. 916; National Bureau of Standards, Washington, DC 20234) to pooled human sera as described elsewhere (13). The standard solutions were stored at −70°C until used.

Controls. These were prepared from DTB as described above for the DTB standards; aliquots were dispensed in vials and lyophilized.

δ-Bilirubin. This was prepared as described elsewhere (15).

Reagents

Phosphate buffer, 0.2 mol/L. Dissolve 27.2 g of anhydrous KH2PO4 and 120 g of urea in 900 mL of water. Adjust the pH of the solution to 4.5 ± 0.05 with 1 mol/L acetic acid and dilute to 1 L with water.

Bilirubin oxidase. The lyophilized reagent, containing 27.5 U of enzyme activity (as measured at 37°C), was reconstituted with 0.5 mL of water. Bilirubin oxidase is also available from Amano International Enzyme Co., Inc., Troy, VA 22974.

De-ionized water was used throughout this study.

We used a Cary 210 spectrophotometer (Varian, Inc., Palo Alto, CA 94303) for absorbance measurements.

Bilirubin Oxidase Method

Add 20 μL of reconstituted bilirubin oxidase solution to 50 μL of sample that has been mixed with 1.0 mL of buffer (test). Prepare a sample blank by substituting either buffer
or water for the bilirubin oxidase. After adding the enzyme to the test, incubate both test and sample blank for 15 min at 37 °C, and then measure their absorbance at 460 nm vs water. Calculate the DBIL concentration in the sample from the difference in absorbance (absorbance of sample blank minus that of test) by use of a calibration curve prepared from DTB standards analyzed by this method.

Comparison Methods

For total bilirubin we used the method of Doumas et al. (13); the diazo method for DBIL has been described elsewhere (12). We used a Kodak Ektachem 700 Analyzer to measure B0, B5, and DBIL. This analyzer measures TBIL by a diazo method and unconjugated bilirubin (B0) and sugar conjugated bilirubins (B5) by differential direct spectrophotometry (B0, B5 slide). Direct-reacting bilirubin (DBIL) and B5 are not measured directly; they are calculated as follows: DBIL = TBIL − B0; B5 = TBIL − (B0 + B5).

Results

Absorption Spectra

Figure 1 shows the absorption spectra of ditaurobilirubin before and after incubation with BOX, and the differential spectrum (sample blank vs test) at pH 4.5. The appearance of absorption peaks near 380 nm and 670 nm indicates that DTB is oxidized to the corresponding biliverdin. The difference in absorbance between test and blank is greatest at 460 nm; this is also the case for the bile isolate (B0) and for patients’ samples containing DBIL.

The spectra of DTB, B0, and sera from jaundiced adults are influenced by the pH of the buffer (Figure 2, A–C); B0 and B5 exhibit broad absorption maxima at 420 and 454 nm, respectively, which do not change appreciably with changes in pH (data not shown). The spectral shifts for DTB and B5 are qualitatively, but not quantitatively, similar; as the pH changes from 4.7 to 4.3, the decrease in absorbance at 460 nm is greater for DTB than for B5. Because DTB is the calibrator for this assay, variations in buffer pH could affect values obtained for DBIL in patients’ samples.

Experimental

Time required for completion of the reaction. This was established by measuring the absorbance of test solutions, at 460 nm, at various intervals. The test solutions were:

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Fig. 1. Absorption spectra of DTB in phosphate buffer (0.2 mol/L, pH 4.5) before and after oxidation with BOX
(- - -) sample blank; (· · ·) test; (---) sample blank vs test
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DTB, B0, and patients’ samples with high DBIL concentrations. Typical data are shown in Figure 3. DTB and B0 are oxidized faster than DBIL in patient’s serum; the slower oxidation of the latter is probably attributable to the high (104 mg/L) content of B0. Because there was no decrease in absorbance after 14 min, we used a 15-min reaction for the BOX procedure.

Reactivity of various bilirubins in the proposed procedure.

The sum of the three direct-reacting bilirubins (mB0, dB0, and B5) is the total bilirubin, as determined by HPLC, is 95% of the total bilirubin concentration, or 168 mg/L (0.95 × 177 mg/L, TBIL). DBIL values for the bile isolate by the BOX and diazo procedures were 162 and 140 mg/L, respectively. Another solution of the bile isolate, for which the calculated DBIL concentration was 126 mg/L, gave DBIL values of 124 and 121 mg/L by the Ektachem and BOX procedures, respectively. These data indicate that the BOX method more accurately measures direct-reacting bilirubins than the direct diazo procedure, which is known to underestimate the concentrations of DTB (6) and dB0 (5).

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Fig. 2. Absorption spectra of bile isolate (A), DTB (B), and patient’s serum (C) in phosphate buffer (0.2 mol/L)
---------- pH 4.3; (→) pH 4.5; (· · ·) pH 4.7

Fig. 3. Rate of oxidation of DTB (→), bile isolate (· · ·), and patient’s serum (———) in the enzymatic method for direct bilirubin
DBIL concentrations: DTB, 221 mg/L; bile isolate, 168 mg/L; patient’s serum, 190 mg/L
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Pure B$_4$ is oxidized slowly and incompletely by BOX; a B$_4$ solution having a TBIL concentration of 75 mg/L (as B$_4$) gave a value of 19 mg/L for DBIL when it was analyzed by the enzymatic method. However, in view of the close agreement of the DBIL values obtained by the BOX and Ekta-chem procedures (see Patient-Comparison Study below), we conclude that the reactivity of native B$_4$ (B$_4$ in human serum) is strikingly different from the reactivity of the same fraction that had been isolated by HPLC.

The reactivity of B$_4$ is negligible and appears to be independent of its concentration (Table 1). We doubt that the small and constant difference in absorbance between sample blank and test is in fact due to oxidation of B$_4$.

**Linearity.** The absorbance (A)–concentration relationships for DTB, bile isolate, and jaundiced human serum (Figure 4) show good linearity and adherence to Beer’s law. This statement is based on the small standard errors of the slopes (ranging from 0.25 to 1.1% of the slope values) and on intercepts that are not significantly different from zero. Solutions of commercial-grade or purified DTB (6) having the same TBIL concentration gave identical ΔA’s at 460 nm; thus, the commercial-grade DTB is suitable for use in calibrating the proposed procedure.

**Interference by hemoglobin.** Known amounts of a hemoglobin solution, prepared according to Sunderman (16), were added to pooled sera from jaundiced adults. The original pools and those containing the added hemoglobin were analyzed by the BOX method. Table 2 shows that hemoglobin in concentrations up to 1.5 g/L caused no interference. Although interference is seen with hemoglobin concentrations >2 g/L, it is much less than that observed with the diazo procedure.

**Turbidity.** Sera from healthy subjects did not cause turbidity when added to phosphate buffer or during the 15-min reaction. However, sera from jaundiced adults or plasma from blood samples collected with heparin almost invariably yielded turbid solutions during the reaction period, which could introduce inaccuracies in the measurement of absorbance and, hence, of DBIL values. Addition of urea to phosphate buffer prevents turbidity which, as determined by electrophoresis of the precipitate, develops from the precipitation of serum globulin or fibrinogen (or both) from such samples. Plasma samples from neonates collected in Carraway tubes (350-μL tubes), containing either Li-heparin or NH$_4$-heparin, often gave turbid solutions when added to the phosphate–urea buffer. Plasma samples obtained with Na-heparin often gave turbid solutions only after they had been introduced into tubes containing Li- or NH$_4$-heparin. It appears that certain types of heparin will cause turbidity, even in the presence of urea in the buffer, and they should be avoided.

**Precision.** Table 3 details the day-to-day precision for the DBIL values of two controls, and the ΔA values for two calibrators. The reproducibility of the BOX procedure is comparable to that obtained by the diazo procedure (6).

**Reference intervals.** Reference intervals for healthy subjects have not been established by the proposed method, but on the basis of the following evidence they are expected to be similar to those obtained by the diazo procedure. We assayed 19 serum specimens from healthy subjects by the BOX and the diazo (12) procedures. Mean DBIL values (and ranges) were 1.5 (0.8–2.6) mg/L and 1.6 (0.0–1.3) mg/L, respectively. Reference DBIL values established previously in our laboratory by the same diazo procedure were (n = 109): X = 1.2 mg/L, SD = 0.4 mg/L; observed range, 0.2 to 2.8 mg/L. Two samples had DBIL concentrations greater than 2

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<th>Table 1. Unconjugated Bilirubin Reacting as Direct Bilirubin in the Enzymatic and Diazo DBIL Methods</th>
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<td>$B_4$, mg/L</td>
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<th>Table 2. Interference by Hemoglobin in the Enzymatic and Diazo DBIL Methods</th>
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<td>Hemoglobin, g/L</td>
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<th>Table 3. Long-Term Precision for Ditaurobilirubin Controls and Calibrators by the Enzymatic DBIL Method</th>
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<td>$B_{DBIL}$, mg/L</td>
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Bilirubin concentrations in calibrators 1 and 2 were 56 and 112 mg/L, respectively.
mg/L (2.2 and 2.8 mg/L), and three less than 0.4 mg/L. It appears, therefore, that reference values from both methods are quite similar.

Patient-Comparison Study

We assayed 51 patients’ samples for DBIL by the BOX and the comparison methods. Of the 51 samples, 43 were from different patients and eight were replicates, collected on different days, from six of the patients. Thirty-nine patients had cholestatic jaundice and four had TBIL values <12 mg/L. Because of occasional discrepancies in the TBIL values obtained by the reference and Ektachem methods and because the DBIL value from the Ektachem is calculated by subtracting B₈ from TBIL, we used the reference TBIL values for calculating the Ektachem DBIL concentrations (TBIL minus B₈).

Values for DBIL by the BOX procedure were 4% lower than those from the Ektachem (Figure 5A), and 50% higher than the B₈ (mB₈ + 3B₀) (Figure 5B). These data indicate that the BOX method measures, in addition to B₈, most of the B₀ present in serum. The underestimation of bilirubin conjugates (5) and B₈ (I) by the direct diazo procedure explains the results shown in Figure 5C; DBIL values by the BOX method are 17% higher than those of the diazo procedure.

The high correlation coefficients indicate a close association among DBIL values obtained by the BOX and the comparison methods. The considerable scattering of the values about the slopes is reflected in the large standard errors of the estimate, and indicates that all of the methods tested do not measure exactly the same thing. The statistically significant intercepts reveal systematic biases between the enzymatic and the comparison methods.

The concentration of B₈ in the 51 serum samples ranged from 3 to 108 mg/L. To check the accuracy of the calibration of the Ektachem for B₈, we used three pure (by HPLC) B₈ solutions (1). TBIL values for these solutions by a diazo procedure (13) were 13, 25, and 75 mg/L; the corresponding B₈ values obtained with the Ektachem were 10, 18, and 62 mg/L. These results suggest that the Ektachem may underestimate the B₈ concentration in serum by about 20%.

Discussion

We developed a new approach for measuring direct-reacting bilirubin in serum by use of bilirubin oxidase. The proposed method is simple and measures almost quantitatively all of the direct-reacting bilirubin fractions. The low pH of the reaction mixture prevents detection of unconjugated bilirubin, a most important requirement for a reliable DBIL method. Failure to prevent the reaction of B₈ could, in certain clinical conditions, lead to erroneous diagnosis. Gilbert’s syndrome is distinguished from the Dubin–Johnson or Rotor syndromes by the absence of conjugated bilirubin in Gilbert’s syndrome. In neonatal jaundice, DBIL concentrations of 15 mg/L or more warrant diagnostic evaluation, because they are usually associated with severe infections, sepsis, biliary atresia, or hepatitis (17). In pure hemolytic (prehepatic) jaundice in the adult, B₈ is the only bilirubin fraction found in serum. In all of the above clinical conditions, falsely abnormal DBIL values resulting from inadequate methods could mislead the physician and obscure the diagnosis.

Calibration is performed with DTB, which is readily available and of sufficient purity (6). As long as commercial DTB preparations are free of B₈, the concentration of DBIL in DTB solutions can be assigned by analysis for TBIL (13). Protein matrices other than human serum must be evaluated before they are used for preparing calibrators for the BOX method. The spectral characteristics of DTB at pH 7.4 vary with the protein matrix (6) and might also be different at pH 4.5. We chose the wavelength of 460 nm because the ΔA (the absorbance of the sample blank minus that of the test) is at a maximum there. Although changes in the pH of the buffer could cause some variability in the ΔA values of either the calibrator or patients’ samples, the SD of the ΔA values for the calibrator (Table 3) indicates that the variability is small when the buffer pH is controlled. The lack of an isobestic point (Figure 2, A–C) limits the choice of the wavelength to that which provides optimum sensitivity. Buffers other than phosphate could be used for the BOX procedure. We have found that acetate buffer at pH 4.5 is also suitable for this assay, and it is currently under evaluation.

The precision of the enzymatic method is similar to that of either the diazo procedure (6) or the Kodak Ektachem (7), and it is clinically acceptable. As with all methods for measuring DBIL, no claim about absolute accuracy can be made for the BOX method. This is because all methods for DBIL have certain limitations. Diazoo procedures underestimate DBIL (5, 6) and are subject to interference by hemoglobin, even when it is present at low concentrations (Table 2). With HPLC it is assumed that the absorption maxima and molar absorptivities of the three direct-reacting bilirubin fractions are identical to those for B₈, although, in fact, they are not known. With the Ektachem slides, values for B₈ and DBIL are calculated rather than directly measured.

As it stands, the proposed method is an endpoint assay with a rather long reaction time. It might be feasible to shorten the reaction time by developing a rate method, which could be adapted to a variety of automated clinical analyzers and thus eliminate the need for a sample blank. Furthermore, by selecting different reaction conditions, it might be possible to avoid measuring the slow-reacting B₈, and detect only the bilirubin conjugates, which provide a better indicator of hepatobiliary cholestasis (relief or persisting impairment) than do DBIL or TBIL (18).

We are grateful to Beckman Instruments, Inc., Carlsbad, CA 92008, for furnishing the bilirubin oxidase used in this study.

References