EDUCATIONAL COMMENTARY – HEMOGLOBIN ELECTROPHORESIS

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LEARNING OUTCOMES

On completion of this exercise, the participant should be able to

- describe separation of the various hemoglobins by electrophoresis.
- identify various abnormal hemoglobins from an electrophoretic pattern compared with a control.
- describe situations when it may be necessary to follow cellulose acetate electrophoresis with citrate agar electrophoresis at an acid pH.
- list advantages and disadvantages of each of the five methods for identifying abnormal hemoglobins described in this commentary.

Hemoglobin electrophoresis is the method of choice to separate and identify abnormal hemoglobins in the blood. The normal composition of hemoglobin in adults consists of approximately 95% hemoglobin A (HbA), less than 3% hemoglobin A2 (HbA2), and the remainder, hemoglobin F (HbF). Changes in the amino acid chains that make up the globin or protein portion of the hemoglobin molecule can cause abnormal hemoglobins that may lead to various diseases, often referred to as hemoglobinopathies. These changes are caused by genetic mutations and may be either qualitative defects in the globin chain, referred to as structural abnormalities, or quantitative defects that alter the rate of globin chain production. Sickle cell disease due to abnormal hemoglobin S (HbS) is an example of a disease caused by a structural abnormality in the globin chain. Less common abnormal hemoglobins due to structural abnormalities are HbD, HbE, and HbG. Thalassemia is an example of a disease caused by a quantitative defect, resulting in decreased production of one type of protein chain. This may result in an increased production of other hemoglobins, such as HbA2 or HbF. In thalassemia, it may be necessary to quantitate the amount of HbA2 or HbF present. In many cases, family history will reveal abnormal hemoglobins present in other family members.

Electrophoresis Principles and Procedures

Electrophoresis may be defined as the movement of charged particles in an electrical field. Hemoglobin electrophoresis uses the basic principles of particle separation used in protein electrophoresis. Charged particles migrate toward the opposite charged electrode. The speed of the migration is determined by the size of the particle, net charge of the particle, strength of the electrical field, and the medium on which the particles are placed.1 Hemoglobin A has a net negative charge at an alkaline pH and moves the farthest toward the positive electrode. Amino acid substitutions in the globin chains of abnormal hemoglobin
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variants alter their charges and, therefore, their velocity. They may be identified by comparison with a control containing abnormal hemoglobin variants.²

The most common type of hemoglobin electrophoresis uses cellulose acetate as a medium and an alkaline pH. This separates out the most frequently encountered abnormal hemoglobins so they may be identified. The procedure begins when EDTA-anticoagulated blood is centrifuged to separate the red blood cells from plasma. The red blood cells are washed and treated with a reagent that lyases the cells and liberates the hemoglobin molecules. The hemolysate is then applied to a cellulose acetate plate and placed in an electrophoresis chamber. Alkaline buffer, pH 8.6, allows the hemoglobin molecules to migrate and separate according to their charge and amino acid composition. When the migration is complete, the plate is stained with a protein binding dye such as ponceau S and cleared to remove the cellulose acetate medium. This leaves a transparent plate with only the hemoglobin bands remaining (Figure). The plate can be evaluated visually or with a densitometer using a 525-nm filter to estimate the percentage of hemoglobin in each band. Although it is fairly labor-intensive, cellulose acetate electrophoresis is still commonly used, especially in smaller laboratories that do not have an abundance of patients with suspected hemoglobinopathies. It does not require expensive equipment and is relatively simple to perform. It supplies both qualitative and quantitative results. In some cases, the densitometer is not adequate to detect small fractions of HbF or HbA₂. In these cases, a different procedure may be used to quantitate these hemoglobins.

![Comparison of Various Hemoglobin Samples on Cellulose Acetate and Citrate Agar.](image-url)
Some abnormal hemoglobins have the same velocity and will migrate together under the previously described electrophoretic conditions. In this case, a second electrophoresis procedure using citrate acetate as the medium and an acidic pH must be performed. The various hemoglobins migrate at different positions under these conditions and usually can be identified using information from both patterns (Figure). For example, Hbs S, D, and G all migrate to the same position using cellulose acetate electrophoresis. Also, an individual with Hb SC disease cannot be distinguished from one with Hb SE disease using only cellulose acetate. Citrate agar electrophoresis can easily separate HbS from HbD and HbG and can differentiate between HbSC and HbSE.

Laboratories that test large numbers of patients with hemoglobinopathies may use advanced techniques, such as isoelectric focusing or capillary electrophoresis, which can separate a number of hemoglobin variants not clearly distinguished by cellulose acetate or citrate agar electrophoresis. Newborn screening programs often use isoelectric focusing due to the large numbers of samples processed. High-pressure liquid chromatography (HPLC) may be used in large-volume laboratories such as reference laboratories. In some cases, definitive diagnosis may require DNA analysis.\(^3\),\(^4\)

Isoelectric focusing separates the various hemoglobins according to their isoelectric point on a gel containing pH gradient. The pH gradient is generated by special molecules called amphoteric electrolytes or ampholytes in an electric field. The hemoglobin proteins migrate in the electric field until they encounter the position that corresponds to their isoelectric point. At this point there is no net charge, and the sample stops migrating. This method is good for large numbers of samples and produces excellent separation, but is costly and requires experienced staff for interpretation. Also, it does not allow for quantitative results.\(^5\)

Capillary electrophoresis separates the sample in an electrical field using small glass capillary tubes filled with buffer. Multiple capillaries may be used simultaneously, making it a good method for large numbers of samples. The recently introduced CapillaryS system (Sebia, Inc.) uses this method in a completely automated system. Besides being capable of walk-away operation, capillary electrophoresis produces high-resolution separation associated with direct quantification of normal and abnormal hemoglobin fractions.\(^5\),\(^6\) The CapillaryS method was compared with an isoelectric focusing method in a neonatal screening program using liquid umbilical cord blood. The researchers concluded that the capillary electrophoresis method was reliable for screening newborns for hemoglobinopathies and it enabled early detection of all major hemoglobinopathies and most minor ones.\(^7\)

Cation-exchange HPLC is the method used by laboratories with a volume of samples large enough to justify the costly equipment. This method uses automated sampling and has the advantage of being able
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to accurately measure small quantities of HbF and HbA₂. It can also be used to quantify glycated hemoglobin (HbA₁C) for monitoring diabetes mellitus.⁴,⁵

Other Tests

The solubility test is often used as a screening test for HbS. It is simple to perform and does not require any expensive equipment. Anticoagulated blood is placed in a solution containing a lysing agent and a reducing agent such as sodium dithionite. The red blood cells are lysed, releasing the hemoglobin molecules. If HbS is present, the solution will be cloudy because it forms crystals; if a majority of HbA is present, the solution will be clear. This solubility test does not distinguish between HbS disease (homozygous: HbS gene inherited from both parents) and HbS trait (heterozygous: one abnormal gene and one normal gene inherited). There are also several other rare hemoglobin variants that can cause sickling (e.g., HbC_As) and may cause a positive test result. Therefore, a positive solubility test should be followed with hemoglobin electrophoresis.² The solubility test is not valid on infants younger than 6 months, due to the amount of HbF normally present.³

Another test often performed to aid in the diagnosis of thalassemia, a quantitative hemoglobinopathy, is ion-exchange column chromatography. A common type of thalassemia, β-thalassemia minor, is characterized by an increased amount of HbA₂. The slight increase may not be accurately quantitated by standard cellulose acetate electrophoresis. In this case quantification of HbA₂ may be performed by this method. Blood is passed through a column lined with a resin containing positively charged molecules. The ionic strength of the buffer and pH levels can be controlled to cause different hemoglobins to possess different net negative charges. The positively charged molecules in the resin bind with the negatively charged hemoglobin molecules. Then different hemoglobins are removed selectively by altering the pH and ionic strength of the elution buffer. HbA₂ is soluble in and removed from the resin by elution buffer. Using a spectrophotometer, the absorbance of the solution of HbA₂ is compared with the absorbance of the remainder of the hemoglobin.² Hemoglobin A₂ may also be accurately measured by HPLC if the equipment is available.

In some cases it is necessary to quantitate the amount of HbF present using the alkali denaturation test. Hemoglobin F is more resistant than other hemoglobins to denaturation by strong alkali solution. After preparing a hemolysate, a strong alkali solution, for example 1.27 M NaOH, is added to the sample. All denatured hemoglobin is precipitated by addition of ammonium sulfate and filtered out. The remaining alkali-resistant HbF is measured spectrophotometrically.²

Flow cytometry may also be used to quantitate HbF. Cells are made permeable so a fluorescent antibody against HbF may enter the cell and attach to any HbF present. One advantage of flow cytometry is that many cells are evaluated, increasing the accuracy. It also allows the amount of HbF within each cell to
be determined by addition of a dye. This method may be useful for determining the amount of mature red blood cells containing mostly HbA and fetal cells containing mostly HbF.  

**Summary**

Hemoglobin electrophoresis is the most common laboratory method to separate and identify abnormal hemoglobins to aid in diagnosis of hemoglobinopathies. Hemoglobin electrophoresis may be performed using different types of media, current, and pH to separate the different hemoglobin proteins according to electrical charge. The most commonly used hemoglobin electrophoresis methods are cellulose acetate at an alkaline pH and citrate agar at an acidic pH. In some cases both types of electrophoresis are necessary to accurately identify the hemoglobinopathy, such as in separation of HbS from Hbs D and G or to differentiate HbA₂ from HbC. These hemoglobin electrophoresis procedures are somewhat time consuming, and in large laboratories that have a greater volume of patients with suspected hemoglobinopathies they may be replaced by newer faster methods using more expensive equipment. Screening of newborns for the most common hemoglobinopathies is also required in many states. These large numbers require some of the more automated methods. It is essential to diagnose hemoglobinopathies as early as possible because in many critical cases, early treatment may prolong life. In addition, such information may be useful for genetic counseling.

**References**


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