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Electrophoresis of Hemoglobin on Polyacrylamide Gels: Precise Method for Measurement of Hemoglobin A_2

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Hemoglobin electrophoresis on polyacrylamide gels is a technique applicable in the clinical laboratory that processes moderately large numbers of specimens. Rapid and precise, it yields reproducible quantitative data when the hemoglobins are eluted from the gel and their concentrations determined spectrophotometrically. Hemoglobin A_2 measurements were reproducible and did not differ from those measured by column chromatography on DEAE-cellulose. Concentrations of hemoglobin A_2 in the blood of patients with sickle cell anemia were significantly higher than in normal individuals and individuals with sickle trait, but significantly lower than is the case for patients with beta-thalassemia minor.

Additional Keyphrase: *sickle cell disease*

Many different methods of hemoglobin (Hb) electrophoresis are used, varying primarily in the type of supporting medium, buffer system, and time required (*1-3*). In the clinical laboratory, the value of some methods is limited by the excessive difficulty of and time required for the procedure, the impracticality of testing large numbers of samples, and the unreliability in the measurement of Hb fractions present.

In contrast, hemoglobin electrophoresis on polyacrylamide gels is efficient, reliable, and quantitatively reproducible.

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Materials and Methods

Hemolysates were prepared from anticoagulated blood by routine techniques. Consistent electrophoretic patterns were obtained by adjusting the Hb concentration of each hemolysate to about 10 g/100 ml and re-extracting old hemolysates with chloroform before testing.

Electrophoresis was carried out in a "Polyanalyst" apparatus (Buchler Instruments, Ft. Lee, N. J. 07025). Buffer temperature was maintained at 10 °C with a circulating water bath. A stock solution of acrylamide (14 g/dl) with methylenebisacrylamide (0.4 g/dl was prepared monthly and stored at 4 °C. Before electrophoresis, 12.5 ml of the stock solution was diluted with 25 ml of Tris-EDTA-borate buffer[1] (pH 8.28), and 0.07 ml of N,N,N′,N′-tetramethylenediamine was added. The acrylamide buffer solution was activated by addition of 12.5 ml of a fresh ammonium persulfate solution (0.48 g/dl) and pipetted into cylindrical glass tubes (75 × 5 mm), sealed by rubber caps. The tubes were filled to within 10 mm of the top and one drop of water was layered over the solution to prevent meniscus formation. After the gels were formed, both electrode chambers were filled with a fivefold-diluted Tris-EDTA-borate buffer (pH 8.28, 75 mmol/liter), and excess ammonium persulfate was removed by prerunning with a current of 6 mA per tube for 15 min.

The volume of hemolysate applied to the gel may vary but Hb A_2 concentration is most accurately measured when 20-μl samples are used. A few crystals of sucrose were dissolved in each hemolysate, to retard diffusion of the sample. Electrophoresis was begun with current of 6 mA per tube and continued for 60 min.

[1]Buffer solution composition is, per liter: 0.36 mol tris(hydroxymethyl)aminomethane, 0.36 mol disodium ethylenediamine tetraacetate, and 0.36 mol sodium borate.

The percentage of Hb fractions present was measured by cutting out the bands, extracting the components with cyanmethemoglobin reagent,[2] and determining their absorption at 420 nm. Because some difficulty was encountered in visually determining the exact width of the Hb A_2 band, we used a simple gel-cutter made of lucite, which held two razor blades 4 mm apart. By centering this band under the blades, a similar amount of gel, containing the entire visible portion of the Hb A_2 band (and therefore, within experimental error, all of the Hb A_2), could be obtained from each sample processed. Each fraction was placed in a 15-ml conical centrifuge tube. Cyanmethemoglobin reagent solution, 2 ml, was added to tubes containing major hemoglobin components such as hemoglobins A, S, or C, and 1 ml to the tubes containing Hb A_2. The gels were finely minced, covered, and allowed to stand at room temperature overnight. After agitation and centrifugation, supernates of the major Hb fractions were diluted 10-fold with cyanmethemoglobin reagent while Hb A_2 extracts were read undiluted. The absorption of each sample was determined at 420 nm.

The percentages of the various hemoglobins present were calculated by the formula of Neerhout et al. (*4*).

Results

After electrophoresis and elution from the gels, the percentages of the major Hb components found for individuals with sickle trait (AS), Hb SC disease and Hb C trait are listed in Table 1.

Table 2 presents the percentage of the minor Hb component, Hb A_2, found in patients with sickle cell anemia (SS), AS, beta-thalassemia minor, and normals. The proportions of Hb A_2 in patients with SS (3.1%) are higher than in those found in normals (2.6%) or those with AS (2.4%) ($P < .05$).[3] A clear distinction exists in the mean Hb A_2 values between normals (2.6%), individuals with AS (2.4%), and patients with SS (3.1%) when compared to those found in patients with beta-thalassemia minor (4.6%) ($P < .05$). Although the concentrations of Hb A_2 in cases of SS (3.1%) and beta-thalassemia (4.6%) are significantly different ($P < .05$), there is some overlap in values.

The reproducibility of this method was tested by making duplicate measurements of Hb A_2 on the same hemolysate. The mean of the differences of replicate determinations for 97 samples was 0.19%. The percentages of Hb A_2 in hemolysates taken from the same individual at different times was measured in 15 patients. There was no significant difference in the percentages of Hb A_2 in the two hemolysates ($P > .05$). To test the precision of our procedure in determining Hb A_2 percentages, we compared our method to one in which chromatography on a diethylaminoethyl-Sephadex column is used (*5*). The results of this comparison (Table 3) indicate no significant difference ($P > .05$) between these methods.

Table 1. Hemoglobins A, S, and C, as Measured Spectrophotometrically after Gel Electrophoresis of Hemolysate from Patients with Sickle Trait (AS), Hemoglobin SC Disease (SC), or Hemoglobin C Trait (AC)

Hemolysate	No. patients	Hemoglobin (±SD), % A	S	C
AS	39	56 ± 4	42 ± 4	—
SC	12		50 ± 3	49 ± 3
AC	34	55 ± 6	—	45 ± 6

Table 2. Percentage of Hemoglobin A_2, as Measured Spectrophotometrically after Gel Electrophoresis, in Hemolysates from Patients with Sickle Cell Anemia (SS), Sickle Trait (AS), Beta-Thalassemia, and Normal Hemoglobin (AA)

Hemolysate	No. patients	A_2, %	Range	±SD
SS	67	3.1	1.8–4.8	0.73
AS	42	2.4	1.2–3.3	0.50
beta-thalassemia	12	4.6	3.6–5.6	0.66
AA	127	2.6	1.1–3.5	0.58

Table 3. Percentages of Hemoglobin A_2 in Identical Hemolysates from 15 Patients, as Measured Spectrophotometrically after Gel Electrophoresis and by DEAE-Sephadex Chromatography

	Electrophoresis	Chromatography
	%	
	1.7	1.6
	3.1	3.4
	2.4	1.6
	1.8	2.3
	2.2	2.2
	2.4	2.1
	2.9	3.1
	2.7	2.7
	2.7	2.7
	3.2	2.3
	2.4	2.7
	1.8	3.9
	2.2	2.4
	1.5	1.2
	3.8	3.9
Mean	2.5	2.5
SD	±0.63	±0.79

Discussion

Electrophoresis of Hb on polyacrylamide gels is sufficiently easy, fast, accurate, and versatile to make this procedure applicable to the clinical laboratory. The technique of Neerhout et al. (*4*) has been modified and simplified by omitting the use of sample and spacer gels. The hemoglobins present have been better resolved by increasing the duration of electrophoresis. Additionally, use of a simple gel-slicing device affords a means of obtaining a similar amount of Hb A_2-containing gel on each determination and thus improving reproducibility.

With dual electrophoresis chambers, as many as 24 samples may be processed at one time, making this procedure feasible when moderately large numbers of specimens are to be screened.

As in all electrophoretic methods in which alkaline buffers are used, the proportion of Hb F cannot be accurately measured because of incomplete separation from Hb's A and S, and Hb A_2 cannot be quantitated when he-

[2] An aqueous solution of, per liter: $NaHCO_3$, (8.4 g), $K_3Fe(CN)_6$ (6.6 g), and KCN (1.1 g). Available from Hycel, Inc., Houston, Texas.

[3] Student's *t*-test was used for all statistical analyses; a *P* value ≤0.05 was considered significant.

moglobin C is present. Gels need not be stained when samples of 10 or 20 μl of hemolysate are applied. The types of Hb present are immediately evident, and without additional separation procedures Hb bands may be cut from the free gels, quantitatively eluted, and measured spectrophotometrically. The most critical need for accurate quantitation arises in determining percentages of the minor Hb component, Hb A_2, which normally comprises 2-3% of the hemolysate and has considerable diagnostic importance in evaluation of patients with hemoglobinopathies. We have determined the reliability of our method for measurement of Hb A_2 percentages by assessing the reproducibility of replicate determinations on the same hemolysate, duplicate determinations on different hemolysates from the same patient, and measurements on the same hemolysate by both our method and DEAE-Sephadex column chromatography. Excellent reliability and reproducibility was documented by all three approaches. Although there is a significant difference in the percentage of Hb A_2 in hemolysates from patients with beta-thalassemia minor and SS, there is occasional overlap that may be a source of diagnostic confusion when an individual is considered to be doubly heterozygous for Hb S and beta-thalassemia and has no detectable Hb A. In such instances, family studies may be necessary for accurate diagnosis. The elevation of Hb A_2 concentration in SS is most likely spurious, resulting from incomplete separation of hemoglobins S and A_2 during electrophoresis.

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Corrections

Correction to *Clinical Chemistry*, Volume 18:

p 1424, Table 2, "units/liter" should read "milliunits/liter."

In reference to the article by Meites, S., and Saniel-Banrey, K., *Clin. Chem.* **19**, 308 (1973), the authors wish to make the following clarification:

The glucose oxidase used was purified from *Aspergillus niger,* and obtained from Sigma Chemical Co., St. Louis, Mo. 63178. It contained 1.5 EU/mg. One unit (EU) will oxidize 1 μmol of glucose to gluconic acid and H_2O_2 per minute at pH 5.1 at 35 °C. The peroxidase used was horseradish peroxidase and was obtained from Sigma Chemical Co. as Type II (RZ 1.0–1.5), approximately 100–150 Purpurogallin (20-s) Units/mg, or as Worthington "Peroxidase D," 626 U/mg (RZ > 1), Worthington Biochemical Corp., Freehold, N.J. 07728.