Estimation of Bilirubin Binding Capacity of Neonatal Serum

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ABSTRACT

One aim of laboratory investigation in neonatal jaundice is to prevent kernicterus from occurring. Estimation of the bilirubin-binding capacity of serum is an important measurement along with total bilirubin determination. With a convenient Sephadex competitive binding procedure, KERN-LUTE® provides information on whether or not the neonate’s bilirubin-binding capacity has been exceeded. Further testing of those serum specimens which show some residual binding capacity estimates whether the remaining bilirubin capacity is equivalent to 0 to 2.5 mg, 2.5 to 5 mg or more than 5 mg bilirubin per dl. References on the utility of this method and other more cumbersome procedures are given.

Introduction

Bilirubin binding capacity of serum has established itself as a useful monitoring procedure for newborn infants who are threatened by kernicterus. The unique physical and chemical properties of bilirubin provide a sound theoretical basis for the creation and utilization of such tests.

Ordinarily, bilirubin in serum is bound to albumin to form a stable large molecule which does not appear in the urine and does not appear to be readily diffusable into tissues. In certain neonates an unbound or “free” bilirubin appears in serum. It is generally agreed that the fraction of bilirubin which is unbound to the albumin in serum is one of the main factors which determines whether or not the development of bilirubin encephalopathy occurs.

Blondheim and his colleagues3,5,6 have described a procedure for the measurement of the bilirubin binding capacity of neonatal serum. This test is now available commercially and is called Kernlute.

In evaluating and differentiating the cause of jaundice and the prediction of kernicterus occurring in neonatal jaundiced patients, the bilirubin binding capacity test provides important information, in addition to total bilirubin determinations.

Principle

The procedure for Kernlute is based on the simple theory of competitive binding of serum albumin and Sephadex for bilirubin. “Free” bilirubin circulates in the blood loosely bound to serum albumin. When the serum bilirubin increases enough to raise the bilirubin : albumin
ratio to more than 0.7, this means that the serum bilirubin binding capacity has been exceeded and the excess unbound bilirubin binds to Sephadex. Kernlute columns contain Sephadex G-25 buffered at pH 7.45. When bilirubin-containing serum is washed through the column, the serum proteins wash through the molecular sieve with all the large molecules, and thus all bilirubin bound to the serum albumin is washed through the column. Any unbound bilirubin remains on the column and binds to the Sephadex.

By adding different amounts of a standard bilirubin solution to further aliquots of serum which possesses residual bilirubin binding capacity, the range of remaining bilirubin binding capacity can be estimated. This principle is illustrated in figure 1 which shows the appearance of the final column reactions with a serum with no bilirubin binding capacity left and one with binding capacity equivalent to a range of 2.5 to 5 mg of bilirubin per dl.

Reagents

Phosphate Buffer. Exactly 2.4 ml of phosphate buffer at pH 7.4 is made to a total volume of 50 ml with distilled water to provide a buffer of approximately 0.07 M concentration.

Sodium Carbonate. This solution is used to dissolve the bilirubin. It is prepared by dissolving the 250 mg quantity of sodium carbonate supplied in 100 ml of water to give a 0.25 percent Na₂CO₃ solution.

Bilirubin Solution. There are 10 little red caps in each Kernlute kit. Each contains 50 µg of lyophilized bilirubin; they are stored in a black vial with a desiccant. To prepare the solution, one cap is dropped into a test tube containing 2.0 ml of sodium carbonate solution. The tube is incubated at 37° C for five min with vortex mixing at one min intervals. The solution should be prepared immediately before use and is not necessary unless the serum gives a negative result (i.e., has some residual bilirubin binding capacity). It is best to make the bilirubin solution up in subdued light. The solution should not be used if it is green; a new solution should be made from a fresh bilirubin cap.

Diazo Tablets. These are individually foil-wrapped. One is used for each column to detect Sephadex-bound bilirubin. They contain p-nitro-benzenediazonium p-toluene sulfonate and are the same as Ictotest® tablets for urine bilirubin.

Sephadex G-25. This acts as a secondary binding site which binds free bilirubin when the serum protein binding sites have been saturated. There are ten columns supplied with each kit.

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**Figure 1.** Appearance of final Kernlute columns with two different serum samples. A shows the test with a serum with no bilirubin binding capacity left. B shows the appearance of the three columns used to estimate the range of residual bilirubin binding capacity equivalent to 2.5 to 5 mg per dl.

<table>
<thead>
<tr>
<th>A. BILIRUBIN BINDING CAPACITY EXCEEDED</th>
<th>B. BILIRUBIN BINDING CAPACITY OF 2.5–5mg BILIRUBIN/dl</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="blue-band" alt="Image of Kernlute columns" /></td>
<td><img src="blue-band" alt="Image of Kernlute columns" /></td>
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<tr>
<td>100 µl serum</td>
<td>100 µl serum</td>
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<tr>
<td>100 µl serum</td>
<td>100 µl serum + 100 µl bilirubin (≥ 2.5 mg/dl)</td>
</tr>
<tr>
<td></td>
<td>100 µl serum + 200 µl bilirubin (≥ 5 mg/dl)</td>
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Standards and Controls

There are no standard solutions involved with the bilirubin binding capacity determination. However, it is important that control serums be used to check the technique, procedure and reagents. Any normal serum can be used as a negative control, but it is important to realize that the residual binding capacity is quite high and will certainly give a result of more than 5 mg bilirubin per dl. Therefore, it is useless to waste extra Sephadex columns by adding bilirubin to the normal control and repeating the test.

To obtain a positive serum, the following steps may be performed:
1. Three bilirubin caps are removed from the vial and each is filled with sodium carbonate solution, and allowed to stand for about 15 min. It takes about six drops of sodium carbonate to fill a cap.
2. While the bilirubin solution stands, a normal serum is diluted with an equal part of water (or old, outdated lyophilized normal control serum is reconstituted with twice the amount of water required). This cuts the protein content to half of normal.
3. To 0.5 ml of this dilute serum, the contents of the three caps are added with a transfer pipet, rinsing the cap contents up and down in the pipet to remove as much bilirubin as possible from each cap. This final solution then contains approximately 15 mg of bilirubin per dl and the serum protein content (and therefore bilirubin binding capacity) is about 25 percent of normal.

This serum should give a positive result (i.e., bilirubin binding capacity exceeded) if the procedure is performed properly and if the reagents are made and used correctly.

Special Apparatus

Columns filled with Sephadex G-25 and buffered at pH 7.45 are required for the test and, as mentioned previously, are supplied with the kit. Also supplied for convenience in performing the procedure are a drain rack and tray. No instruments or other apparatus are required.

Procedure

1. The top cap of a column is removed and discarded and the buffer discarded from the top of the column.
2. Phosphate buffer is added to the lower black line on the column (0.75 ml).
3. Exactly 100 µl of serum are added to the column and mixed with the buffer by gentle swirling.
4. The bottom cap of the column is removed (and saved) and the buffer-serum mixture allowed to enter the column. Eluate is drained off.
5. When the column has drained, phosphate buffer is added to the top black line of the column (2 ml) and allowed to drain. This washes the serum protein and bound bilirubin through the column into the eluate.
6. When the column has again drained, the bottom cap is replaced and water is added to the bottom black line (0.75 ml).
7. One diazo tablet is added to the water and allowed to dissolve (some residue will remain, but when effervescence has stopped, the procedure may be continued).
8. The bottom cap is removed from the column and the diazo reagent allowed to enter the column.
9. When the column has drained, it is observed for a blue band of color in the Sephadex. This indicates the presence of unbound bilirubin on the Sephadex and therefore means that the bilirubin-binding capacity of serum has been exceeded.

If no blue band is evident on the column, the procedure is repeated with two more aliquots of patient serum in order to estimate the range of the residual binding capacity.
Using two new columns, the procedure is repeated. To each column are added 100 μl of serum. To one column are added 100 μl of bilirubin solution, and to the other are added 200 μl of bilirubin solution. The draining and washing steps previously noted are performed as is the color formation step with the diazo reagent. If a blue band forms on the first and second new columns the residual bilirubin binding capacity is equivalent to less than 2.5 mg of bilirubin per dl. If a blue band does not form on the first column but does form on the second, the reserve bilirubin binding capacity is equivalent to a range of 2.5 to 5 mg of bilirubin per dl. If a blue band does not form on either new column, the reserve bilirubin binding capacity is equivalent to more than 5 mg of bilirubin per dl.

The procedure is illustrated in simple form in figure 2, and the interpretation of results for a negative and positive serum has been shown in figure 1.

Discussion

Kernicterus is a term first used in the early 1900's to describe the yellow staining found at autopsy in the basal ganglia of infants who died after severe jaundice attack. Premature infants suffer kernicterus more often than full-term infants. This very serious condition can occur not only in those infants with hemolytic disease of the newborn (Rh or ABO incompatibility) but also in various kinds of infections, metabolic disorders and familial disorders. In physiologic jaundice of the newborn, the upper serum bilirubin concentration of 5 to 6 mg per dl is mainly unconjugated bilirubin and is probably due to delayed development of glucuronyl transferase activity of the normally immature liver of all newborns. This transient jaundice usually disappears by the fifth day of life as liver functional capacities develop. In order to differentiate those jaundiced infants who

![Figure 2. Directions for Kernlute. The Kernlute procedure should be performed under subdued lighting conditions.](image-url)
may develop kernicterus from those who have the usual "physiological jaundice," it is also important to determine the bilirubin binding capacity of the infant's serum.

Investigators have combined the Sephadex gel filtration step with the analysis of the eluate for bilirubin to determine the amount of bound and unbound bilirubin in serum. Pays and Beljean\(^8\) cut the Sephadex column apart and used the area in which the unbound serum bilirubin attaches to the Sephadex to perform a colorimetric assay for bilirubin. Other investigators\(^10\) determined the bilirubin content of the eluate (which is albumin-bound bilirubin). Then the bilirubin attached to the Sephadex from the column was eluted by running more albumin through to remove bilirubin from the column. Analyzing the second eluate gave unbound bilirubin. McCluskey et al\(^7\) reported on albumin-titratable bilirubin in jaundiced neonates. This was determined by adding albumin to bilirubin and measuring the albumin-bilirubin complex by fluorescence. Odell\(^8\) has reported on the saturation index which he determined by using salicylates or comparable drugs to displace the bilirubin bound to the protein of the serum of neonates. Athanassiadis et al\(^2\) used electrophoresis to separate bound from unbound bilirubin.

**Sources of Error**

Kernlute provides estimation of the range of residual bilirubin binding capacity left. Accordingly, small differences in the procedure should not give erroneous answers. However, if the bilirubin solution is allowed to oxidize to biliverdin, serious errors may result since the measurement of the amount of bilirubin must be accurately made to estimate the ranges of residual binding capacity. For best results the entire procedure should be carried out in subdued light. In addition, complete draining of the column must take place before the next addition of water or buffer occurs so that each elution or wash of the column is not contaminated by the previous solution.

**Normal Ranges**

Normal neonatal serum has a residual bilirubin binding capacity equivalent to more than 5 mg of bilirubin per dl as determined by Kernlute.

**Résumé of Clinical Interpretations**

Kernlute determines the "loose binding" or the weak affinity of albumin for bilirubin. Griffiths et al\(^4\) have reported a procedure for determining both the "tight" and "loose" bilirubin binding capacity of serum. This would be of particular value in infants whose bilirubin binding capacity is further complicated by the presence of drugs or respiratory conditions interfering or competing with the albumin binding of bilirubin.

For "screening" of jaundiced neonates, the procedure describing combined with total bilirubin determinations would suffice in most cases. If serial total bilirubin levels continue to increase, a conjugated bilirubin determination should be made to eliminate such rare inherited disorders as the Rotor or Dubin-Johnson syndromes. If unconjugated (total) serum bilirubin continues to rise, serial bilirubin binding capacity determinations (even during phototherapy if that is the chosen treatment) aid in determining when exchange transfusion becomes necessary to avoid kernicterus.

The aim of laboratory investigation is to prevent kernicterus from occurring. As a last resort, exchange transfusion may be the only satisfactory route if phototherapy, avoidance of competitive drugs

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and albumin administration prove unsuccessful.

References


