

The Separation of Hemoglobin Variants by Capillary Zone Electrophoresis

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1 Introduction

Screening hemoglobin (Hb) variants is generally performed with the intention of detecting abnormal species. Abnormalities in the structure of the hemoglobin molecule can result in various blood disorders, collectively referred to as 'hemoglobinopathies' [1]. There are currently two major approaches utilized to detect Hb variants: slab gel electrophoresis and gel isoelectric focusing. Whereas both techniques offer excellent resolution, they are inherently cumbersome and time-consuming.

High performance capillary electrophoresis (HPCE) has attracted great interest as a highly efficient separation technique for analyzing complex biological macromolecules; the technique also has the merits of speed, ease of operation, and direct data acquisition and analysis. Clinical applications of HPCE for the analysis of proteins in serum [2-4], urine [2], and saliva [5,6] have been reported and different research groups have, furthermore, shown that the separation of model mixtures of hemoglobins A, F, S and C can be performed by capillary isoelectric focusing (CIEF) [7,8].

Hb A, F, S, and C, have the same molecular weight, 64500 Da, and only slightly different isoelectric points (pI, the pH at which the net charge on the protein is zero). Literature values for the pI of Hb C, S, F, and A, are 7.45, 7.20, 7.00, and 6.95, respectively [9]. Although the pI values of these proteins may vary from one separation system to another, the resolution of Hb A and F, the pI values of which differ by as little as 0.05 pH units, serves as a challenge for testing the resolution of a separation technique.

The separation of Hb variants by capillary zone electrophoresis (CZE) was first demonstrated by Chen *et al.* who used a proprietary buffer, 240 mM barbituric acid at pH 8.6, to discriminate between the Hb variants [2,10]. In their studies, the Hb variants C, S, F, and A were separated in bare fused silica capillaries.

In this study, we report an alternative method for separation of model mixtures of hemoglobin variants by CZE. The essential elements of this method are the use of a neutral, hydrophilic coated capillary with no electroosmotic flow (EOF), and separation at a pH near the range of pI values of the variants. Compared with bare silica capillaries, the employment of a hydrophilic coated capillary reduces protein adsorption during the course of the separation, and alleviates the need for buffers of high ionic strength [11,12] or basic pH [13,14]. Thus, the buffer can still remain an adjustable parameter for optimizing the separation. Migration time reproducibility is discussed, as is the use of the method to predict the relative amounts of the Hb variants present in mixtures.

2 Experimental

2.1 Chemicals

HEPES (*N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid]), bis-tris propane (1,3-bis[tris(hydroxymethyl)methylamino]propane), benzyl alcohol, hydrochloric acid, and sodium hydroxide were purchased from Sigma (St Louis, MO, USA). Mixtures of Hb C, S, F, and A, of Hb A and F, and of Hb A and S were purchased from Isolab (Akrón, OH, USA).

2.2 CZE

A P/ACE 2210 capillary electrophoresis instrument controlled by System Gold[®] software (Beckman Instruments, Fullerton, CA, USA) was used in this study. Neutral, hydrophilic coated capillaries of 50 μ m i.d. from eCAP[®] Neutral Capillary Methods Development Kit/Proteins (Beckman) were used for separations of hemoglobin variants. Hemoglobin mixtures were dissolved in double deionized water at a concentration of 4 mg/ml, and were detected at 214 nm. The proteins were introduced into the separation capillary by pressure injection (0.5 psi for 2 s). Between runs the column was rinsed with the running buffer for 2 min at high pressure (20 psi). Capillary length was 27 cm; the inlet to detector distance was 20 cm. The running buffer was 20 mM bis-tris propane hydrochloride at pH 6.65. Unless specified, the separation field strength was 500 V/cm.

3 Results and Discussion

In CZE the resolution, *R*, between two peaks, as defined by Giddings, is calculated using eq. (1) [15].

$$R = (N^{0.5}/4)[(\mu_2 - \mu_1)/(\mu_{avg} + \mu_{eo})] \quad (1)$$

where *N* is the separation efficiency, μ_2 and μ_1 are the electrophoretic mobility of species 1 and 2, respectively, μ_{avg} is the average electrophoretic mobility of the two species, and μ_{eo} is the electroosmotic mobility. This expression suggests that for two species with similar electrophoretic mobilities, reducing both the electroosmotic flow and the electrophoretic mobilities of the two species can maximize the resolution, at the expense of longer separation time.

The strategy of this study to resolve Hb F, A, S, and C (especially F, and A) was to perform the CZE in a neutral, hydrophilic coated capillary at a pH near the pI values of the Hb variants. The electroosmotic flow in the neutral coated capillary, measured with a neutral marker (benzyl alcohol) in 20 mM HEPES – sodium hydroxide at pH 7, was 0.014×10^{-4} cm²/Vs. This value is two orders of magnitude less than the EOF of bare fused silica capillaries, and can be regarded as zero for normal CZE separations.

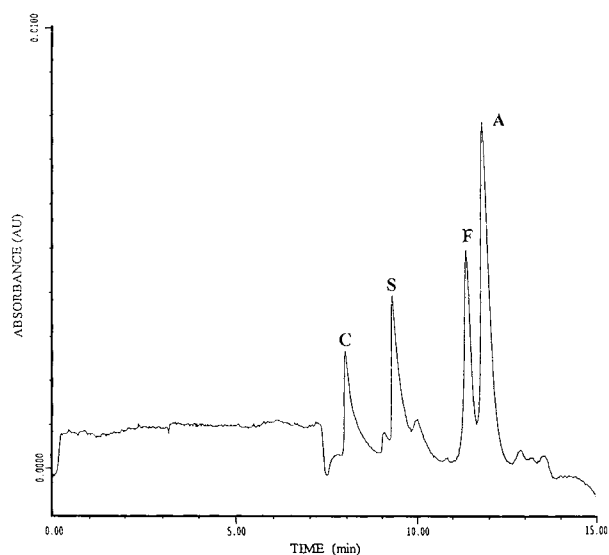


Figure 1
Electropherograms of Hb A, F, S, and C. The running buffer was 20 mM bis-tris propane at pH 6.65.

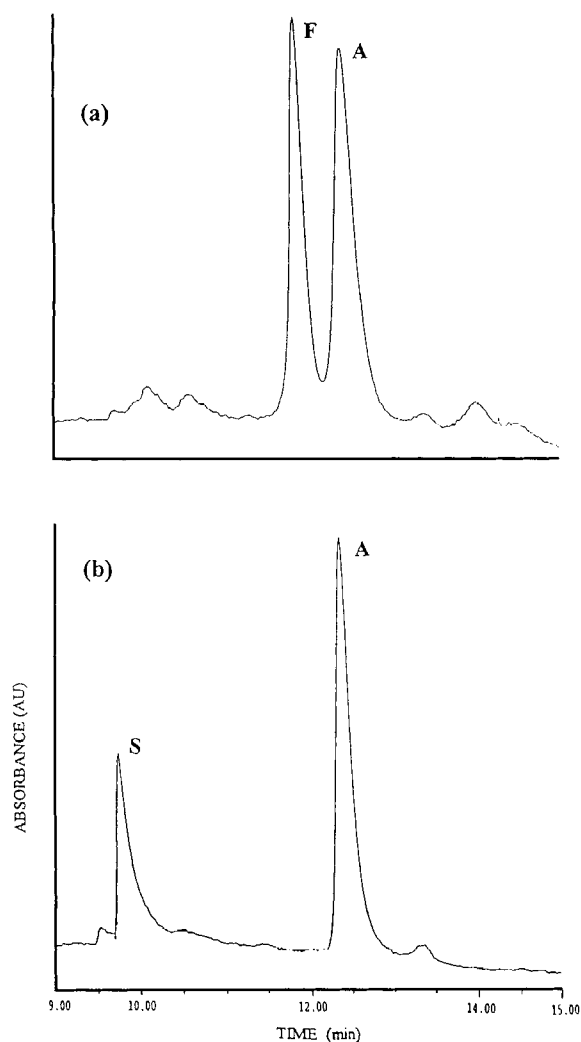


Figure 2
Electropherograms of (a) Hb F and A, and (b) Hb S and A. Conditions were as for Figure 1.

The optimum pH for the separation was determined by increasing the pH of running buffer from 6.0 in increments of 0.1 pH units until the best resolution was observed. Increasing the pH of the running buffer from 6.0 to 6.5 improved the separation of Hb C and S but Hb F and A still could not be separated. When the pH was increased to 6.65, however, Hb F and A were well separated (**Figure 1**). The identification of the hemoglobin peaks was confirmed by comparing the electropherograms of separations of the pairs Hb F and A and Hb S and A under the same conditions; the electropherograms obtained for these pairs are shown in **Figure 2**. As expected, the Hb variants migrated as a function of decreasing pI values.

The relative standard deviations (*RSD*) of the migration times of the Hb variants are listed in **Table 1**; all are all less than 2 %. It is of interest to note that the reproducibility decreased with increasing migration time and with reduced pI value. When the pH of the running buffer is adjusted to a value near the pI of a protein, the numbers of charged sites on the protein molecules and the electrophoretic mobility both approach zero. In such a circumstance, fluctuation of the potential at the protein – solution interface as a result of re-equilibration of electrolytes between runs and/or variation of buffer viscosity caused by local *Joule* heating will significantly change the electrophoretic mobility, leading to poor migration time reproducibility.

Table 1
Reproducibility of migration time for hemoglobin variants (*n* = 9).

Variant	pI	Average migration time [min]	<i>RSD</i> [%]
C	7.45	8.02	0.8
S	7.20	9.32	0.9
F	7.00	11.29	1.5
A	6.95	11.67	1.9

Further increasing the buffer pH from 6.65 did not help resolve Hb F and A. For the results shown above, samples were prepared by dissolving hemoglobin variants in double deionized water. It was found that the resolution of Hb F and A became worse when the samples were prepared by dissolving proteins in the running buffer (electropherogram not shown). Obviously, focusing of protein zones by sample stacking did improve the separation.

In the absence of electroosmosis, *i.e.* when longitudinal diffusion is the only process causing peak dispersion [15], the resolution, *R*, defined by eq. (1), can be expressed in the form of eq. (2):

$$R = 0.25[(\mu_2 - \mu_1)/(\mu_{\text{avg}})](\mu_{\text{avg}} \times V/2D_{\text{avg}})^{0.5} \quad (2)$$

where *V* is the separation voltage, and *D_{avg}* is the average diffusion coefficient of the two species. Eq. (2) describes the resolution, *R*, as a combination of a selectivity term (with the difference between the electrophoretic mobilities defined as the selectivity coefficient) and an efficiency term. Eq. (2) implies that resolution can be linearly increased with increased efficiency by increasing the separation voltage. In this study, however, an optimum field strength was discovered for resolution of Hb A and F. As is shown in **Figure 3**, the resolution between A and F was optimized by applying a field strength of 600 V/cm. At higher field strengths (700 V/cm), the peaks for Hb A and F merged. This phenomenon was quite reproducible, and was observed with another capillary. It was also found that the peak efficiencies of Hb C and S were lower at 700 V/cm than at 600 V/cm. It is speculated that *Joule* heating might be the cause of these phenomena. A high field strength, 700 V/cm, raises

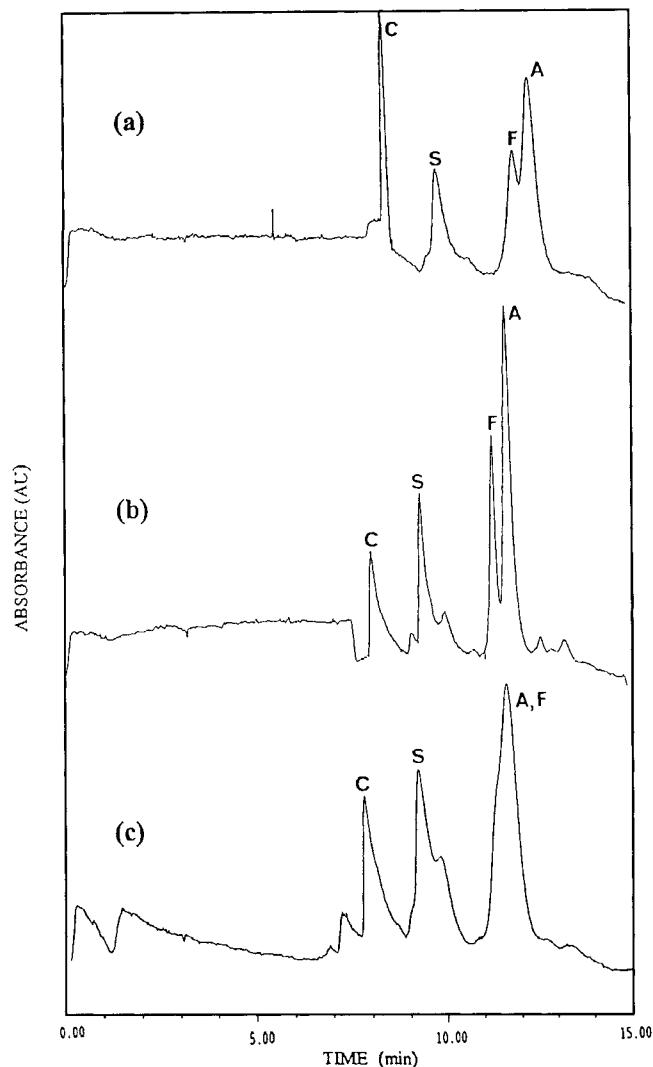


Figure 3
Electropherograms of Hb A, F, S, and C under different field strengths: (a) 500 V/cm, (b) 600 V/cm, and (c) 700 V/cm. Conditions as for Figure 1.

the temperature of the running buffer to such an extent that peak broadening resulting from the diffusion process becomes the factor dominating peak efficiency (eq. (2)).

The hemoglobin variants have almost the same chemical constitution and UV absorbance extinction coefficients. Thus, the UV absorbance can be used for quantifying the relative amounts of each variant in the mixture. **Table 2** lists the results predicted, from the electropherograms, for the relative amounts [%] of the Hb variants. Two different prediction methods were employed, one based on peak area and the other on peak height. As is apparent from Table 2, the values predicted from peak area are not consistent with literature values for Hb S and A. Tailing and overlapping peaks may be the causes of the low accuracy. The predictions based on peak heights are, on the other hand, in excellent agreement with literature values.

Table 2
Prediction of the relative amounts [%] of hemoglobin variants in a mixture of Hb C, S, F, and A.

Variant	Value predicted from:		Literature value ^{a)}
	peak area	peak height	
C	10	14	15 ± 5
S	15	20	22 ± 5
F	27	26	26 ± 5
A	48	40	38 ± 5

^{a)} Target values of the supplier.

4 Conclusion

In these preliminary studies a strategy has been demonstrated for resolution and quantification of Hb A, F, S, and C in model mixtures by CZE. The effects of complex sample matrices on the separation efficiency of this methodology are presently under investigation. Owing to the merits of simplicity and ease of operation, this approach is expected to be applicable to other protein mixtures and might be regarded as an alternative to CIEF for the resolution of proteins with similar pI values.

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