Automated Jendrassik–Grof Method for Measurement of Bilirubin in Serum with the Greiner Selective Analyzer (GSA II D), and Comparison with the Method Involving Diazotized 2,4-Dichloroaniline

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We automated the Jendrassik–Grof method for measurement of bilirubin with the Greiner Selective Analyzer GSA II D. Comparison with the manual Jendrassik–Grof method showed a highly significant correlation coefficient of 0.994 for total and 0.980 for "direct" bilirubin measurement. The automated method showed good precision and was linear to 667 μmol/L, the highest concentration tested. Addition of hemolysate caused a greater effect on the measurement of direct bilirubin than on total bilirubin. The inhibitory effect on measurement of total bilirubin, though small, was statistically significant at hemoglobin concentrations >2 g/L. Comparison with the method involving diazotized 2,4-dichloroaniline showed the Jendrassik-Grof method to be relatively less affected by hemolysis and turbidity in the measurement of total bilirubin.

Most methods for determination of bilirubin in serum involve coupling diazotized sulfanilic acid with bilirubin to produce a colored product, which is measured colorimetrically. Substitution of 2,4-dichloroaniline for sulfanilic acid reportedly improves the stability of the diazo reagent and the speed of the reaction (1). Küffer et al. (2) have automated this method. Thus, in the method (3) currently available for use with the Greiner Selective Analyzer (GSA II), total bilirubin concentration is determined from its reaction with diazotized 2,4-dichloroaniline in the presence of "Brij-35" (polyoxyethylene lauryl ether), the reaction being inhibited in the blank tube by added sodium azide (4). To determine "direct" bilirubin concentration, the Brij-35 is omitted, and the color developed is measured vs a blank consisting of serum and 2,4-dichloroaniline in hydrochloric acid (5).

However, hemoglobin in concentrations as low as 400 mg/L interferes with the 2,4-dichloroaniline procedure, and hemolysed samples are common from pediatric patients, so we developed and evaluated an automated Jendrassik–Grof method for use on the Greiner Selective Analyzer. In the evaluation we assessed the precision, accuracy, and range of linearity of the proposed method, and compared results with the 2,4-dichloroaniline procedure (4) with respect to interference due to hemolysis and turbidity.

Materials and Methods

Instrument

The GSA II is a multichannel analyzer that is programmed to transfer a pre-determined volume of sample and 100-μL water rinse into each of two process tubes (designated "blank" and "test"), which are set into an incubator maintained at 37 °C. During their 10-min travel to the spectrophotometer, pre-set volumes of reagents are dispensed automatically into appropriate process tubes. The difference in absorbances between the test and the blank is multiplied by a factor to yield results in desired units. The factor, theoretically, is based on the ratio of total to sample volume and the molar absorptivity of the product measured (2, 4, 5).

Methods

2,4-Dichloroaniline method. The 2,4-dichloroaniline method was set up as described in the Greiner Handbook (4).

Jendrassik–Grof method. The Jendrassik–Grof method was set up with the GSA II and was later transferred to the GSA II D. Use of the method on the GSA II D required a minor modification of the wiring (done with the help of Greiner Electronics, Langenthal, Switzerland) to permit the use of five dispensers needed for each measurement of total and direct bilirubin concentration.

Reagents for the Jendrassik–Grof method. The caffeine-sodium benzoate, sulfanilic acid, sodium nitrite, diazo, and alkaline tartrate reagents were prepared as described by Doumas et al. (6). The ascorbic acid (ACS grade, Fisher Certified) and hydrochloric acid solutions were prepared in the same concentrations as those used by Gambino and Di Re (7, 8).

Measurement of total bilirubin. Reagent dispensers were located in the analyzer at the positions shown in Table 1. To improve the stability of the diazo reagent and of the ascorbic acid solution, both were kept in closed-type dispensers, with which aeration of the reagent is minimized, and they were freshly prepared every 72 h. The ascorbic acid was added 1 min after addition of diazo reagent, and the alkaline tartrate reagent was dispensed 0.7 min after the delivery of ascorbic acid. The readings were taken 2.2 min after addition of alkaline tartrate.

The method was calibrated with Validate-A control material. Accuracy and reliability of the calibration were previously established in separate studies by using Versatol Pediatrics and bilirubin standards prepared in bovine serum albumin as published by Doumas et al. (6). Validate-A and Versatol Pediatrics were purchased as lyophilized materials from Warner-Chilecott General Diagnostics of Canada Ltd.

Measurement of direct bilirubin. The same procedure and dispensers as those used for measurement of total bilirubin were used, with two alterations: the volume of specimen was 60 μL for each process tube (blank and test); and 0.05 mol/L HCl was placed in the dispenser at position 62, which was set to dispense 200 μL into each process tube. This was used instead of the caffeine–benzoate reagent.

The concentrations of total and direct bilirubin in serum as measured by the automated method were compared with those measured manually.

Manual method. The reagents were the same as those used for the automated method. The procedure (Table 2) is based on the method for total bilirubin determination of Doumas
Table 1. Position of Dispensers and Volumes of Sera and Reagents for Measurement of Total Bilirubin in the GSA II D

<table>
<thead>
<tr>
<th>Reagent no.</th>
<th>Sample and reagents</th>
<th>Position</th>
<th>Volume, µL</th>
<th>Blank</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td></td>
<td></td>
<td>20</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>De-Ionized water</td>
<td></td>
<td></td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>R-1</td>
<td>Caffeine reagent</td>
<td>57</td>
<td>200</td>
<td>200</td>
<td></td>
</tr>
<tr>
<td>R-2</td>
<td>Sulfanilic acid</td>
<td>40</td>
<td>110</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R-3</td>
<td>Diazro reagent</td>
<td>39</td>
<td>—</td>
<td>110</td>
<td></td>
</tr>
<tr>
<td>R-4</td>
<td>Ascorbic acid</td>
<td>29</td>
<td>20</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>R-5</td>
<td>Alkaline tartrate</td>
<td>22</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Total volume</td>
<td></td>
<td></td>
<td>550</td>
<td>550</td>
<td></td>
</tr>
</tbody>
</table>

wavelength: 578 nm

* To obtain the number of minutes that the process tube takes to reach the spectrophotometer from R-1, etc., divide the position number by 10. * Highest wavelength available.

e et al. (6), with the following modifications:

- The volumes were scaled down proportionately to one-fifth, to decrease specimen requirement.
- The volume of alkaline tartrate reagent was further decreased by a third, to increase the absorbance. This reduction decreased the final pH by no more than 0.1 unit.
- Ascorbic acid was used systematically for all measurements.
- Hydrochloric acid was used in the measurement of direct bilirubin as reported by Gambino and Di Re (7, 8).

Immediately after addition of the alkaline tartrate reagent, absorbances at 600 nm were recorded with a Spectronic 100 spectrophotometer (Bausch & Lomb), and multiplied by a factor derived from calibrations with bilirubin standards prepared in bovine serum albumin.

Analytical Variables

Within-run precision. Total bilirubin determinations were made in 20 consecutive replicates of Calibrate I, Calibrate III, and Versatol Pediatrics, purchased as lyophilized materials from Warner-Chilcott General Diagnostics of Canada Ltd. Direct bilirubin was measured in 10 to 14 replicates of sera at two different concentrations. Their means and coefficients of variation were then calculated.

Between-day precision. Single measurements of total bilirubin were made each day on freshly prepared Calibrate I and Versatol Pediatrics during four weeks. Their means and coefficients of variation were calculated.

Linearity. Versatol Pediatrics was reconstituted in 1.5 mL of de-ionized water and serial diluted with de-ionized water to provide a range from 15.4 to 687 µmol/L. Duplicate measurements were made and the mean values were plotted on the graph.

Effect of hemolysis. Hemolysate was prepared from washed, packed erythrocytes, and four different aqueous dilutions of the hemolysate were added to sera. The sera consisted of: (A) patients' sera and (B) pooled sera to which was added Versatol Pediatrics to obtain two bilirubin concentrations.

Hemoglobin concentrations in these specimens were measured by the method of Hunter et al. (9).

Total and direct bilirubin concentrations were measured in replicates of patients' sera (i.e., A) by the Jendrassik-Grof method. The total bilirubin concentrations were measured in replicate samples of pooled sera (i.e., B) by the Jendrassik-Grof and 2,4-dichloroaniline methods.

Effect of turbidity. Turbid samples were prepared by adding Intralipid® to patient's serum and to pooled sera with added Versatol Pediatrics, as mentioned previously. The total concentrations of added Intralipid in the sera were 1000, 2000, and 4000 mg/L. Measurements were made of total and direct bilirubin concentrations in patients' sera by the Jendrassik-Grof method, and of total bilirubin concentration in pooled sera by both methods.

Turbidity, expressed in absorbance units (10), was determined in the GSA II D at 578 nm vs. the initial non-turbid sample, which served as the blank. The samples were diluted in isotonic saline in the same proportion as in the Greiner method.

Comparison with Manual Method

To study the correlation between the automated and manual methods, measurements were made by both methods on 53 patients' sera for total, and 40 patients' sera for direct bilirubin concentrations. A linear regression was performed by the method of least squares and the correlation coefficient was calculated.

Statistical Analyses

The statistical significance of interference due to hemo-

Table 2. Manual Method for Measurement of Total and Direct Bilirubin Concentration in Serum

<table>
<thead>
<tr>
<th>Reagent no.</th>
<th>Sample and reagents</th>
<th>Volume, µL</th>
<th>Blank</th>
<th>Direct</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>Caffeine</td>
<td>800</td>
<td>—</td>
<td>800</td>
<td></td>
</tr>
<tr>
<td>b</td>
<td>HCl, 50 mmol/L</td>
<td>—</td>
<td>800</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>c</td>
<td>Serum</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>d</td>
<td>Diazro</td>
<td>—</td>
<td>200</td>
<td>200</td>
<td></td>
</tr>
<tr>
<td>e</td>
<td>Sulfanilic acid</td>
<td>200</td>
<td>—</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>f</td>
<td>Ascorbic acid</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td></td>
</tr>
</tbody>
</table>

Immediately add:

| g          | Alkaline tartrate   | 400        | 400   | 400    |       |
| f          | Ascorbic acid       | 50         | 50    | 50     |       |
| g          | Alkaline tartrate   | 400        | 400   | 400    |       |
| Total      | Volume              | 1550       | 1550  | 1550   |       |

wavelength: 600 nm

Fig. 1. Linearity of measurement of bilirubin by the Jendrassik-Grof method

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lysat was established by comparing the means of results with and without hemolysate by Student's t-test when the variance ratio was not significant and by the Welch t-test when the variance ratio was significant (11). Likewise, we calculated the significance of interference due to hemoglobin and turbidity.

Results

Analytical Variables

Within-run precision. The coefficients of variation for total bilirubin concentration were 4.3, 1.3, and 1.3% for concentrations of 17.1, 94.0, and 338.6 μmol/L, respectively. For direct bilirubin measurement the CVs were 2.6 and 1.1% for concentrations of 3.4 and 97.5 μmol/L, respectively.

Between-day precision. The CVs were 5.8 and 2.6% for total bilirubin concentrations of 17.1 and 342 μmol/L, respectively.

Linearity. Figure 1 shows that the measurement is linear to a concentration of 667 μmol/L, the highest concentration tested.

Effect of hemolysate. Table 3 lists the results on measurements of total and direct bilirubin concentrations by the automated Jendrassik–Grof method. They show a progressive decrease in the apparent concentration of direct bilirubin with increasing concentrations of hemoglobin. The 2.8% decrease at a hemoglobin concentration of 480 mg/L, though small, was statistically significant (p <0.001). It further decreased by 5.1 and 9.7% at hemoglobin concentrations of 720 and 1200 mg/L, respectively. On the other hand, there was no significant effect on measurement of total bilirubin up to a hemoglobin concentration of 1200 mg/L.

The results for total bilirubin concentrations as determined by the automated Jendrassik–Grof and 2,4-dichloroaniline methods are presented in Table 4. Concentrations of hemoglobin higher than 2000 mg/L caused a small but statistically significant decrease in the measurement of total bilirubin concentration by the Jendrassik–Grof method. The maximum decrease was 7% at a hemoglobin concentration of 8040 mg/L. The decrease was more pronounced when the measurements were made by the 2,4-dichloroaniline method so that at a hemoglobin concentration of as low as 600 mg/L there was a small but significant decrease in the measurement of total bilirubin concentration. It decreased further on further increases of hemoglobin concentrations. At 2220 mg/L, the statistically significant decrease (p <0.001) in the measurement of total bilirubin concentration ranged from 12.8 to 16.8%. At a hemoglobin concentration of 8040 mg/L, there was a 32.6% fall in the apparent concentration of total bilirubin.

Effect of turbidity. Turbidity appeared to affect the measurement of direct bilirubin more than that of total bilirubin, as seen from Table 5. When the amount of Intralipid was raised by approximately 2000 mg/L the direct bilirubin concentration showed a significant decrease by 7% (p <0.001). When the Intralipid concentration was increased by 4000 mg/L, the direct bilirubin concentration decreased significantly by 17.9% while the total bilirubin concentration decreased significantly by only 5.7% (p <0.01).

Comparisons between the Jendrassik–Grof and the 2,4-dichloroaniline methods (Table 6) confirms the slight interference in measurement by the Jendrassik–Grof method at Intralipid concentrations of 4000 mg/L. The effect was more pronounced on the 2,4-dichloroaniline method, which showed significant decreases (p <0.001) even when the Intralipid concentrations were increased by only about 1000 mg/L.

Correlation between Manual and Automated Methods

The linear regression relating the two methods for total bilirubin measurement is shown in Figure 2. The correlation...
Table 5. Total and Direct Bilirubin Concentrations after Addition of Intralipid: Automated Jendrassik-Grof method (n = 10)

<table>
<thead>
<tr>
<th>No.</th>
<th>Material</th>
<th>Conc of Intralipid, mg/L</th>
<th>A_{578}mm</th>
<th>Total Mean</th>
<th>CV, %</th>
<th>Direct Mean</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Serum</td>
<td>—</td>
<td>0.050</td>
<td>47.2</td>
<td>1.4</td>
<td>31.3</td>
<td>2.8</td>
</tr>
<tr>
<td>2</td>
<td>Serum + Intralipid*</td>
<td>1000</td>
<td>0.261</td>
<td>47.4</td>
<td>1.2</td>
<td>30.8</td>
<td>0.8</td>
</tr>
<tr>
<td>3</td>
<td>Serum + Intralipid</td>
<td>2000</td>
<td>0.525</td>
<td>46.9</td>
<td>2.6</td>
<td>29.1**</td>
<td>2.4</td>
</tr>
<tr>
<td>4</td>
<td>Serum + Intralipid</td>
<td>4000</td>
<td>1.150</td>
<td>44.5*</td>
<td>4.5</td>
<td>25.7</td>
<td>6.2</td>
</tr>
</tbody>
</table>

* n = 5. * 1 vs 4: p < 0.01. ** 1 vs 3: p < 0.001.

Table 6. Total Bilirubin Concentration after Addition of Intralipid: Comparison between Automated Jendrassik-Grof and 2,4-Dichloroaniline Methods (n = 10)

<table>
<thead>
<tr>
<th>No.</th>
<th>Material</th>
<th>Conc of Intralipid, mg/L</th>
<th>A_{578}mm</th>
<th>Conc of total bilirubin, μmol/L</th>
<th>Jendrassik-Grof Mean</th>
<th>CV, %</th>
<th>2,4-Dichloroaniline Mean</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>S-VP*</td>
<td>—</td>
<td>0.069</td>
<td>55.9</td>
<td>59.5</td>
<td>2.7</td>
<td>59.5</td>
<td>0.9</td>
</tr>
<tr>
<td>2</td>
<td>S-VP + Intralipid</td>
<td>1000</td>
<td>0.263</td>
<td>55.4</td>
<td>55.6**</td>
<td>1.6</td>
<td>55.6</td>
<td>3.1</td>
</tr>
<tr>
<td>3</td>
<td>S-VP + Intralipid</td>
<td>2000</td>
<td>0.523</td>
<td>54.9</td>
<td>51.6</td>
<td>2.5</td>
<td>51.6</td>
<td>6.1</td>
</tr>
<tr>
<td>4</td>
<td>S-VP + Intralipid</td>
<td>4000</td>
<td>1.253</td>
<td>54.2*</td>
<td>51.0</td>
<td>2.0</td>
<td>51.0</td>
<td>2.9</td>
</tr>
<tr>
<td>5</td>
<td>S-VP</td>
<td>—</td>
<td>0.196</td>
<td>143.5</td>
<td>150.5</td>
<td>0.4</td>
<td>150.5</td>
<td>0.7</td>
</tr>
<tr>
<td>6</td>
<td>S-VP + Intralipid</td>
<td>1000</td>
<td>0.277</td>
<td>143.3</td>
<td>146.5**</td>
<td>0.7</td>
<td>146.5</td>
<td>0.6</td>
</tr>
<tr>
<td>7</td>
<td>S-VP + Intralipid</td>
<td>2000</td>
<td>0.538</td>
<td>141.8</td>
<td>143.5</td>
<td>2.3</td>
<td>143.5</td>
<td>2.1</td>
</tr>
<tr>
<td>8</td>
<td>S-VP + Intralipid</td>
<td>4000</td>
<td>1.048</td>
<td>142.4</td>
<td>140.7</td>
<td>1.5</td>
<td>140.7</td>
<td>2.2</td>
</tr>
</tbody>
</table>

* S-VP: Serum with added Versatol Pediatrics. * 1 vs 4: p < 0.01. ** 1 vs 2, 5 vs 6: p < 0.001.

coefficient, r, was 0.994, indicating a highly significant correlation between the two methods. The mean value for the manual method was 71.3 μmol/L and for the automated method 80.7 μmol/L. Figure 3 shows the linear regression relating the two methods for direct bilirubin measurement. The correlation coefficient was 0.980, indicating a highly significant correlation between the two methods. The mean value by the manual method was 35.9 μmol/L and by the automated method 37.6 μmol/L.

Discussion

The Jendrassik-Grof method automated on the Greiner Selective Analyzer GSA II D compared favorably with the manual method. The bias between the manual and automated methods for the measurement of total bilirubin concentration may be attributed to the differences in calibration procedures.

The high precision, good linearity, ease of operation, speed of measurement, and minimal interference due to hemolysis and turbidity facilitates the determination of total and direct bilirubin concentrations in the laboratory.

Sera obtained from cord blood and those from capillary-blood collections in neonates and infants are often hemolized. Thus, interference by hemolysis is an important factor to be considered in any measurement of bilirubin. Therefore, the method chosen should be such that the effect of hemoglobin is minimal. Hemoglobin has been shown to have a suppressive effect in several diazo procedures, reviewed by Michaelsson (12), who claimed that addition of ascorbic acid in the Jendrassik-Grof method eliminated the suppressive effect of hemoglobin up to 14000 mg/L.

In their method in which 2,4-dichloroaniline and Brij-35 are used with the Greiner Selective Analyzer, Kuffer et al. (2) reported that a hemoglobin concentration of 400 mg/L decreased the apparent concentration of bilirubin by 5%. At
higher concentrations of hemoglobin the suppressive effect was greater, such that at 1130 mg/L the bilirubin concentration decreased by 11.9%, at 1880 mg/L it decreased by 22.8%, at 2790 mg/L it decreased by 39.3%, and at 9050 mg/L it decreased by 78.4%. We have confirmed in this study that hemolysis has a significant and marked effect on the 2,4-dichloroaniline method (4). Addition of hemolyte caused significant decreases in the measurement of bilirubin at hemoglobin concentrations as low as 600 mg/L. It decreased steadily with further increases of hemoglobin such that at 8040 mg/L there was a 32.5% decrease in the measured bilirubin (Table 4).

In contrast, the Jendrassik–Grof method showed relatively little interference by hemolysis. Hemoglobin concentrations of 1200 mg/L did not interfere with the measurements (Table 4). There was only a marginal effect at 2220 mg/L and a 7.1% decrease at 8040 mg/L. Thus, though hemolysis interferes with the Jendrassik–Grof method, as has also been documented elsewhere (13–15), the effect is marginal and of minor consequence.

Turbidity is yet another factor to be considered. The laboratory may receive specimens from infants receiving intravenous infusion of Intralipid. Shennan et al. (17) reported that total bilirubin as measured by the Jendrassik–Grof method is not affected by Intralipid concentration up to 4000 mg/L, while the direct fraction showed an apparent increase. In this manual procedure the tube designated “blank” contained caffeine–sodium benzoate as one of the reagents, while that designated “test” for direct bilirubin contained, instead, 0.05 mol/L HCl. This discrepancy has been removed in the automated method presented here, so that both tubes—the blank and the test—contained 0.05 mol/L HCl. Turbidity equivalent to that produced by an Intralipid concentration of 2000 mg/L had no effect on the total and only a small effect on direct bilirubin measurement. Specimens with greater turbidity should be cleared by methods such as high-speed centrifugation (17) before bilirubin concentration is measured.

Turbidity appeared to affect the 2,4-dichloroaniline method more than the Jendrassik–Grof method. Intralipid concentrations of 1000 mg/L produced a statistically significant decrease in the apparent concentration of total bilirubin as measured by the 2,4-dichloroaniline method. The differences in response between the two methods may be attributed to the greater dilution undergone by the specimens in the Jendrassik–Grof method as compared to that of the 2,4-dichloroaniline method. In the Jendrassik–Grof method the specimens underwent a six-fold dilution with water before any addition of reagents, and the ratio of specimen to total volume was 1:27.5, whereas the corresponding values in the 2,4-dichloroaniline method were 2.7-fold dilution and a ratio of 1:8.7.

A single measurement of total bilirubin requires 40 μL with the Jendrassik–Grof method, 120 μL with the 2,4-dichloroaniline method. The greater range of linearity in the Jendrassik–Grof method is also an added advantage for measurements in pediatric patients.

In light of the foregoing discussion documenting the relatively minor interferences due to hemolysis and turbidity, and in consideration of other features such as smaller sample volume and greater range of linearity, it is apparent that the automated Jendrassik–Grof method is superior to the 2,4-dichloroaniline method.

We thank Dr. D. M. Goldberg for helpful criticism of the manuscript.

References
5. Determination of direct bilirubin in 60 μl sample by a 2,4-dichloroaniline method. Ibid., No. 76.095, pp 1–4.