HEMOGLOBIN CONCENTRATION

Principle

Whole blood is diluted in cyanmethemoglobin reagent. This reagent hemolyzes the erythrocytes which releases hemoglobin into the solution. The ferrous ions (Fe²⁺) of the hemoglobin molecules are oxidized by potassium ferricyanide to ferric ions (Fe³⁺). This oxidation results in the formation of methemoglobin. Methemoglobin combines with the cyanide ions (CN⁻) to form cyanmethemoglobin, a stable compound. All hemoglobin derivatives except sulfhemoglobin are converted to cyanmethemoglobin.

When measured spectrophotometrically at 540 nm, the absorbance of cyanmethemoglobin follows Lambert-Beer's law and is directly proportional to the concentration of hemoglobin in the blood. A reference (standard) curve is prepared using cyanmethemoglobin standard solutions of known hemoglobin concentrations (Figure 7-10). An unknown hemoglobin concentration may be calculated from the measured absorbance, read from a standard calibration curve, or read directly from the instrument scale of specialized instruments.

Reagents and Equipment

1. Cyanmethemoglobin standard, 80 mg/dL - available commercially
2. Cyanmethemoglobin reagent – available commercially as a dry powder or liquid reagent containing potassium ferricyanide (K₃Fe(CN)₆), potassium cyanide (KCN), and sodium bicarbonate. Cyanmethemoglobin reagent should be stored in a brown bottle to prevent deterioration.
3. Test tubes, 13 x 100 mm
4. Micropipet, 20 μL (0.020 mL)
5. Micropipet tips
6. Volumetric pipets: 1 mL, 2 mL, 3 mL, 4 mL
7. Serologic pipets, 5 mL
8. Matched cuvets
9. Spectrophotometer
10. Graph paper

Quality Control

Commercial controls with established control limits should be run with each hemoglobin determination.

Specimen

Whole blood, anticoagulated with EDTA, or free-flowing capillary blood may also be used.
Procedure

1. Preparation of a Standard Hemoglobin Curve: Prepare duplicate dilutions of the cyanmethemoglobin standard representing 4 g/dL, 8 g/dL, 12 g/dL, 16 g/dL, and 20 g/dL.

<table>
<thead>
<tr>
<th>Tube</th>
<th>Cyanmethemoglobin Standard</th>
<th>Cyanmethemoglobin Reagent</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 g/dL</td>
<td>1 mL</td>
<td>4 mL</td>
<td>1/5</td>
</tr>
<tr>
<td>8 g/dL</td>
<td>2 mL</td>
<td>3 mL</td>
<td>2/5</td>
</tr>
<tr>
<td>12 g/dL</td>
<td>3 mL</td>
<td>2 mL</td>
<td>3/5</td>
</tr>
<tr>
<td>16 g/dL</td>
<td>4 mL</td>
<td>1 mL</td>
<td>4/5</td>
</tr>
<tr>
<td>20 g/dL</td>
<td>5 mL</td>
<td>0 mL</td>
<td></td>
</tr>
</tbody>
</table>

a. Label 13 x 100 mm test tubes.
b. Using the appropriate volumetric pipets, pipet the cyanmethemoglobin standard into the lower third of each test tube.
c. Using a 5 mL serologic pipet, add the proper amount of cyanmethemoglobin reagent to each tube.
d. Mix contents of each tube thoroughly.
e. Transfer the standard solutions into matched cuvets.
f. Read the absorbance of each solution at 540 nm using a reagent blank. Record the results.
g. Prepare a standard hemoglobin curve using graph paper. Plot the absorbance on the y-axis and the hemoglobin concentration in g/dL on the x-axis (Figure 7-10). 

2. Hemoglobin Procedure:
a. Pipet 5.0 mL of cyanmethemoglobin reagent into appropriately labeled 13 x 100 mm test tube.
b. Draw 0.02 mL of well-mixed whole blood into the micropipet. Carefully wipe the excess blood from the outside of the pipet.
c. Expel the blood from the micropipet into the cyanmethemoglobin reagent.
d. Rinse the micropipet several times to remove all remaining blood from the pipet.
e. Mix the contents of the test tube thoroughly by inversion.
f. Allow the diluted hemoglobin solution to sit at room temperature for 10 minutes. This ensures that full color development occurs.
g. Mix the test tube thoroughly and transfer the contents to a matched cuvet.
h. Record the absorbance of the diluted hemoglobin solution using a reagent blank at 540 nm.
i. Determine the hemoglobin concentration (g/dL) of the diluted solution using the standard calibration curve established for that particular spectrophotometer and set of reagents. The hemoglobin concentration may also be calculated using Lambert-Beer's law.

Interpretation of Results

Using linear graph paper, a standard curve is prepared by plotting the concentration of each cyanmethemoglobin standard on the x-axis and its corresponding absorbance on the y-axis. A
straight line that best fits the plotted data points is drawn. The results for patients and controls are read from this curve using their respective absorbance readings.

**Reference Intervals**

<table>
<thead>
<tr>
<th></th>
<th>Conventional Units (g/dL)</th>
<th>SI Units (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adult Males:</td>
<td>13.5 - 17.5</td>
<td>135-175</td>
</tr>
<tr>
<td>Adult Females:</td>
<td>12.0 - 16.0</td>
<td>120-160</td>
</tr>
</tbody>
</table>

**Comments**

1. The 80 mg/dL cyanmethemoglobin standard is equivalent to 20 g/dL hemoglobin.
2. A new standard curve should be established whenever a new batch of cyanmethemoglobin reagent is prepared, a change in spectrophotometers is made, or spectrophotometer light source is changed.
3. Cyanmethemoglobin reagent is unstable and deteriorates upon exposure to light; therefore, it should be kept in a brown bottle. Cyanmethemoglobin reagent is stable for several months when stored at room temperature.
4. Technical sources of error include inadequate mixing of specimen; inaccurate pipets; pipeting error; use of dirty, scratched, or unmatched cuvets; and use of deteriorated reagents.
5. Physiologic sources of error are given in Table 7-3. 

**References**