Pitfalls in the American Monitor Kit Methods for Determination of Total and "Direct" Bilirubin

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We evaluated the American Monitor Corporation kit for total and direct-reacting bilirubin and found that it suffers serious deficiencies, which lead to inaccurate and imprecise results. The main problem with the total-bilirubin procedure is that the short reaction time (2 min) is inadequate for completion of the reaction. The poor precision of the direct-bilirubin method is due to the short reaction time and the inability of the "stabilizer" (hydroxylamine sulfate) to completely destroy the diazo reagent. Depending on when Fehling's reagent is added, the reaction time may vary from 2 min to 7 min. Values for direct bilirubin at 7 min exceed those obtained at 2 min by 17 to 29%. The short reaction time makes color development temperature dependent, an additional source of imprecision. The suboptimal concentration of the diazo reagent results in underestimation of direct-reacting bilirubin. We recommend changes that improve both precision and accuracy of the kit procedures.

For several years, we have served as a reference laboratory for the assignment of primary values for commercial quality-control sera. For bilirubin one of the methods we were asked to use is that of the American Monitor Corporation, Indianapolis, IN 46268. It is based on the Jendrassik–Grob principle (7).

We report our experience with the American Monitor kit for the determination of total and "direct" bilirubin (TBIL and DBIL).

Materials and Methods

Equipment

We used a Cary 210 spectrophotometer (Varian, Inc., Palo Alto, CA 94303) and "Suprasil (QS)" cuvets (specified light-path 10 ± 0.01 mm; Hellma Cells, Inc., Jamaica, NY 11424) for absorbance measurements.

Materials

We used the following BIL preparations: (a) Bilirubin control (lot no. C111045; Beckman Instruments, Inc., Brea, CA 92621). This control contains both unconjugated and conjugated bilirubin; the latter is taurine-conjugated, and is available from Lee Scientific, Inc., St. Louis, MO 63144. (b) A homemade control (Control A) containing conjugated bilirubin isolated from human bile by the method described by Lucassen (2). (c) Jaundiced sera from adults.

Bilirubin standard solutions. These were prepared by adding "reference" grade bilirubin (Pfanstiehl Laboratories, Inc., Waukegan, IL 60085) to human serum pools as described elsewhere (3). We analyzed two lots of Pfanstiehl "reference" grade bilirubin (nos. 8407 and 12622) and Standard Reference Material 916 (U.S. Natl. Bureau of Standards) several times by the method of Doumas et al. (3). The mean molar absorptivities at 600 nm were: lot 8407 (n = 7), 75 600 (L mol⁻¹ cm⁻¹); lot 12622 (n = 5), 75 700; SRM 916 (n = 7), 75 800.

Methods

American Monitor. We used the TBIL and DBIL procedures ("Macro- Methods") as described by the manufacturer. Unless otherwise indicated, we added the Fehling's reagent 20 s after adding the "stabilizer" (hydroxylamine sulfate). Other methods. For TBIL we used the method of Doumas

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et al. (3). For DBIL we used a combination of the methods of Michaelsson (4) and Gambino (5).

The procedure is as follows: Pipet 0.25 mL of sample into 1.0 mL of 50 mmol/L HCl. Add 0.5 mL of diazo reagent and after a 10-min incubation (room temperature), add 0.1 mL of a 20 g/L solution of ascorbic acid, followed immediately by 1.5 mL of alkaline tartrate and 2.0 mL of caffeine reagent. The concentrations of diazo, alkaline tartrate, and caffeine reagents are those described in ref. 3.

The concentration of DBIL was calculated against a standard (unconjugated bilirubin) analyzed by the same procedure except that the sample was added to caffeine reagent and HCl was added at the end.

Sample blanks were prepared by substituting sulfanilic acid for diazo reagent. All absorbance measurements were made at 600 nm.

Results

Total bilirubin. Use of the 2-min reaction time specified by the manufacturer results in serious underestimation of TBIL (Table 1). By prolonging the reaction time to 10 min, TBIL values obtained with the American Monitor kit were much closer to those obtained by our procedure.

Direct bilirubin. The main problem with the American Monitor procedure was poor day-to-day precision; typical results are shown in Table 2. We identified three sources responsible for the irreproducible results: (a) the short coupling time (2 min); (b) the dependence of the coupling reaction rate on temperature; and (c) the choice of hydroxylamine as "stabilizer," which presumably is used to destroy excess diazo reagent before Fehling's reagent is added. DBIL values also depend on the interval between addition of hydroxylamine and of Fehling's solution; the user is instructed to add Fehling's solution no later than 5 min after adding hydroxylamine. It is the combination of these variables that gives rise to irreproducible results.

Effect of Temperature

Results in Table 3 show that with the American Monitor method temperature has a remarkable effect on color development, and therefore on DBIL values. In contrast, DBIL values by our method are unaffected by temperature. Color development in our TBIL procedure is also unaffected by temperature.

Effect of Hydroxylamine on the Diazot Reagent

We studied this initially by adding Fehling's reagent at various time intervals after adding hydroxylamine. As shown in Table 4, DBIL values depend on the time elapsed between the addition of hydroxylamine and of Fehling's solution. These results suggest that hydroxylamine does not completely destroy the diazo reagent and that the coupling reaction proceeds until Fehling's solution is added. The following experiment showed us that hydroxylamine has little effect on the diazo reagent. We prepared a mixture of HCl (50 mmol/L), hydroxylamine, and diazo reagent, using the volumes rec-
Table 5. Lack of Effect of Hydroxylamine on the Diazol Reagent in the American Monitor Procedure for “Direct” Bilirubin

<table>
<thead>
<tr>
<th>Sample</th>
<th>2 min a</th>
<th>10 min a</th>
<th>Regular procedure b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum 1</td>
<td>12.5</td>
<td>11.8</td>
<td>20.0</td>
</tr>
<tr>
<td>Serum 2</td>
<td>16.1</td>
<td>15.6</td>
<td>18.1</td>
</tr>
<tr>
<td>Serum 3</td>
<td>20.1</td>
<td>19.7</td>
<td>30.1</td>
</tr>
<tr>
<td>Beckman calib.</td>
<td>40.5</td>
<td>39.8</td>
<td>46.7</td>
</tr>
<tr>
<td>Control A</td>
<td>72.4</td>
<td>71.1</td>
<td>85.4</td>
</tr>
</tbody>
</table>

a Interval of reaction between hydroxylamine and diazo reagent before addition of sample. b Hydroxylamine added after the samples had reacted with the diazo reagent for 10 min.

Ascorbic acid and diazo reagent were mixed, the diazo peak at 268 nm disappeared within 30 min. The product of the reaction between ascorbic acid and diazo reagent absorbs maximally at 245 nm.

Comparison of “Direct” Bilirubin Values by the American Monitor Method and Our Procedure

It is apparent from the data in Table 6 that DBIL is seriously underestimated by the American Monitor method. Although the accuracy of DBIL measurements cannot be ascertained at this time (owing to the lack of reference preparations for conjugated bilirubins), it is reasonable to assume that the most nearly correct results are obtained by procedures that allow the reaction of DBIL to be nearly complete while keeping the unconjugated bilirubin from reacting. We analyzed bilirubin standards in serum by our DBIL procedure and found the contribution of unconjugated bilirubin to be quite small; at concentrations of 20, 50, 100, and 200 mg of unconjugated bilirubin per liter, the DBIL values were 0.6, 0.9, 1.3, and 1.8 mg/L, respectively.

Ascorbic Acid and the Diazol Reagent

Michaelsson (4) proposed that ascorbic acid be used to destroy excess diazo reagent in the DBIL procedure and to decrease interference by hemoglobin in the measurement of TBIL. Thompson (6) and Simmons (7) used ascorbic acid in preparing a single serum blank for both TBIL and DBIL procedures, by adding ascorbic acid to serum and then diazo and caffeine reagents.

We examined the efficiency with which ascorbic acid destroys the diazo reagent under various conditions. With sera containing high concentrations of DBIL, the approach of Simmons and Thompson is not valid. We found that the diazo reagent is not destroyed instantly and that some DBIL reacts. The consequent high blank results in underestimation of both TBIL and DBIL. For two serum specimens having DBIL values of 120 and 102 mg/L, sample blanks prepared according to Thompson had corrected absorbance values (corrected by subtracting the sulfanilic acid blanks) equivalent to bilirubin concentrations of 14 and 9.5 mg/L, respectively. We had the same experience with Simmons’ continuous-flow procedure. The fact that ascorbic acid does not completely destroy the diazo reagent does not mean that it can be omitted in the DBIL procedure; any remaining diazo reagent is destroyed instantly by ascorbic acid (or hydroxylamine) when alkaline tartrate is added. If ascorbic acid (or hydroxylamine) is omitted in the DBIL procedure, a substantial fraction of unconjugated bilirubin will react when alkaline tartrate is added. For example, when we analyzed a 100 mg/L solution of unconjugated bilirubin by the DBIL procedure without ascorbic acid, the color produced was equivalent to 50 mg of bilirubin per liter. To prevent destruction of the azopigment, Michaelsson (4) recommends adding the alkaline tartrate immediately after ascorbic acid. We added the alkaline tartrate 20 s, 1 min, 2 min, 5 min, and 10 min after ascorbic acid, and found no evidence of azopigment destruction.

Precision

We evaluated the long-term precision of our procedures for total and direct bilirubin by analyzing two control sera (lyophilized). For total bilirubin (n = 21), the CVs were 1.3 and 1.5% for concentrations of 68.4 and 178.5 mg/L, respectively. For direct bilirubin (n = 15), the CVs were 1.6 and 2.1% for concentrations of 24.6 and 50.7 mg/L, respectively.

Discussion

Although the American Monitor kit for TBIL and DBIL is based on a sound principle, the procedures currently recom-
mended by the manufacturer result in serious inaccuracies and imprecision in both determinations.

The accuracy of the TBIL procedure can be improved by extending the time of the coupling reaction to 10 min (Table 1). In our experience a 2-min reaction suffices for bilirubin standards in human or bovine serum albumin, but not for bilirubin in serum.

The DBIL procedure suffers from several deficiencies that cause poor precision and accuracy. One of the problems is the short reaction time, which does not allow the reaction to go to completion; it has been well documented (4, 8) that short reaction times (1 or 2 min) tend to cause underestimation of DBIL, especially when its concentration is high. Failure to allow the reaction to go to completion makes the color development temperature-dependent, an additional source of imprecision and inaccuracy.

We emphasize that we use the term "completion of the reaction" rather loosely here. For specimens containing high concentrations of DBIL, a 10-min incubation is inadequate to bring the reaction to completion. For example, a specimen having a very high concentration of DBIL (about 280 mg/L) gave the highest result for DBIL when we extended the coupling time to 30 min. The 1-, 10-, 20-, and 30-min DBIL values were 180, 263, 270, and 280 mg/L, respectively. Our experience with other specimens having high DBIL concentrations is that the 10-min values are about 95% as high as those for 30 min. Thus, we chose the 10-min reaction time as a practical compromise.

Caffeine reagent is added in the direct procedure to prevent overestimation of DBIL. In its absence the molar absorptivity of azobilirubin at 600 nm is considerably higher than in its presence (4, 8) and, because this reagent is necessary for the reaction of the standard (unconjugated bilirubin), it must be added in the direct procedure.

It is the combination of the use of hydroxylamine and the instruction to add the Fehling's reagent up to 5 min after the "stabilizer" that is chiefly responsible for the irreproducible results. Depending on when the Fehling's reagent is added, the reaction time may vary anywhere from 2 to 7 min. Thus, DBIL values at 7 min can be higher than those obtained at 2 min by 17 to 29% (Table 4).

DBIL values obtained with the American Monitor kit are only 64 to 75% as high as those obtained by our method—a rather serious underestimation. Both precision and accuracy of the DBIL method can be improved substantially by extending the reaction time to 10 min (hydroxylamine and Fehling's reagent should be added in that order after this time).

We have two other suggestions for improving the method: (a) Increase the concentration of the diazo reagent by 150%, i.e., 0.05 mL of sodium nitrite to 3 mL of sulfuric acid. This will provide 1.2 μmol per tube or a molar ratio of bilirubin to diazo of about 5.8 for a 200 mg/L concentration of bilirubin. We found this ratio to be optimal for linearity and completeness of the reaction. When the diazo is prepared according to the manufacturer's directions, this ratio is about 2.3, which is rather low because some of the diazo reacts with serum proteins and caffeine. (b) Change the reagent volumes from the prescribed 2.4 to 2.0 mL and from 1.1 and 0.7 mL to 1.0 mL each. Because volumetric pipets are not available for the fractional volumes called for by the kit manufacturer, this change, which allows the use of such pipets, should improve the precision of the method.

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References