Quantitation of A₂ Hemoglobin by Polyacrylamide Gel Disc Electrophoresis: A Method with Individual Specimen Standardization

HENRY G. SCHRIEVER, M.D. AND DANUTE M. LEVECKIS, B.A.

Department of Pathology, John F. Kennedy Community Hospital, Edison, NJ 08817

ABSTRACT

A polyacrylamide gel electrophoresis technic for the determination of hemoglobin A₂ is presented. Rapid separation is an advantage. The use of a diluted hemolysate as a 4 percent standard avoids overestimation of the A₂ fraction by densitometry and also provides a reference for visual comparison. Normal range of A₂ hemoglobin by this method is 1.12 to 4.13 g per dkg. Coefficient of variation was 5.84 percent.

Introduction

The diagnosis of beta thalassemia minor can be established in three steps. The first step is measuring red cell indices to see if they are in the thalassemic range. The second is checking the blood smear for the red cell targeting and basophilic stippling which are typical of the condition. The third and final step is measurement of the A₂ hemoglobin fraction.¹²,¹³ Elevation of A₂ hemoglobin is specific for beta thalassemia minor, with the possible exception of pernicious anemia¹⁴ which is easily excluded by morphology and red cell indices.

Methods for the determination of A₂ hemoglobin must provide for both separation and quantitation. Electrophoresis on starch gel,¹⁰ starch block,¹⁵ polyacrylamide gel,³,⁵ cellulose acetate,²,⁸,⁹,¹⁶,¹⁷,¹⁸ paper⁴,¹¹ and agar gel²⁰ have been used for separation. Column chromatography has also been used.¹,⁷,¹⁰ Quantitative comparison of the two fractions is usually accomplished by either direct densitometry or elution and colorimetry, with A₂ hemoglobin being reported as a percent of total hemoglobin. Many of the methods are either time-consuming or inaccurate or both.

Method

The method given here has been used for three years in the diagnosis of more than 100 cases of thalassemia minor. It has two main advantages. The separation by polyacrylamide gel disc electrophoresis is rapid and distinct. A 4 percent dilution of each original hemolysate is used as a standard for comparison with the A₂ band. This avoids the densitometric comparison of a tiny A₂ band with a huge A₁ fraction, which tends to give false high values for hemoglobin A₂. A third advantage is that with the use of the 4 percent "standard", elevated A₂ fractions can usually be spotted easily by inspection if densitometry is not available.
HB QUANTITATION BY POLYACRYLAMIDE GEL DISC ELECTROPHORESIS

PRINCIPLE

The normal hemoglobin fractions A\textsubscript{1} and A\textsubscript{2} are separated by electrophoresis in polyacrylamide gel, in about 20 minutes. Once clearly separated, the A\textsubscript{2} fraction is quantitated by densitometry. Two gel columns are electrophoresed for each A\textsubscript{2} determination. The extra column carries a 4 percent dilution of the original hemolysate which serves as a standard for densitometric comparison to the A\textsubscript{2} peak.

REAGENTS\textsuperscript{8}

Temed solution. To 0.12 ml of NNN'N' tetramethylethylene-diamine (Canalco #204) are added 18.1 g of 2-amino-2-hydroxymethyl-3-propanediol (tris) (Canalco #210), 24 ml of 1 N HCl and enough distilled water to make 100 ml. The pH range is 8.8 to 9.0. The solution is stable indefinitely at 4°.

Acrylamide solution. To 28.0 g of acrylamide monomer (Canalco #201) are added 0.735 g of N,N'methylenebisacrylamide (bis) (Canalco #202) and enough distilled water to make 100 ml. The solution is stable indefinitely at 4°.

Catalyst solution. To 0.14 g of ammonium persulfate (Canalco #209) are added enough distilled water to make 100 ml. Store solution at 4° and make fresh monthly.

Upper gel component 1. To 48 ml 1 of N HCl are added 5.98 g tris, 0.46 ml temed and enough distilled water to make 100 ml. Adjust the pH to 6.6 to 6.8. The solution is stable indefinitely at 4°.

Upper gel component 2. To 20.0 g of acrylamide are added 5.0 g bis and enough distilled water to make 100 ml. The solution is stable indefinitely at 4°.

Riboflavin solution. To 0.004 g of riboflavin (Canalco #208) are added enough distilled water to make 100 ml. Store solution at 4°.

Sucrose solution. To 40 g of sucrose are added enough distilled water to make 100 ml. Solution is good for one month at 4°.

Upper gel solution. To one part component 1 are added one part component 2, one part riboflavin solution, four parts sucrose solution and one part distilled water. The solution is stable indefinitely at 4°.

Glycine buffer. To 6.0 g of tris are added 28.8 g of glycine (Canalco #203) and enough distilled water to make one liter. The solution is stable for approximately six months at 4°.

Saponin solution. Zap-Isoton (Coulter Electronics, Hialeah FL).

Chloroform. AR grade.

SPECIAL APPARATUS

The necessary equipment includes:
(1) Disc electrophoresis cell with stacking buffer chambers;\textsuperscript{*}
(2) Power supply capable of supplying 60 ma current;
(3) Densitometer and integrator capable of scanning gel columns;\textsuperscript{f}
(4) Glass Columns; 8 X 60 mm, o.d. cut from glass tubing;
(5) Polymerization rack,\textsuperscript{t} or equivalent constructed from 7 ml Vacutainer stoppers glued to a solid base;
(6) Disposable plastic syringe, 10 cc, fitted with curved #18 blunt ended lumbar puncture needle and
(7) Micropipet to deliver 0.025 ml.\textsuperscript{§}

PROCEDURE

MAKING THE HEMOLYSATE

Two to seven ml anticoagulated whole blood (EDTA, heparin or citrate) are centrifuged at 2500 rpm for five minutes; the plasma and buffy coat are aspirated and discarded. The red cells are washed three

\textsuperscript{*} Model 1200 bath with #1807 Safety interlock adapter, Canalco, Rockville, MD 20852.
\textsuperscript{f} Densicord and Integraph, Photovolt Corporation, New York, NY 10010.
\textsuperscript{t} Canalco #1812.
\textsuperscript{§} Unimetrics, Anaheim, CA 92801.
times with normal saline solution, centrifuging and discarding the washes each time. One drop of Zap-Isoton is added to the packed cells and the mixture is shaken vigorously for one minute.

One-half ml of chloroform is added to the mixture which is shaken vigorously for one minute and then centrifuged at 2500 rpm for 15 minutes. The supernatant hemolysate is then aspirated and transferred to a clean test tube. If not sparkling clear, the procedure is repeated. The hemoglobin concentration should be 18 to 25 gm per dl. A 4 percent "standard" hemolysate solution is prepared by adding 0.1 ml of the original hemolysate to 2.4 ml 40 percent sucrose solution in a test tube.

**Making the Gel Columns**

One part Temed solution, one part acrylamide solution and two parts catalyst solution are drawn into a 10 ml syringe through the curved needle. The solutions are mixed, handwarmed and degassed by pulling a vacuum in the syringe with a finger over the outlet. Two glass columns for each specimen are placed in the polymerization rack and one ml of gel solution is added.

Distilled water is carefully layered over the gel mixture using a disposable Pasteur pipet. No mixing of gel and water should occur. The columns are allowed to stand for 20 to 30 minutes for gelling to occur. Columns may now be capped with polyethylene film and stored at 4°. When the columns are to be used, the water layer is shaken off, 0.15 ml upper gel solution is added and layered with water. The columns are placed in ultraviolet, fluorescent or sunlight for polymerization, which takes about 20 minutes.

**Electrophoresis**

The water layer on the columns is shaken off and the columns are filled with glycine buffer. With the long-tipped micropipet 0.015 ml hemolysate is carefully layered on top of the upper gel so that it forms a thin band. The same is done for the 4 percent "standard" hemolysate on a separate column (figure 1). The columns are inserted in the disc electrophoresis cell, the chambers filled with glycine buffer and electrophoresed at 5 ma per column until the A₁ and the slower moving A₂ fractions are separated by two mm (figure 2) which takes approximately 20 minutes.

**Densitometry**

The columns are removed and wiped clean with tissue. The densitometer is set for log-linear response (L5 on the Densi-

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*Figure 1. Gel columns ready for electrophoresis. Hemolysate has been layered under the glycine buffer on top of the upper gel. The greater density of the hemolysate retards diffusion and keeps it a thin band.*
HB QUANTITATION BY POLYACRYLAMIDE GEL DISC ELECTROPHORESIS

Figure 2. Full strength hemolysate and diluted 4 percent “standard” on left after 20 minute electrophoresis. The hemoglobin A2 band on right is much more intense than the band of the standard, indicating thalassemia minor.

Figure 3. Densitometric tracings of the A2 peak (1), the A2 peak with zero set in the valley between A1 and A2 bands (2), and the 4 percent “standard” (3). Calculation consists of averaging the tooth count for 1 and 2 and comparing with 3. Result of 2.3 percent is in normal range.

Figure 4. Densitometric tracings in thalassemia minor. Total tooth count of A2 band (1) and A2 band with zero set in A1-A2 valley (2) is 95, the average of which is 47.5, giving a 5.0 g per dkg A2 hemoglobin result when compared to the total tooth count of 38 for the 4 percent “standard” (3).

Calculation

The integrator marks (teeth) under the 4 percent “standard”, and each of the two tracings of the full strength A2 fraction are counted. The average tooth count of the two tracings of the A2 fraction is determined and compared with the 4 percent standard (figure 3 and 4).

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\text{Percent A2} = \frac{\text{average teeth}}{\text{standard teeth}} \times 4
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Sources of Error

Bad Hemolysate

Contamination with red cell stroma will cause a fuzzy separation. Re-extraction, however, with chloroform will correct this. Hemolysates are generally stable at 4° for months, but they may deteriorate to a brownish color and give poor separation.

Delay in Electrophoresis

After adding the glycine buffer, the hemolysates should be layered immediately and electrophoresed without delay. If the
buffer is permitted to diffuse into the gel, fuzzy separation will result.

**Faint Bands**

The standard and $A_2$ bands should be dense enough to give a fairly high tooth count on the integrator (30 to 90) to avoid the inaccuracy inherent in low numbers. Amount of hemolysate added to the columns may be adjusted.

**Normal Values**

In 72 normal patients, the mean value of $A_2$ hemoglobin was 2.62 g per dkg ± 0.75 giving a 95 percent range of 1.12 to 4.13. In 86 patients with thalassemia minor, the mean was 6.2 g per dkg and the $A_2$ hemoglobin had a range of 4.2 to 11.5.

**Precision**

In 19 replicate determinations, the mean was 3.84 g per dkg ± 0.225, giving a coefficient of variation of 5.84 percent.

**References**