Bilirubin–Protein Interactions Monitored by Difference Spectroscopy

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Difference spectroscopy is used to monitor bilirubin–protein interactions, to assess the residual binding capacity of proteins for bilirubin. A change in the difference spectra monitored at 482 nm is directly proportional to bound bilirubin up to a molar ratio of bilirubin to albumin of approximately 1; increasing bilirubin beyond the 1:1 molar ratio does not further change the difference spectra. After excess free bilirubin is added, the change in the difference spectrum is proportional to the residual binding capacity of the serum for bilirubin. The risk of kernicterus among neonates may be assessed by monitoring the residual bilirubin binding capacity of serum. This report summarizes our research effort leading to an assay method which requires only 40 µl of serum and can be completed in less than 10 min.

Additional Keyphrases: pediatric chemistry • kernicterus • residual bilirubin-binding capacity of serum proteins • bilirubin encephalopathy

At birth, the blood/brain barrier may not be fully developed, and it becomes important to monitor and control bilirubin concentrations in neonates who are at risk of developing bilirubin encephalopathy. Serum bilirubin exists mainly in two forms, conjugated and unconjugated (1); the unconjugated or indirect form is more closely associated with the development of kernicterus. The danger of neurological damage is frequently assessed by monitoring unconjugated bilirubin in serum, often measured as total bilirubin. This practice results from the work reported by Meyer in 1956 (2) wherein he demonstrated that the incidence of kernicterus dramatically increases when serum bilirubin concentrations approach 180 to 200 mg/liter. Infants with high total bilirubin concentrations are often treated by exchange transfusions.

It is now generally accepted that it is not the concentration of unconjugated bilirubin per se that causes encephalopathy but rather the increased “free bilirubin” in serum before the blood/brain barrier develops (3, 4). Normally, unconjugated bilirubin is tightly bound to serum proteins, mainly albumin. However, when high-affinity binding sites become saturated, concentrations of free bilirubin increase, increasing the risk of bilirubin encephalopathy. The danger of kernicterus is determined by at least three factors: the concentration of unconjugated bilirubin, the patient’s capacity to bind and metabolize bilirubin, and the presence of substances that can alter the bilirubin binding interactions. The balance between these factors can be assessed by monitoring the residual binding capacity of serum for bilirubin. This concept has been emphasized by Levine (5), who stated, “It seems clear that the serum bilirubin concentration cannot be used alone to evaluate the effect of drugs or to assess the risk of kernicterus. We have to assess the binding of bilirubin to albumin.”

Investigations summarized in this report demonstrate the feasibility of utilizing difference spectroscopy to monitor bilirubin–protein interactions as a means to assess the residual binding capacity of sera for bilirubin. The work of Wennberg and Cowger published in 1973 (6) provided a starting point for our investigations.

Materials and Methods

Instrumentation. Spectrophotometry was done with either a recording Cary 118C or a Zeiss PM-6 spectrophotometer.

Reagents. Bovine albumin Fraction V, human albumin Fraction V, and crystalline bilirubin were obtained from Sigma; disodium ethylenediaminetetraacetate (EDTA) and sodium borate were from J. T. Baker Chemical Co.; sodium hydroxide was from Mallinckrodt, Inc. Sephadex experiments were done by the Kernlute method (Ames Co.).

The sodium borate buffer (pH 10.5) concentration was 53 mmol/liter. Sodium hydroxide used for the standards was 0.1 mol/liter. Unless specified otherwise, 5 mmol/liter EDTA was included in both the buffer and the NaOH solutions. Bilirubin standards were prepared by dissolving bilirubin in NaOH that contained EDTA; the working bilirubin standards were 10-fold dilutions with the borate buffer.

Procedure. Changes in the difference spectra, ΔA, are determined as follows: ΔA = ASB - AS - AB. ASB = the absorbance of the reaction mixture containing the test proteins (either bovine serum albumin, human serum albumin, human cord serum, infant serum, or adult human serum), borate buffer, and bilirubin standard; generally, sufficient bilirubin is added to saturate the binding sites on the proteins. AS = the absorbance of a second aliquot of the same test proteins diluted with buffer to the same final volume. AB = the absorbance of the bilirubin standard diluted with buffer only. In a typical experiment the matched cuvets contain:

<table>
<thead>
<tr>
<th>ASB</th>
<th>20 µl of test solution</th>
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<tbody>
<tr>
<td></td>
<td>2.00 ml of buffer</td>
</tr>
<tr>
<td></td>
<td>0.40 ml of working bilirubin standard</td>
</tr>
<tr>
<td>AS</td>
<td>20 µl of test solution</td>
</tr>
<tr>
<td></td>
<td>2.40 ml of buffer</td>
</tr>
<tr>
<td>AB</td>
<td>0.40 ml of working bilirubin standard</td>
</tr>
<tr>
<td></td>
<td>2.02 ml of buffer</td>
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After the pipetting is complete, the contents of each cuvet are mixed by inversion and the absorbances measured. The entire procedure including pipetting, mixing, and absorbance measurements can be completed in less than 10 minutes and uses only 40 µl of serum.

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Results

Absorbance

Absorbance spectra with use of cord-serum aliquots for solutions ASB, AS, and AB over the wavelength range of 350–600 nm are shown (Figure 1) along with the difference spectrum, ΔA. Identical experiments with use of either purified serum albumins (human or bovine), infant sera, or adult sera yield very similar curves, but not identical ones. Purified albumins give bilirubin interaction spectra, ASB, with the absorbance maxima shifted by 5 to 10 nm toward the ultraviolet; ΔA maxima are shifted in the same direction by 3 to 5 nm. The free bilirubin solution, AB, shows the characteristic (6, 7) absorbance maximum at 435–437 nm (dotted line in Figure 1) at pH 10.5. When bilirubin is bound to serum albumin the absorbance maximum shifts to 460–470 nm (dashed line). This shift of approximately 30 nm was utilized in our studies to monitor bilirubin–protein interactions. The absorbance shift generates a difference spectra, ΔA, which peaks at 482–485 nm.

Dilute bilirubin solutions follow Beer's law (1, 8); under the conditions used in our experiments the absorbance of the diluted serum, ASB, is proportional to the bilirubin concentration to at least 200 mg/liter. When the bilirubin concentrations determined by absorbance were compared to those determined by the diazo method (8) routinely used in our laboratory, the correlation coefficient was 0.996. A standard curve is established, measuring the absorbance at 482 nm; this wavelength is not at the absorbance peak for bilirubin but is the optimum wavelength to measure ΔA. By selecting 482 nm, it is possible to monitor both the bilirubin concentration and the difference spectra without changing the wavelength.

Stabilizing with EDTA

Instability of bilirubin is a familiar problem to laboratory workers. Difference spectra are reproducible only when the reaction mixtures contain EDTA to stabilize the bilirubin solutions. The presence of EDTA is critical when ΔA is measured because a decrease in absorbance of the bilirubin solution, AB, causes an increase in ΔA not attributable to the bilirubin–protein interactions. The absorbance of the bilirubin solutions in the dark only slightly decreases over a 2-h measuring period in the presence of 6.7 × 10^{-5} mol/liter EDTA (Figure 2). Zero-time absorbance (Figure 2) is determined by extrapolating the absorbance of the bilirubin solution containing 6.7 × 10^{-5} mol/liter EDTA to time 0. Higher concentrations of EDTA have no detectable influence on the bilirubin–protein interactions monitored by difference spectroscopy. We now routinely include 5 mmol of EDTA per liter in the reaction mixture, as suggested by Fog and Bakken (7).

pH

The effects of pH on the absorbance characteristics of bilirubin and bilirubin–protein solutions are quite complex. Absorbance spectra vary considerably over the pH range 5.6 to 11.2 included in our investigations. Qualitatively, our observations agree with those of Wennberg and Cowger (6). In the more acidic media, the absorbance maximum of free bilirubin shifts to about 450 nm and a shoulder develops at about 490 nm, which may be due to the formation of colloidal bilirubin. The solubility of bilirubin at pH 7.4 and ionic strength 0.1 mol/liter is reportedly only 0.05 mg/liter (9). This very low solubility possibly accounts for the difficulty we experience in reproducing the spectral determinations at lower pH values.

In the more acid media the ASB absorbance peak shifts to approximately 455 nm and at the same time the absorbance maximum of the free bilirubin, AB, also shifts from 436 nm to about 450 nm; these changes combine to significantly decrease the ΔA generated by the bilirubin–protein interactions. The largest and most reproducible absorbance changes that are attributable to bilirubin–protein interactions are those measured in an alkaline medium. In agreement with Wennberg and Cowger (6), we find very little change in the spectra of either free bilirubin or protein-bound bilirubin over the pH range 8 to 11. We routinely buffer to pH 10.5.

Bilirubin–Albumin Relationships

Measurement of ΔA provides a signal for monitoring specific bilirubin–protein interactions to a molar ratio of bilirubin to albumin of about 1. We conducted experiments in which the concentration of either bilirubin or binding proteins was varied independently while holding the other variable constant.

The results shown in Figure 3 are representative of experiments in which the binding protein is held constant; at the lowest bilirubin concentrations binding sites are present in
Ligand Saturation or Paucity

Residual binding capacity for bilirubin, the natural ligand, is estimated by difference spectroscopy by determining $\Delta A$ after adding sufficient free bilirubin to saturate the available binding sites. As endogenous bilirubin concentrations increase in sera (Figure 5), the binding sites become pre-occupied with bilirubin; $\Delta A$ is inversely proportional to the initial bilirubin concentration of the sera. The slope of the linear regression line is $-0.0035$.

If $\Delta A$ is to be used to assess the danger of bilirubin encephalopathy, it is important to determine whether bilirubin bound when it is present at very low concentrations in serum generates the same $\Delta A$ as bilirubin bound when the concentration is near the saturation point. In other words, does $\Delta A$ reflect a single type of bilirubin–protein interaction? Figure 6 summarizes experiments in which $\Delta A$ was determined after incremental addition of bilirubin to serum aliquots having initially very low bilirubin concentrations; $\Delta A$ was determined for each amount of added bilirubin. As the bilirubin increases there is seen a corresponding increase in $\Delta A$, attributable to the increased number of bilirubin–protein interactions. The $\Delta A$/mg of bilirubin at low concentrations, which is indicated...
by the slope in Figure 6, is numerically the same but opposite in sign from Figure 5. A comparison of these experiments (Figures 5 and 6) indicates that the binding of a molecule of bilirubin at low concentrations generates the same ΔA as when the bilirubin is bound at high concentrations. Therefore, the ΔA measured after saturation of the protein with excess bilirubin can be used to estimate the residual binding capacity—and the danger of bilirubin encephalopathy.

Discussion

Bilirubin Absorbance

The characteristic absorbance properties of bilirubin have prompted the development of several techniques for direct spectrophotometric assay (10, 11). Even though these direct absorbance-based methods are usually quite simple, they have not replaced the popular diazo techniques, because of their general lack of specificity. However, the absorbance, AS, can be used to estimate serum bilirubin; no additional serum over the 40 μl needed for determination of ΔA is required.

Binding Interactions

Normally, unconjugated bilirubin, the species considered to be responsible for kernicterus (4), is bound tightly to serum proteins, mainly to albumin. Investigators claim that each mole of albumin binds from one (6, 12, 13) to three (14) moles of bilirubin. Under certain conditions the bilirubin binding capacity in newborns is even less than 1 mole of bilirubin per mole of albumin (15). In vivo, this situation is further complicated by extravascular albumin (16). The equilibrium constant for the high-affinity binding sites as determined by several different studies in which a variety of techniques was used is in the range 1.5-3.0 × 10^3 per mole (17, 19). Changes in ΔA monitored in our experiments appear to be proportional to the reaction of bilirubin with "high-affinity" binding sites on the serum proteins. The results do not exclude secondary binding reactions, which probably take place but do not cause measurable absorbance changes.

Gel Filtration

Results of our spectroscopy investigations agree with those of experiments in which gel filtration on Sephadex was used to separate the bound bilirubin. When the molar ratio of bilirubin to albumin exceeds approximately 1, the high-affinity binding sites become saturated and the gel filtration test becomes positive, indicating increased concentrations of free or loosely bound bilirubin. The Kernlute test (A mes Co.) has been used for some time to assess the possible danger of kernicterus among newborns; our findings are generally in agreement with those reported by Kaputulnik et al. (3).

Causes of Kernicterus

The risk of kernicterus is considered to increase if for any reason neonates show high concentrations of "free" bilirubin in their serum. Values for total bilirubin correlate with neurologic dysfunction (2, 20), but this is only one of several factors that together determine the likelihood of kernicterus. A decrease in the number of available binding sites for any reason may increase the risk, even at moderate bilirubin concentrations (21). During treatment of newborns for various disorders, the therapeutic agents used may influence bilirubin toxicity. The following is a partial listing of potential interfering chemical agents reported in the literature: amino acids (23), caffeine (4), chlorothiazid (24), diazepam (25), ethacrynic acid (24), furosemide (24, 26), hematin (4), Intralipid (27), oxytocin (29), phenobarbital (30), salicylate (4, 13, 31), sodium benzoate (4, 13, 25), sulfisoxazole (11, 13, 32), and gentamicin (33). To the degree these substances are able to influence the "high-affinity" binding sites, they may actually promote transfer of bilirubin into the brain (5, 32).

Several approaches are proposed for assessing the risk of bilirubin encephalopathy. Methods based on Sephadex gel filtration (3, 34, 35), thin-layer chromatography on Sephadex (36), and fluorometry (37) to monitor bilirubin–protein interactions are reported in the literature. Our investigations suggest that difference spectroscopy can be used to monitor the residual binding capacity for bilirubin among newborns. Only 40 μl of serum is required; with use of microcuvetas the quantity of serum can be even less. Total serum bilirubin is estimated at the same time, and both values can be obtained in less than 10 min. The assay procedure is now being studied in our laboratory to verify its clinical utility.

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