Detection and Measurement of Total Bilirubin in Serum, with Use of Surfactants as Solubilizing Agents

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We describe a new method for measuring total bilirubin in serum. Nonionic, cationic, or anionic surfactants can be used as solubilizing agents to promote the diazo coupling of indirect-reacting bilirubin. A representative surfactant–azobilirubin system is illustrated, in which absorbance is maximum at 560 nm and linear to a concentration of 200 mg of bilirubin per liter. At pH 2.5 and in the presence of Duponol (an anionic surfactant), bilirubin is completely coupled within 6 min at 37 °C. All ingredients required for an assay are combined in two dry reagents, which are stable for a year at room temperature. Sensitivity is comparable to that of procedures routinely used in clinical laboratories; precision, linearity, and stability of the reagents in solution are excellent. Results for fresh sera correlated well with those obtained by the Jendrassik–Grof method. Hemolysis is minimized and turbidity eliminated.

Additional Keyphrases: Jendrassik–Grof method compared • error evaluation as compared with four methods

The methods most widely used to determine bilirubin in serum are based on the discovery of Ehrlich (1) that bilirubin in urine reacts with 1-diazobenzene-sulfonic acid to form a chromophore. Van den Bergh and Snapper (2) applied this discovery to the quantitation of bilirubin in serum. Later, van den Bergh and Muller (3) described the “accelerator” effect of ethanol on this reaction. Malloy and Evelyn (4) described a method in which a smaller alcohol concentration was used, in an effort to avoid loss of bilirubin by protein precipitation. Adler and Strauss (5) found caffeine–sodium benzoate could be used to replace alcohol. Jendrassik and Grof (6) combined caffeine–sodium benzoate with sodium acetate as an “accelerator” to form alkaline azobilirubin at pH 13.4.

As early as 1916, van den Bergh and Muller (3) described the direct and indirect diazo reactions of serum bilirubin. Studies by Cole et al. (7, 8), Billings and Lathe (9), Schmid (10), and Talafant (11) helped elucidate the fundamental differences between “direct”- and “indirect”-reacting bilirubin. The water-soluble pigment called “direct-reacting” bilirubin has been identified as a mixture of bilirubin monoglucuronides and diglucuronides (9), both of which are formed by conjugation of lipid-soluble bilirubin with highly water-soluble glucuronic acid through an ester linkage. The water-insoluble pigment called “indirect-reacting” bilirubin is unconjugated and is bound almost entirely to albumin. For the coupling reaction to occur, bilirubin must be freed from its attachment to albumin and rendered water soluble. Alcohols do this by breaking the salt linkage; caffeine–sodium benzoate solubilizes the bilirubin–albumin complex by a displacement reaction (12).

Turbidity has always been a major disadvantage in bilirubin analysis where alcohol is used as the solubilizing agent. Van den Bergh and Grotepass (13) tried unsuccessfully to overcome this difficulty by precipitating the proteins from an alkaline solution. The methods of Jendrassik and Czike (14) and Haslewod and King (15) also suffer from the loss of bilirubin in the protein precipitate. Several methods have been developed that avoid the precipitation of proteins (4, 16-19). In the technique of Malloy and Evelyn (4) (and its modifications), which has had wide acceptance in the United States, sufficient methanol is added to allow coupling but not enough to precipitate the serum proteins. The serum is diluted 25-fold in this technique, often resulting in cloudy filtrates and turbid serum blanks. Consequently, poor precision at the normal concentration of bilirubin in serum is inevitable. Some other substances—caffeine, sodium benzoate, gum arabic, salicylate, pyridine, and bile salts—promote the diazo coupling of the “indirect-reacting” bilirubin without
the use of alcohol; however, large quantities of these substances are required for complete reaction.

When alcohol is used as a solubilizing agent, temperature effects, often overlooked, may be a significant source of error. Meites and Hogg (21), using the method of Malloy and Evelyn, have shown that when the temperature is lowered from 22 to 10 °C, production of azo pigments decreases by about half. This technique, then, requires either careful temperature control or standardization at different temperatures, procedures inconvenient for routine use. In addition, interference by hemoglobin1 may cause results to be decreased by as much as 45% (20).

In an effort to overcome these difficulties, we undertook to determine if anionic, cationic, or nonionic surfactants could be used as solubilizing agents in the determination of total bilirubin.

Materials and Methods

Reagents


Other reagents: dL-malic acid, sulfanilic acid, sodium nitrite, disodium hydrogen phosphate (Na2HPO4), and sodium carbonate. All were Mallinckrodt AR-grade chemicals.

Bilirubin standard. Dissolve 20 mg of bilirubin (reference standard; Pfister Labs. Inc., Waukegan, Ill. 60085) in 100 ml of a mixture containing sodium carbonate (1 g/liter) and bovine albumin (50 g/liter) (Miles Laboratories, Inc., Kankakee, Ill. 60901).

Buffer mixture. The buffer contains malic acid, 212 mmol/liter, and disodium hydrogen phosphate (Na2HPO4) 15.4 mmol/liter. This buffer system, together with the active optimized components, exhibited a pH of 2.48 ± 0.02 at 25 °C. Adding 200 μl of serum to the buffer system produced a nominal change of 0.09 pH at 25 °C, indicating adequate buffer capacity at this concentration.

Reagent No. 1. Dissolve 53.60 mg of malic acid, 0.25 mg of sodium nitrite, and 4.80 mg of disodium hydrogen phosphate (Na2HPO4) in 2.0 ml of distilled water.

Reagent No. 2. Dissolve 53.60 mg of malic acid, 75.00 mg of “Duponol,” 1.20 mg of sulfanilic acid, and 4.00 mg of disodium hydrogen phosphate (Na2HPO4) in 2.0 ml of distilled water.

Equipment

Absorption spectra were obtained with a Model 124 Double Beam Spectrophotometer equipped with a Model 165 Recorder (both from Perkin-Elmer, Coleman Instruments Division, Maywood, Ill. 60153). All other spectrophotometric measurements were made with a Model B Spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif. 92634).

Procedure

To each “test” and “serum blank” tube was added 2.0 ml of reagent No. 1, followed by 2.0 ml of reagent No. 2 to the “test” tube and 2.0 ml of distilled water to the “serum blank” tube. The contents of both tubes were thoroughly mixed and allowed to stand 1 min at ambient temperature (25 °C). A 200-μl2 aliquot of untreated serum or standard was added to both tubes, mixed, and incubated in a 37 ± 0.5 °C water bath for 6 min. The “serum blank” was adjusted for zero absorbance and the “test” absorbance read at 560 nm within 10 min of the end of the incubation.

Results

Diazoitized sulfanilic acid and bilirubin were coupled with the aid of surfactants as “solubilizing agents” at pH 2.0, 4.5–4.8, and 13. The absorption spectrum of azobilirubin varies with pH, but not with surfactant; absorption spectra, recorded at the same pH with different surfactants, show great similarities (Figure 1). The color of the reaction mixture is blue at pH 2.0, red at pH 4–5, and bluish–green at pH 13.0–13.6, a phenomenon attributed to the changing number of conjugated double bonds in the various azo pigments (22).

To determine whether Beer's law was obeyed if various surfactants were used, we prepared standard curves for several such surfactants at selected pH's. Although the chemical systems were not optimized, the anionic, cationic, and nonionic surfactant systems we used yielded data that were linearly related to concentration to 15–20 mg/100 ml (Figure 2). Our review of the literature indicated that specifications of optimum requirements for diazotization varied among authors (12, 21, 26). We therefore decided to determine optimum conditions experimentally. For optimization and comparison with a reference method, we selected Duponol, as it conveniently promotes the coupling reaction at pH 2.0, the pH at which diazotization of sulfanilic acid occurs. The Duponol system displayed better linearity than other surfactant systems that also solubilize at pH 2.0, especially when hemolyzed serum was used.

Optimization Experiments

The system was optimized with respect to pH, diazotization time, color–development time, and concentration of Duponol, sulfanilic acid, and nitrite. All variables except the one under scrutiny were held constant. Preliminary studies indicated a working pH range for concentration optimization to

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1 Hemoglobin concentration of 10 g/liter of serum.

2 A 100-μl sample may be used if the volume of the test is decreased by half.
be between pH 2.50 and 2.70 at 25 °C. Bilirubin concentration was maintained at about 20 mg/100 ml, to observe response at abnormally high bilirubin concentrations. A nominal concentration of 0.15 mg of nitrite and 0.45 mg of sulfanilic acid per test was included in the starting formulation.

**Duponol.** When Duponol concentration was varied from 10 to 100 mg/test, a plateau between 70-80 mg/test was observed. We chose to use a concentration of 75 mg/test (Figure 3).

**Sulfanilic acid.** On varying this from 0.1 to 1.3 mg/test, an optimum of 1.2 mg/test was chosen (Figure 3).

**Nitrite.** Nitrite was varied from 0.05 to 0.35 mg/test while maintaining optimum concentrations for Duponol and sulfanilic acid; the optimum concentration was found to be 0.25 mg/test (Figure 3).

**pH.** The pH was measured at 25 °C before and after incubation at 37 °C, a nominal change of 0.10 pH unit was observed. At optimum active component concentration, a peak was obtained at pH 2.48 at 25 °C. A pH of 2.48 ± 0.02 at 25 °C was used in the final formulation.

**Diazotization time.** Absorptivity studies of diazotization between nitrite and sulfanilic acid at 240 nm indicate 1 min at 25 °C and slightly less than 1 min at 37 °C is necessary for diazotization to be complete.

**Color development time.** At 560 nm (20 mg of bilirubin per 100 ml) absorptivity is constant between
6–10 min at 37 °C, and 15–18 min at 25 °C. With 5 mg of bilirubin per 100 ml and 37 °C, a plateau between 5–10 min was observed (Figure 4).

**Stability of optimized formula.** Combined reagents, when stored dry, are stable for one year. In solution, reagent 1 is stable for six days at 25 °C, and reagent 2 is stable for at least three months at 25 °C.

**Linearity of optimized test.** Linearity was tested over the range 0–20 mg/100 ml, with use of pure bilirubin reference standard. Two standard curves were prepared, one covering the range 1–5 mg/100 ml (for normal to slightly abnormal sera), and the other 5–20 mg/100 ml (extremely abnormal). Both curves showed good linearity (0.999 linearity coefficient) with only slight curvature at 20 mg/100 ml (Figure 5).

**Interference Effects**

**Serum absorption effect.** Studies of hemolysis-free serum indicated spurious absorption at 560 nm. Absorptivities ranged from 0.2 to 0.5, equivalent to 0.4 to 1.0 mg of bilirubin per 100 ml. To overcome the interference from nonbilirubin pigments, we recommend that a serum blank determination be made with each sample.

**Interference by hemoglobin.** To determine the effect of hemoglobin on the Duponol–azobilirubin system, we did the following: Simulated icteric sera were prepared by supplementing pooled serum with measured amounts of bilirubin reference standard. Nonicteric and the simulated icteric sera were assayed by three independent methods: the "Duponol"–azobilirubin method, an in-house modification of the Jendrassik-Grof method, which correlates with the Malloy and Evelyn method.

3 An in-house nonalkalinized modification of the Jendrassik-Grof method, which correlates with the Malloy and Evelyn method.
the Jendrassik–Grof method, and a modified Jendrassik–Grof method proposed by Meites (23). All sera were assayed before and after addition of hemoglobin in the form of lysed erythrocytes. Recovery data are given in Table 1.

Performance

Comparison with reference method. Sera from 50 patients, containing normal and abnormal concentrations of bilirubin, were assayed by the Duponol–azobilirubin method and by a reference method, that of Jendrassik and Grof (23). This method has been well studied (24-28) and has been found to be highly specific and reproducible. Sensitivity was found to be similar for the two methods (Figure 6).

Precision. Within-day precision was determined by 10 replicate assays on nonicteric sera and pooled serum supplemented at two concentrations. Results showed a CV of 2–3% for bilirubin with a mean concentration of 0.4 mg/100 ml, of 1.8% for 4.7 mg/100 ml, and of 0.9% for 9.4 mg/100 ml. In all cases the standard deviation was <0.1 mg/100 ml.

Day-to-day assays of nonicteric sera and two supplemented pooled sera over a 20-day period, values showed (for mean bilirubin concentration in mg/100 ml, SD, and CV, respectively) of 0.4 ±<0.1, 6.8% (n

<p>| Table 1. Effect of Hemoglobin Interference: A Comparative Study of Three Methods Used on Nine Sera |
|-----------------------------------------------|-----------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Bilirubin mg/100 ml, without hemoglobin</th>
<th>Hemoglobin conc, mg/ml</th>
<th>Bilirubin mg/100 ml, with hemoglobin</th>
<th>Recovery, %</th>
</tr>
</thead>
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<td>0.5</td>
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<td>0.5</td>
<td>100.0</td>
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<tr>
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<td>100</td>
<td>4.7</td>
<td>97.9</td>
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<td>250</td>
<td>0.5</td>
<td>100.0</td>
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<td>4.7</td>
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<td>8.9</td>
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<td>0.5</td>
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* A nonalkalinized in-house modification of the Jendrassik and Grof method which correlates with the Malloy-Evelyn method.

* Spurious negative results, owing to hemoglobin interference at low bilirubin concns.
Table 2. Error Comparison Study: Summary of Statistical Analysis

<table>
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<tr>
<th>Method</th>
<th>Range, mg/100 ml</th>
<th>Proportional error, %</th>
<th>Constant error, mg/100 ml</th>
<th>Random error, mg/100 ml</th>
<th>Correlation coefficient</th>
<th>Number of samples</th>
<th>Error significant?</th>
</tr>
</thead>
<tbody>
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<td>0.5</td>
<td>0.6</td>
<td>0.9981</td>
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<td>&lt;0.1</td>
<td>0.1</td>
<td>0.6</td>
<td>0.8</td>
<td>0.9779</td>
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<tr>
<td>Method of Stone &amp; Weisberg</td>
<td>0.2-10.0</td>
<td>17.8</td>
<td>0.2</td>
<td>0.1</td>
<td>0.4</td>
<td>0.982</td>
<td>5</td>
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<tr>
<td>Method of Powell (23)</td>
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<td>&lt;0.1</td>
<td>0.1</td>
<td>0.2</td>
<td>0.3</td>
<td>0.9947</td>
<td>5</td>
</tr>
<tr>
<td>Method of Powell (23) modified by Leth &amp; Ruthven (24)</td>
<td>0.2-1.5</td>
<td>&lt;0.1</td>
<td>0.1</td>
<td>0.2</td>
<td>0.3</td>
<td>0.9954</td>
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Discussion

There are many published methods for determining bilirubin in serum, which indicates the difficulties of the estimation and suggests that improved methods are needed. Use of a surfactant as a solubilizing agent minimizes many of the difficulties inherent in methods currently being used.

Hemoglobin interference in bilirubin assay has long been a concern. With several diazo methods, the value for total bilirubin is diminished by varying degrees if hemolysate is added (20-23, 30-33). We also find (Table 1) that hemoglobin causes a diminished total bilirubin value, directly in proportion to the hemoglobin and bilirubin concentrations. The effect is more pronounced with the nonalkalinized modification of the Jendrassik-Grof procedure. Additionally, this method gave spurious negative results at low bilirubin concentrations. Results for hemoglobin interference with the Jendrassik-Grof and our method were about the same.

To compare the Duponol-azobilirubin method with methods currently in use, we did an error comparison. Data generated from this study were statistically analyzed and the various types and magnitudes of error determined. From correlation and regression studies based on the method of least squares, random error was calculated as the standard error of estimate in the y direction. Proportional error was measured from the slope of the line and constant error determined directly from the y intercept. Table 2 summarizes the types and magnitudes of error found in our method and compares them with those of four methods calculated from raw data found in the literature (34). A t-test value calculated for our method proved erroneous because of the small constant and random errors (bias = 0.078, SDₜ = 0.170). Application of Student's t-test provides information only on the relative magnitude of the constant and random errors (35). For this reason, all data were evaluated as to individual errors.

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References


* The standard deviation of the differences of the actual y value from the y value calculated from the least-squares equation (y = mx + b) (35).