

Zak K. Shihabi
Mark E. Hinsdale
Harry K. Daugherty, Jr.

Pathology Department,
Wake Forest University
Baptist Medical Center,
Winston-Salem, NC, USA

Hemoglobin A₂ quantification by capillary zone electrophoresis

Hemoglobin A₂ (HbA₂) comprises about 2.2% of the total hemoglobin in the erythrocytes. The separation and quantitation of this minor hemoglobin by capillary electrophoresis (CE) using an arginine Tris buffer is described. Some of the variables affecting the accuracy and precision of HbA₂ quantification are investigated. Furthermore, the quantification of this hemoglobin by CE is compared to that of a microcolumn chromatography method. The CE method is better suited than the microcolumn method for measuring HbA₂ in the sickle cell trait.

Keywords: Hemoglobin A₂ / β -thalassemia / Hemoglobin variants

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1 Introduction

Hemoglobin A (Hb A) is the most common hemoglobin in the normal adult. It is composed of two normal α - and two normal β -chains and it is present in most individuals at about 95–98% of the total hemoglobin. On the other hand, HbA₂ is composed of two α - and two δ -chains. In the normal individual it comprises only about 1.5–3% (median 2.2%) of the total hemoglobin. Because of the lack of β -chain synthesis in β -thalassemia there is an increase in HbA₂ with a decrease in the normal mean corpuscular volume (MCV). A mild hypochromic and microcytic anemia characterizes this clinical condition. An elevated HbA₂ level, in the presence of a normal serum iron level, is used clinically to aid in the diagnosis of β -thalassemia [1]. HbA₂ can also increase slightly in other clinical disorders such as in hyperthyroidism, hemoglobin S (Hb S) trait, and megaloblastic anemia in absence of β -thalassemia. There is also a reciprocal relationship between HbA₂ and Hb F (the fetal hemoglobin). From the analytical aspects, HbA₂ separates well on agarose gel electrophoresis; however, the densitometric scans are not accurate enough to detect the small changes in the quantity of HbA₂ [2, 3]. Microcolumn chromatography [2, 3] and HPLC [4, 5] are also used but they are either complicated or time-consuming.

Previously, HbA₂ as well as the major hemoglobin variants (A, F, S, and C) were separated by CE using a concentrated Tris buffer in a narrow diameter capillary [6]. However, the two major problems in HbA₂ are the accuracy and the reproducibility of the quantification of a very

minor peak (~ 2%) in the presence of a very large peak. The precision of the HbA₂ should be < 6% for clinical diagnosis. Jenkins *et al.* [7] found CE to be suitable for Hb A₂ quantification with a CV of 8.4%. Cotton *et al.* [8] used a commercial buffer of unknown composition which coats the capillary with special propriety polyions to achieve good reproducibility. The quantification of a minor small peak in the presence of a large one is problematic in most separation methods and especially CE. In CE, several factors affect the precision and accuracy of such quantification, e.g., sample size, wall interaction, integrator software, and voltage [9]. Here it is demonstrated that the major hemoglobin variants A, F, S, C, and A₂ can be separated well by CE in an arginine-Tris buffer. Some of the variables, which affect the precision and accuracy of hemoglobin A₂ quantification, are investigated and, furthermore, the result of the CE method is compared to that of a commercial microcolumn method.

2 Materials and methods

2.1 CE Instrument

A 33 cm $\times$ 50 μ m (ID) capillary was used in a Model 4000 Quanta (Waters Associates, Milford, MA) equipped with a 405 nm filter. The capillary was washed for 1.5 min between samples with 2 M NaOH and for 5 min before the instrument was shut down. The capillary was also rinsed for 1.5 min with the electrophoresis buffer between sample runs. The sample was injected hydrodynamically for 7 s and electrophoresed at 6 kV for 15 min for quantitation (or 9 kV for 10 min for qualitative separation). The data were collected using the software Peak Simple of SRI Instruments (Torrance, CA).

2.2 Electrophoresis buffer and sample preparation

Arginine (2.8 g) and Tris (2.4 g) were dissolved in 100 mL water and the pH was adjusted to 8.5 (if needed). Hemolysis solution (20 mL) was added to the buffer (stored

Correspondence: Professor Z. K. Shihabi, Department of Pathology, Bowman Gray School of Medicine, Wake Forest University, Winston-Salem, NC 27157, USA
E-mail: zshihabi@wfubmc.edu
Fax: +910-716-9944

Abbreviations: Hb, hemoglobin; MCV, mean corpuscular volume

refrigerated). Hemolysis solution was prepared as follows: Saponin (100 mg) was added to 4.8 g/L Tris buffer and the pH was adjusted to 8.5 with 6 N HCl (stored refrigerated). For sample preparation red blood cells were collected in EDTA tubes (10 μ L) and vortex-mixed with 400 μ L hemolysis solution. The hemolysates were injected on the capillary. For comparison, some samples were measured with the Beta-Thal HbA₂ Quick Column Kit (Helena Laboratories, Beaumont, TX).

3 Results and discussion

The separation of the major hemoglobin, including A, F, S, and C, is illustrated in Fig. 1. In CE as well as in the majority of the electrophoretic and chromatographic systems, HbA₂ comigrates with Hb C. Few previous studies investigated the separation of the hemoglobin variants by CE (6–8, 10, 11). The Hb F variant, which did not separate well from Hb A at pH 8.5 in the Tris buffer [6], separates better in the arginine-Tris buffer.

For the qualitative analysis of the major variants, a 9 kV voltage for 10 min gave a good separation within a short

time. However, for quantification of the HbA₂, the electrophoresis voltage has to be decreased to 6 kV. As a result, of course, the separation time is lengthened to 15 min; this was necessary, however, in order to increase the relative area under the HbA₂ peak for better precision of the integration. Usually, the larger the area under the peak, the better the precision, especially for analytes at low concentration [8]. However, in spite of these conditions, we found the precision of the peak height to be much better than that of the area (Table 1) as has been found previously, mainly because of the integrator software [9]. Increasing the sample size to 20 s to further increase the peak area led to a decrease in precision due to the inadequate capillary wash of the excess adsorbed proteins. However, as far as accuracy is concerned, the peak area is more accurate and more linear than the peak height. Since the peak area was constantly lower than the peak height by an average of 8% (with the software we used) this factor was used to correct the peak height for better accuracy. Cotton *et al.* [8] found a similar CV for HbA₂ using proprietary reagents, while Jenkins *et al.* [7], using a borate buffer, found a CV of 8.4%. To speed up and improve the separation, a narrower capillary, 30 μ m $\times$ 33 cm, was tried but the precision of the quantification

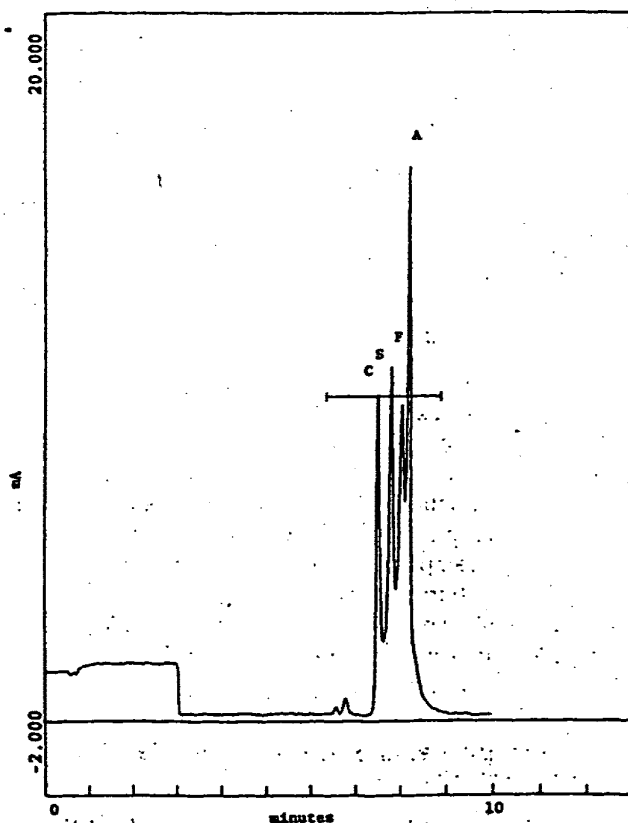


Figure 1. Separation of the major hemoglobin variants by CE at 9 kV.

Table 1. CV for HbA₂ based on area (corrected for the migration) 6tj; and height at 7 s sample at 6 and 9 kV (n = 10).

kV	Area (corrected)		Height	
	Percentage	CV	Percentage	CV
6	2.5	9.6	2.7	5.4
9	2.6	10.7	2.7	5.7

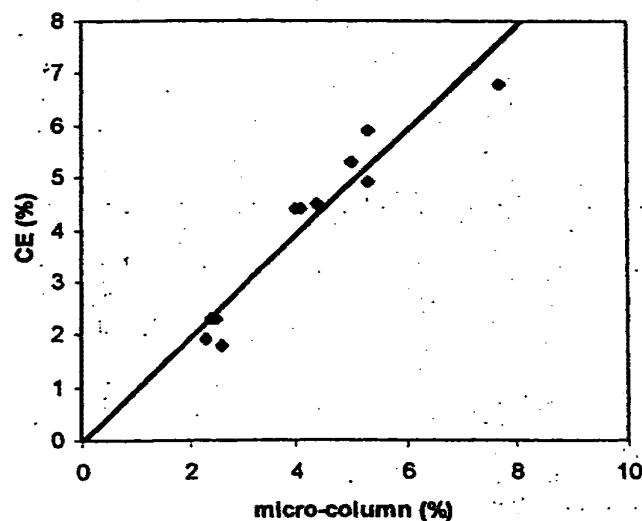


Figure 2. Comparison of HbA₂ by CE and the micro-column method.

was not as good as that of the 50 μ m because of the decreased signal-to-noise ratio.

Since Hb C behaves similarly to hemoglobin HbA₂ in migration in this method, it can be used for checking the linearity, with some reservations. Note that when HbA₂ is over 10% this high ratio indicates that the hemoglobin is not HbA₂. Hemoglobin E, which is very common in the Far East, comigrated with Hb C and also with HbA₂ in this system, similar to that observed in the agarose electrophoresis. The described CE method was compared to that of the microcolumn (Helena Lab) for patients with normal Hb A. The correlation is relatively good ($r = 0.95$; Fig. 2) similar to the findings of Jenkins *et al.* [7]. HbA₂, which eluted from the microcolumn from a normal individual, gave a corresponding single peak on the CE as expected (Fig. 3A). However, the eluent of the microcolumn from patients who carry the Hb S variant gave two

closely migrating peaks, one for HbA₂ and another one for a small amount of the Hb S (Fig. 3).

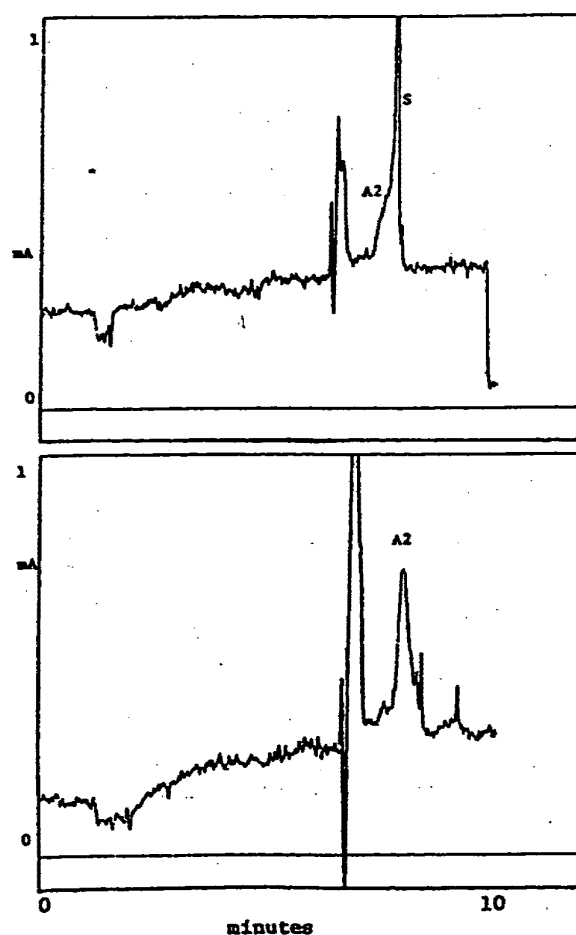


Figure 3. Separation of HbA₂ eluted from the microcolumn by CE from (top) a patient with S trait (Hb S); and (bottom) a normal patient (50 s injection).

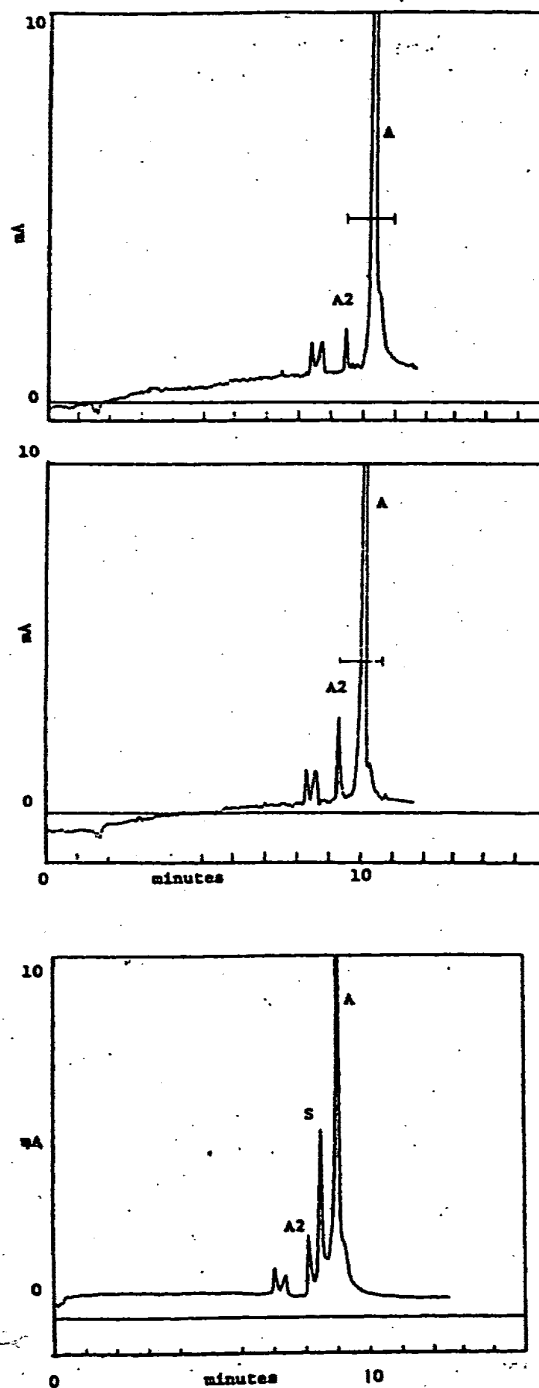


Figure 4. HbA₂ from (top) a normal patient; (middle) a patient with β -thalassemia with a low MCV, and (bottom) a patient with S trait.

This indicated that small amounts of Hb S coeluted with the HbA₂ on the microcolumn. Peak trailing for HbA₂ has been reported earlier [3]. The coelution in the microcolumn probably depends on several factors, such as temperature, speed, lot number, etc. Because of the very low concentration of HbA₂ in the elution, the injected sample size on the CE was increased to 50 s, leading to poor separation between HbA₂ and Hb S in addition to a noisy baseline (Fig. 3). Thus, the CE method is more suited for measuring HbA₂ in the sickle cell trait than the microcolumn method.

The reference interval for 30 individuals with normal MCV is 1.6–3.1, which is close to that reported for this hemoglobin [1]. Figure 4 illustrates HbA₂ from a normal patient, a patient with β -thalassemia (low MCV), and a patient with the S trait. A good separation was obtained in all these cases. Several workers [12–14] have used CE with isoelectric focusing for separation and quantitation of the hemoglobin variants. These methods usually give better resolution than capillary zone electrophoresis but the quantitation and reproducibility, especially of HbA₂, can be more problematic. The advantages of CE over the agarose and the microcolumn are the speed, the low cost of operation, and the full automation. Another advantage of CE is the ability to detect visually any abnormal or possibly interfering peaks, especially Hb S.

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4 References

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