Direct Spectrophotometry of Bilirubin in Serum of the Newborn, with Use of Caffeine Reagent

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We recently described a direct spectrophotometric method for unconjugated bilirubin, with caffeine reagent (Clin Chem 1986;32:1389-93). Because this method is independent of the protein matrix we used it for the preparation of bilirubin standards (Clin Chem 1987;33:1817-21). Now, in this paper, we utilize the caffeine reagent in setting up a bilirubin method for serum from neonates. This resulted in a two-wavelength (465 and 528 nm) equation, which fully corrects for HbO₂ interferences. In combination with a bilirubin standard, this equation may be transformed into a simple relative formula for use with this simple dilution method. We studied this two-wavelength method with 55 neonates’ sera, comparing results with those by both the diazo method of Doumas et al. (Clin Chem 1985;31:1779-89) and the borate method of Hertz et al. (Scand J Clin Lab Invest 1974;33:215-30). We found this new method is independent of hemolysis and of the matrix of the sera. Therefore, it is very suitable for use in neonatology.

For more than 60 years, caffeine reagent has been used to accelerate the coupling of p-diazobenzene sulfonate with unconjugated bilirubin in serum (1). In 1986, we introduced a method for using this caffeine reagent in direct spectrophotometry of unconjugated bilirubin (2). Two other properties of this reagent are that it yields a molar absorptivity for bilirubin that is independent of the protein matrix, and it better clears the turbidity of human sera than is the case for direct spectrophotometry involving dilution with borate buffer (3). The matrix independence of the molar absorptivity of bilirubin in caffeine reagent allowed us to prepare bilirubin standards (4) with various human and bovine serum albumins as a protein base.

Here we report the use of the caffeine reagent to determine bilirubin in serum from neonates, the effects of (e.g.) carotenoids being negligible at that age (3, 5, 6). To correct for hemolysis, we had to develop a calculation involving absorbances at least two wavelengths (2λ). To study the reliability of the new 2λ method, we have determined the bilirubin concentration in serum samples from neonates by each of three methods: (a) the diazo method of Doumas et al. (7), (b) the borate method of Hertz et al. (3), and (c) the new 2λ method with caffeine reagent.

Materials and Methods

Equipment

The equipment was as described in our previous paper (2).

Bilirubin Standards

We used bilirubin Standard Reference Material (SRM) no. 916 (batches 455 and 1806; National Bureau of Standards, Washington, DC 20234) as the primary standard. Its stated purity is 99.0% and it is supplied in ampules, under argon.

We also used “reference grade” bilirubin (batch 12622; Pfannstieh Lab. Inc., Waukegan, IL 60085); the lots we used showed no spectrophotometric difference from the NBS preparations.

In the analyses for bilirubin in patients’ sera by all three methods, we included an assay of one of our standards, 850625 P300, prepared as recently described (4). This standard has a mean (eight determinations) bilirubin content of 281.0 μmol/L, a result not significantly different when measured with the method of Doumas et al. (7) or the caffeine method (2). These lyophilized standards, which are packed under reduced pressure in brown vials to shield the bilirubin from the effects of light, have good stability (4).

Diaz Method

We used the diazo method (7) to quantify bilirubin, except that we used the original caffeine reagent of Jendrassik and Grof (8), as in our one-wavelength caffeine method (2; see below).

Caffeine Method

Because there is no true blank to use for direct spectrophotometry of bilirubin in native sera, we correct for interferences by using a two-wavelength formula, as follows. Because oxyhemoglobin (HbO₂) strongly interferes in this type of bilirubin analyses, we did not use one of the two wavelength maximums, 432 or 457 nm, to determine bilirubin in caffeine reagent (2). Rather, we preferred to use 465 nm, because this wavelength is situated near the top of the absorption curve and because the molar absorptivity of HbO₂ at that wavelength appeared to be reduced by about 20% and that of bilirubin by only 1.44%, with respect to values at 457 nm. Therefore 465 nm seemed to us to be optimal for the first wavelength.

In direct spectrophotometry of serum from neonates, the influence of carotenoids, etc., was negligible (3, 5, 6); therefore, we corrected only for hemolysis. Because, for HbO₂, A₄₃₂ = A₄₆₅ the second wavelength chosen was 528 nm. We found for the molar absorptivity of unconjugated (UB) bilirubin at 465 nm and 528 nm, respectively, 48 000 (SD = 100, n = 10) and 970 L mol⁻¹ cm⁻¹ (SD = 15, n = 6), the latter measured at high bilirubin concentrations. Thus, the 2λ formula becomes:

\[ C_{UB} = f \times \frac{A_{465} - A_{528}}{48 000 - 970} \times 10^6 \mu mol/L \]

or

\[ C_{UB} = f \times 21.26 (A_{465} - A_{528}) \mu mol/L \]  

where \( f \) is the reciprocal volume fraction of serum, and \( A \) is the absorbance in a cuvet with a 10-mm pathlength. Equation 1 is derived from theory rather than an empirical statement. This type of equation should always be validated with a reliable standard.

Caffeine method with 2λ calculation. Dilute about 40 μL of serum and of standard precisely and accurately with 1 to 1.6 mL of caffeine reagent. Measure the absorbances at 465 and 528 nm.
528 nm, with the caffeine reagent as blank. Calculate the bilirubin concentrations by using equation 1. Analytical recovery of bilirubin of the standard was held within 100% ±1.3% by regularly calibrating the dilutor (2). However, if it is difficult to determine the reciprocal volume fraction accurately, or if the cuvet pathlength differs from 10 mm, use of equation 1 with both the test and the standard yields:

\[ \frac{(A_{450} - A_{628})_{\text{stand}}}{(A_{450} - A_{628})_{\text{test}}} \times \text{concn of standard, } \mu\text{mol/L} \] (2)

This is an easy, accurate, and simple modification of our test.

Caffeine reagent. Dissolve 125 g of CH₃COONa · 3H₂O (Merck, Darmstadt, F.R.G.; no. 6267), 75 g of sodium benzoate (Merck; no. 6290), and 1.0 g of Na₂EDTA (Merck, no. 8418) in 500 mL of doubly distilled water. Add 50 g of caffeine (Merck, no. 2584), stir until the caffeine is completely dissolved, and dilute to 1 L with water. Filter this solution and store at room temperature; it is stable in a brown bottle for months.

Borate Method

This direct spectrophotometric method for bilirubin in borate reagent (100 μmol/L, pH 9.30) is based on the "best fit" formula of Hertz et al. (3). We used about 40 μL of serum with an f value of about 40 with the equation of Hertz et al. (3):

\[ C_{TB} = f \times \left[ \frac{21.6 \times A_{450} - (27.4 \times A_{628})}{28.3A_{550\text{stand}} - 34.7A_{628\text{stand}}} \right] \mu\text{mol/L} \] (3)

where TB is total bilirubin, and f and A are as defined above. Because the borate method appears to depend on the protein matrix of our standards (2, 4), quality control of this bilirubin test was based on the constancy of the apparent bilirubin concentration of the standard in borate reagent (100% ±1.3%).

Sera from Neonates

About half of the blood samples from neonates were venous blood and half were obtained by heel puncture. The blood was kept in a dark box, shielded from light, and centrifuged soon after clotting. The serum was stored at -20 °C.

In all, 55 serum samples were collected from infants younger than four days post partum. We divided the samples into three groups, according to the degree of hemolysis. Only four sera were obtained after phototherapy, but the mutual relations between the three bilirubin concentrations in each serum as measured by the three analytical methods did not differ significantly from those of the other serum samples. Each serum was analyzed in duplicate by each of the three methods. No sera from infants with disease of the liver, gall bladder, or bile ducts were included in this study.

Check on Our 2x Formula

With bilirubin standards. To check equation 1, we assayed two of our bilirubin standards—850726 P300 (bilirubin content 289.8 μmol/L) and 850625 P300 (281.0 μmol/L). Each of these standards was diluted fivefold precisely and accurately with caffeine reagent (f = 20). With the aid of equation 1, factors of 21.34 and 21.18, respectively, were found for these standards (see Results).

With HbO₂ solutions. To study the influence of hemolysis on the reliability of the 2x formula, we added HbO₂ solutions to three different protein solutions: (a) to the blank of one of our bilirubin standards (4); (b) to one of our bilirubin standards (4) (bilirubin concentration 208 μmol/L); and (c) to serum from exchange-transfusion blood obtained from a neonate. The HbO₂ solutions were prepared from EDTA-treated blood after we had washed the erythrocytes two or three times with phosphate-buffered isotonic saline (9). The erythrocyte suspension was then hemolyzed with water, frozen at -20 °C, and centrifuged for 10 min at 3000 rpm after thawing. After determining the HbO₂ concentration of the supernate, we prepared a set of eight samples, including a blank, by serial dilution with phosphate-buffered saline. We mixed 1 mL of each of these samples with 4 mL of each of the three protein solutions. The HbO₂ content of these "synthetic" sera ranged from 0 to about 1000 μmol/L. We then determined the bilirubin content of each of these mixtures, in duplicate, with our 2x caffeine method and with the method of Doumas et al. (7).

Comparison of Bilirubin Concentrations in Neonates’ Sera as Measured by All Three Methods

Analytical set-up. We included in each batch of sera from jaundiced neonates our bilirubin standard 850625 P300 and assayed all samples by the method of Doumas et al. (7) and by both direct spectrophotometric methods. All analyses were made in duplicate, and the absorbances of the standards were measured twice, with a Lambda 5 UV/VIS spectrophotometer (Perkin-Elmer Corp., Norwalk, CT) equipped with a printer–plotter (2). The analyses and calibrations of both direct spectrophotometric methods were performed as described above. In one of each duplicate of the 2x-caffeine method the concentration of HbO₂ was determined from the absorbance at 540 nm (λmax), compared with that of a HbO₂ standard. In all, for assay by all three methods, we used about 240 μL of serum per neonate.

Classification of jaundiced sera from neonates. As is generally known, serum from neonates is hemolytic, although not always visibly so. Although both the 2x-caffeine method and the borate method (3) appear to be nearly independent of the presence of HbO₂, the method of Doumas et al. (7) is susceptible to such interference. Therefore we divided our sera into three groups (A, B, and C), according to the degree of hemolysis.

In 24 of the sera (group A), the HbO₂ concentrations were 0–39 μmol/L (mean: 29 μmol/L); in 18 (B), between 40 and 79 μmol/L (mean 54 μmol/L); and in 13 (C), between 80 and 156 μmol/L (mean 118 μmol/L). This division is especially important for group A, because in this group the method of Doumas et al. (7) is hardly disturbed by HbO₂ (7, 10, 11; and see our 'Table 3 concerning neonates' serum) and may therefore be considered a reference method. The bilirubin concentrations measured by each of the three analytical methods in groups A, B, and C were inter-compared by means of linear regression analysis.

Results

Check on Our 2x–Equation 1

With bilirubin standards. We found with our bilirubin standard 850726 P300 a mean absorption factor of 21.18 (SD 0.13, n = 5), with our standard 850625 P300 a mean value of 21.34 (SD 0.12, n = 5) (see equation 1).

With HbO₂ addition. (a) We found (Table 1) that neither with the diazo nor with the 2x-caffeine method could "bilirubin" be detected in a synthetic albumin standard blank to which HbO₂ had been added to give concentrations of 1000
Table 1. Effect of HbO₂ Concentration on Measurement of Bilirubin in Albumin Solution*

<table>
<thead>
<tr>
<th>HbO₂ concn, μmol/L</th>
<th>Measured “bilirubin” concn, μmol/L</th>
<th>Doumas et al. (7)</th>
<th>Present method</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.5</td>
<td>0.6</td>
<td></td>
</tr>
<tr>
<td>48</td>
<td>1.0</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>71</td>
<td>0.9</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>136</td>
<td>1.5</td>
<td>0.8</td>
<td></td>
</tr>
<tr>
<td>250</td>
<td>0.8</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>500</td>
<td>−0.5</td>
<td>−0.8</td>
<td></td>
</tr>
<tr>
<td>750</td>
<td>0.5</td>
<td>−0.8</td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td>0.4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Albumin, 40 g/L. Values for bilirubin are means of duplicate determinations.

μmol/L. This means that, for the 2α-caffeine method, in equation 1 $A_{528}$ completely corrects for HbO₂. (b) Clearly (Table 2), the 2α method also reasonably corrects for HbO₂ added to a synthetic bilirubin albumin standard, even for HbO₂ concentrations >200 μmol/L, which are clinically irrelevant. (c) The 2α-caffeine method showed excellent analytical recovery of bilirubin (Table 3) in serum from neonates to which HbO₂ was added, whereas the diazo method demonstrates a clear destruction of the azobilirubins when HbO₂ concentrations exceed 50 μmol/L.

Correlations between Bilirubin Concentrations in Sera from Jaundiced Neonates, as Measured with the Three Analytical Methods

In Table 4 we give some general information on the three serum groups A, B, and C expanded with linear regression analyses of x, y, and z in each group. We find for the group with the lowest hemolysis (A) a very good agreement between the bilirubin concentrations y and z, as shown by a slope of 0.999 and an intercept of −0.18. However, for greater hemolysis (groups B and C), we observe similar slopes with increasing intercepts in the regressions between y and z. Analogously increasing intercepts were found in the three regression equations between x and z for groups A to C.

In the three relations between z and x and in the three between y and x we found (Table 4) rather constant, but increased, slopes, ranging from 1.081 to 1.089. In groups A, B, and C we measured (Table 4) very low intercepts in the three regression analyses between x and y combined with increased, but constant, slopes. The ratios of the arithmetic means of y and x and of z and x (Table 5) appear to increase with a higher degree of hemolysis, a phenomenon hardly seen in $z/y$.

Discussion

Developing and checking the new method. We conclude from the above-described checks that the new 2α-caffeine method is so reliable that it can be used in neonatal investigations, especially as the clinically relevant concentrations of HbO₂ in these sera are <200 μmol/L.

Clinical investigation. The division into three hemolysis groups was especially important in order to isolate group A, in which hemolysis was so restricted that the method of Doumas et al. (7) still holds as the reference method for bilirubin (7 and Table 3).

Effect of hemolysis. Table 4 shows a very close agreement between the 2α-caffeine and the diazo method for Group A. From this we conclude that the 2α-caffeine test is accurate for this purpose. However, at higher degrees of hemolysis (groups B and C), the regression intercepts increased from −0.18 to 1.92 and 4.98, respectively, whereas the slopes of the regressions between y and x did not change. The increasing intercepts resulted from decreasing concentrations of x, caused by oxidation of the azobilirubins in the diazo method (7, 10, 11). This loss of azobilirubins agrees roughly with the values in Table 3. This agreement also may be deduced from the similarly increasing intercepts found in the relations between x and y (Table 4). That there is greater destruction of the azobilirubins with increased hemolysis also may roughly be concluded from the increasing ratios for the mean bilirubin concentrations: $y/x$ and $z/x$ (Table 5).

As is generally known, the borate method of Hertz et al. (3) is insensitive to hemolysis, and this property will also be inherent in the present method, as may be deduced because the three relations between z and y show both minimal intercepts, constant slopes (Table 4), and constancy of the ratios $z/y$ (Table 5). Summarizing, it may be concluded that the 2α-caffeine method is accurate and insensitive to HbO₂ because results agree with those by the reference method (7) for low-hemolysis samples, and because results agree with those by the hemolysis-insensitive borate method (3) for all degrees of hemolysis. The Doumas method was shown to be reliable when hemolysis is minimal; however, we could not confirm that interference by HbO₂ up to 2 g/L (124 μmol/L) was negligible. Moreover, our results agree with those of other authors (10, 11) who used the diazo method according to Jendrassik and Gröf (8). Any discrepancy might be explained by a different ascorbic acid content in the sera used, and or in the different composition of the caffeine reagent (see above).

Matrix effects. The recently described matrix effects (2, 4)
Table 4. Bilirubin Concentrations in Neonatal Sera, as Measured by the Method of Doumas et al. (x), by the Present Method (y), and by the Method of Hertz et al. (z)

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of neonates</th>
<th>HbO₂ Conc, μmol/L</th>
<th>Bilirubin Linear regression*</th>
<th>r</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Range</td>
<td>Mean</td>
<td>Range</td>
<td>̄x</td>
</tr>
<tr>
<td>A</td>
<td>24</td>
<td>0-39</td>
<td>29</td>
<td>50-300</td>
</tr>
<tr>
<td>B</td>
<td>18</td>
<td>40-79</td>
<td>54</td>
<td>43-329</td>
</tr>
<tr>
<td>C</td>
<td>13</td>
<td>80-156</td>
<td>118</td>
<td>100-265</td>
</tr>
</tbody>
</table>

SDs of slope and intercept are listed in parentheses. Arithmetic means of x, y, and z are given.

Table 5. Ratios for the Mean Bilirubin Concentrations, with Their SDs and CVs, as obtained with the Present Method (y) and the Methods of Doumas et al. (x) and Hertz et al. (z)

<table>
<thead>
<tr>
<th>Group</th>
<th>̄y/ ̄x*</th>
<th>̄y/ ̄z</th>
<th>̄x/ ̄z</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.9977</td>
<td>—</td>
<td>—</td>
<td>2.12</td>
</tr>
<tr>
<td>B</td>
<td>1.0089</td>
<td>—</td>
<td>—</td>
<td>2.48</td>
</tr>
<tr>
<td>C</td>
<td>1.0295</td>
<td>—</td>
<td>—</td>
<td>1.78</td>
</tr>
</tbody>
</table>

*SDs of ratios are listed in parentheses.

on the borate method (3) appear also to have occurred with sera from neonates, as judged from the six slopes (1.061 up to 1.089) in the relations between x and x, as well as z and y (Table 4) and from the three ̄y/ ̄x values (Table 5). We find from these results that the borate method yields a matrix effect of about 8.5% with neonatal sera. A similar effect of about 8% has recently been described by Blijenberg et al. (12). It is probable that the borate method of Sigggaard-Andersen and Komarmy (13) would have given better results for neonates' sera (see formula 29 in 3). However, this formula is a purely empirical approximation for overcoming the matrix effect of the borate buffer.

Although the caffeine reagent has a clear influence on sample turbidity with the new method (2), it is important to note that the neonatologist must be aware of the fact that blood sampling must not coincide with the intravenous infusion of lipids.

Summarizing: we conclude that the 2x-coffee method is influenced neither by hemolysis nor by albumin. Therefore it is to be preferred in neonatology. The method requires a simple dilution for direct spectrophotometry, and a serum blank is not needed.

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References