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Journal of Chromatography A, 767 (1997) 231–239

JOURNAL OF CHROMATOGRAPHY A

Capillary isoelectric focusing with anionic coated capillaries

Donna M. Whynot[a], Richard A. Hartwick[b], Susan Bane[a,*]

[a]*Department of Chemistry, State University of New York at Binghamton, Binghamton, NY 13902-6016, USA*
[b]*PharmAssist Analytical Laboratory, Inc., RD1 Box 248A, Whitestore Road, South New Berlin, NY 13843, USA*

Received 23 September 1996; revised 12 November 1996; accepted 11 December 1996

Abstract

Capillary isoelectric focusing (cIEF) is a powerful analytical technique for the separation of proteins. cIEF can be performed in capillaries with reduced electroosmotic flow, or in capillaries with controlled electroosmotic flow. This work introduces an alternative method for cIEF with controlled electroosmotic flow through the use of an anionic coated capillary. The method is shown to be applicable to basic, neutral and acidic proteins, with some peak broadening for acidic proteins. It also proves to have good reproducibility with R.S.D.s around 3%, a linear relationship between migration time and isoelectric point and quantitative capability. The total analysis time is shorter and sample preparation is simplified over other cIEF methods.

Keywords: Isoelectric focusing; Coated capillaries; Proteins

1. Introduction

Isoelectric focusing (IEF) is a powerful technique used for the separation of protein mixtures based on differences in isoelectric points (p*I*). In this method, the proteins to be analyzed migrate through a pH gradient until they reach the pH at which they have zero net charge, i.e., the isoelectric point. Historically, IEF was most commonly performed in a gel media, either in a tube or in a slab format. These methods are limited by a number of factors such as Joule heating, difficulty with quantitation, and the time and labor required for analysis.

Hjertén and coworkers introduced IEF in the capillary format (cIEF) in an attempt to overcome the problems with the conventional method [1–5]. Higher field strengths can be used in cIEF since the small diameter of the capillaries, 50–100 μm, can dissipate the Joule heating efficiently. These high field strengths can lead to higher resolution and faster analysis. Hjertén speculated that cIEF should be performed in capillaries in which the electroosmotic flow (EOF) is completely eliminated in order to maintain stable focused protein zones [1]. Coatings such as linear polyacrylamide and methylcellulose were used to eliminate the EOF [2]. The coated capillaries were then filled with a mixture of the ampholytes to be used to create the pH gradient and the proteins to be analyzed. An acidic buffer was placed at the anode and a basic buffer at the cathode. When the electric field was applied, the pH gradient was formed with the most acidic ampholyte at the anode and the most basic ampholyte at the cathode. The proteins then moved to the position in the gradient where the pH is equal to its p*I*. As the focusing process occurred, the current decreased to a minimum when the focusing was complete [6]. Detection of the protein zones can be achieved by

*Corresponding author.

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PII S0021-9673(96)01101-6

mobilizing the zones past a detection window [1,2] through salt mobilization, which involves turning off the electric field after focusing is complete and changing the buffer at the cathode or anode to a salt before turning the electric field back on. This additional step for mobilization is a major disadvantage of this method since it adds to the complexity of the technique. Other polymeric coatings have been described for cIEF, some of which maintain some EOF [7,8].

In an attempt to simplify the technique, cIEF has been performed in bare capillaries [9–12]. Small amounts of polymer additives such as hydroxypropylmethylcellulose or methylcellulose are added to the sample, and a dynamic coating of these polymers forms on the capillary wall when voltage is applied. An EOF can be created that is slow enough to permit the focusing of proteins but also allows for the mobilization of the focused zones past a UV detection window. This method offers the advantage of performing both focusing and detection in one step. A drawback is that the capillary must be washed with harsh solutions to create a new surface between each analysis. Also, sample preparation is more involved, with the addition of the polymer to the ampholyte–protein mixture.

An alternative method to control EOF in capillaries is through ionic coatings. To be useful for cIEF, the coating must be relatively pH stable and have an EOF that is independent of pH, as well as reproducible. An anionic coating that shows these characteristics is one in which sodium 2-acrylamido-2-methyl-propanesulfonate (NaAMPS) is polymerized or copolymerized with acrylamide on the capillary wall to form a monolayer of polymer [13]. Depending on the ratio of NaAMPS to acrylamide, the EOF of capillaries coated with this anionic polymer can vary from near zero to a maximum value of 60% of the maximum flow of a bare silica capillary at alkaline pH.

Here, we report cIEF in anionic coated capillaries using NaAMPS–acrylamide polymer mixture as the coating. The relationship between p*I* and migration time, the effect of EOF on separation, the effect of capillary length on separation, reproducibility and quantitation are discussed. The applicability of this method to acidic proteins and to a commercial sample of hemoglobin is illustrated.

2. Experimental

2.1. Instrumentation

All experiments except the quantitation experiments were performed on a laboratory constructed instrument including a CZE 1000 PN30 high power supply (Spellman, Plainview, NY, USA), a digital controller for the CZE 1000 unit, designed by Chamonix Industries (Johnson City, NY, USA) and a Plexiglas box. Detection was achieved with a Spectra 100 UV detector (Thermo Separations Products, San Jose, CA, USA). Electropherograms were obtained using a SP-4400 Integrator (Thermo Separations Products). Detection wavelength was at 280 nm.

The quantitation experiments were performed on a Spectraphysics SpectraPHORESIS 1000 commercial instrument (Thermo Separations Products). Data acquisition was performed with PC1000 System Software (version 3.0).

2.2. Reagents and materials

Fused-silica capillary of 75 μm I.D. was purchased from Polymicro Technology (Phoenix, AZ, USA). Protein standards were purchased from Sigma (St. Louis, MO, USA). Acrylamide, *N,N,N′,N′*-tetramethylenediamine (TEMED) and ammonium persulfate (APS) were purchased form Bio-Rad Labs. (Richmond, CA, USA). 2-Acrylamido-2-methyl-1-propanesulfonic acid and mesityl oxide were purchased from Aldrich (Milwaukee, WI, USA). 3-Methacryloxypropyltrimethoxysilane was purchased from United Chemical Technologies (Bristol, PA, USA). All other chemicals were obtained from Fisher Scientific (Fair Lawn, NJ, USA).

2.3. Capillary coating

The capillary coating used here was described by Sun et al. [13]. Fused-silica capillaries were conditioned with 1 *M* sodium hydroxide for 30 min, then rinsed with deionized water. The capillaries were treated with a bifunctional reagent by filling them with a 1% (v/v) 3-methacryloxypropyltri-

methoxysilane solution (adjusted to pH 3.5 with acetic acid), and allowing it to react for 1 h at room temperature. The capillary was then rinsed with water.

The coating was prepared by making a 0.39 *M* solution of NaAMPS and a 0.28 *M* solution of acrylamide, mixing these in proper proportions and polymerizing it in the treated capillary. The NaAMPS solution was prepared by dissolving 0.80 g of 2-acrylamido-2-methylpropanesulfonic acid in 8.0 ml of deionized water. Concentrated sodium hydroxide was added to neutralize the solution to pH 7.0. The solution was then brought to 10 ml with deionized water. The 0.39 *M* acrylamide solution was made by dissolving 0.28 g acrylamide in 10 ml deionized water.

Ammonium persulfate (1 mg/ml of solution) and TEMED (1 μl/ml of solution) were added to the NaAMPS–acrylamide solution to initiate polymerization. The treated capillary was filled with the mixed solution and allowed to react for 1 h. The capillary was then rinsed with water and dried with air. The capillary was allowed to dry overnight before use.

To achieve the proper EOF for the cIEF, the NaAMPS and acrylamide were mixed with different volume ratios to coat the capillaries. A 2% (v/v) solution of mesityl oxide was used as a neutral marker to measure the EOF.

2.4. Analysis of proteins

Proteins analyzed included cytochrome *c* (p*I* 9.6), chymotrypsinogen A (p*I* 9.1), myoglobin (p*I* 7.2 and 6.8), carbonic anhydrase (p*I* 6.6 and 5.9), bovine serum albumin (p*I* 4.8) and bovine hemoglobin. NaAMPS–acrylamide coated capillaries of 75 μm I.D. were rinsed with deionized water for 5 min between runs. The capillary length in most cases was 60 cm with 40 cm between the anode and the detection window. The anolyte solution was 10 m*M* H_3PO_4 and the catholyte solution was 20 m*M* NaOH. The applied voltage was either 15 or 20 kV. Sample solutions consisted of 1–2 mg/ml protein dissolved in 5% Pharmalyte 3-10, as used in Refs. [9,10]. In some cases, 1% TEMED was added to the sample solutions as a pH gradient extender.

3. Results and discussion

3.1. Effect of electroosmotic flow

The first parameter studied was the effect of the EOF of the anionic coating on cIEF separations. This was done by performing the same separation on three NaAMPS–acrylamide coated capillaries with different EOFs. Fig. 1 shows the separation of cytochrome *c* (p*I* 9.1) and myoglobin (p*I* 7.2 and 6.8). Electropherogram A shows the separation on a NaAMPS–acrylamide coated capillary with an EOF of $4.86 \cdot 10^{-4}$ cm^2 s^{-1} V^{-1}. Under these conditions, none of the peaks are resolved. Electropherogram B shows the separation on a coated capillary with a lower EOF of $2.13 \cdot 10^{-4}$ cm^2 s^{-1} V^{-1}. The cytochrome *c* peak is resolved, but the two myoglobin peaks are not. Lastly, electropherogram C shows the separation on a coated capillary with an even lower EOF of $1.76 \cdot 10^{-4}$ cm^2 s^{-1} V^{-1}. All three peaks are resolved under these conditions. In this example, slowing the EOF from $4.86 \cdot 10^{-4}$ cm^2 s^{-1} V^{-1} to $1.76 \cdot 10^{-4}$ cm^2 s^{-1} V^{-1} resulted in a significant increase in resolution but only a 5 min increase in analysis time. Therefore, a specific EOF may be chosen to optimize each particular separation depending on the resolution desired.

Visually, the resolution of the protein peaks is not always obvious. However, as can be seen from Fig. 2, the two myoglobin peaks, differing in p*I* by 0.4 pH units, are baseline resolved. With a wide-range pH gradient of 3 to 10, this resolution is essentially the same as what is achieved with other cIEF methods with EOF [9,10]. Higher resolution may be achieved by using a narrow-range pH gradient as needed for a particular analysis.

3.2. Reproducibility

The reproducibility of migration times was evaluated using the cIEF of myoglobin and carbonic anhydrase as shown in Fig. 2. The capillary coating used for this analysis created an EOF of $5.18 \cdot 10^{-4}$ cm^2 s^{-1} V^{-1}, which proved to be optimal for the separation of myoglobin and carbonic anhydrase. The EOF is slow enough for focusing to occur before the protein zones pass by the detection window, but also fast enough to allow for the analysis to occur in

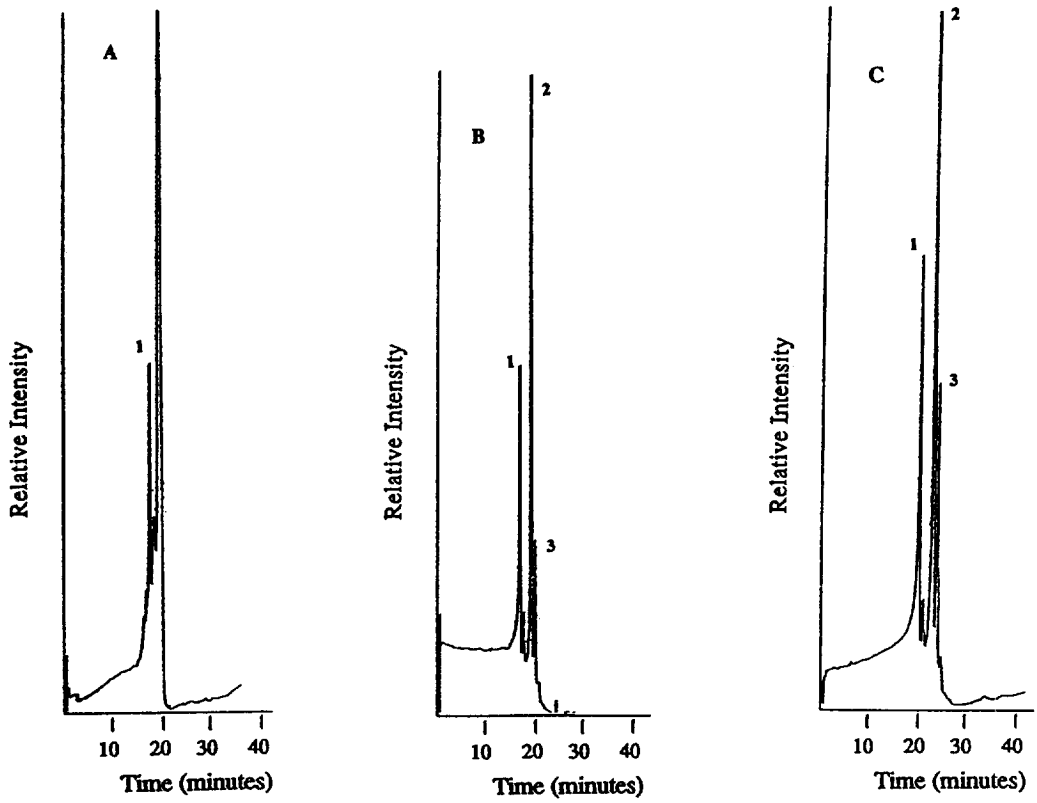

Fig. 1. The effect of EOF on cIEF shown by the separation of cytochrome *c* (p*I* 9.1) and myoglobin (p*I* 7.2 and 6.8). Sample: 1 mg/ml each protein with 5% Pharmalyte 3-10. Capillary: 60 cm (effective length 40 cm)×75 μm I.D. IEF: anolyte 10 m*M* H_3PO_4, catholyte 20 m*M* NaOH, 15 kV voltage. Detection: UV, 280 nm. Peaks: (1) cytochrome *c*, p*I* 9.1; (2) myoglobin, p*I* 7.2; (3) myoglobin, p*I* 6.8. EOFs of capillaries: A: $4.86 \cdot 10^{-4}$ cm^2 s^{-1} V^{-1}; B: $2.13 \cdot 10^{-4}$ cm^2 s^{-1} V^{-1}; C: $1.76 \cdot 10^{-4}$ cm^2 s^{-1} V^{-1}.

a reasonable amount of time. Table 1 shows the average migration times for five injections with the percent relative standard deviation of the migration times for each peak.

Capillary coatings that use Si–O–Si bonds in the coating chemistry, such as the linear polyacrylamide coating, tend to have poor pH stability [14]. Since the NaAMPS–acrylamide anionic coating uses this type of chemistry, the stability of the coating was a major consideration. However, the capillary coating used for these experiments proved to be stable and give reproducible results for the duration of the experiments, which usually consisted of more than twenty injections per capillary. The anionic coating has previously been shown to have pH independent flows in the pH range of 3 to 9, which remain stable after 18 days. The coating was also shown to have a lifetime of up to 60 h with applied field strengths comparable to those used here [13]. Under the conditions used in these experiments, no degradation of the capillary coating was experienced. Occasionally, the proteins would precipitate and cause the loss of current and, less frequently, blockage of the capillary. Under optimized conditions, these problems rarely occur.

3.3. Linearity of pH gradient

The linearity of the pH gradient was evaluated using the cIEF of chymotrypsinogen A (p*I* 9.6), cytochrome *c* (p*I* 9.1) and myoglobin (p*I* 7.2 and 6.8) on a NaAMPS–acrylamide coated capillary with an EOF of $4.52 \cdot 10^{-4}$ cm^2 s^{-1} V^{-1}, as shown in Fig. 3.

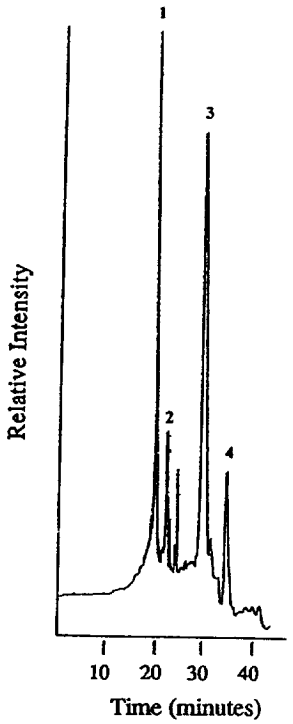

Fig. 2. Reproducibility of cIEF shown by the separation of myoglobin (p*I* 7.2 and 6.8) and carbonic anhydrase (p*I* 6.6 and 5.9). Sample: 1 mg/ml each protein with 5% Pharmalyte 3-10. Capillary: NaAMPS–acrylamide coated with EOF of $5.18 \cdot 10^{-4}$ $cm^2\ s^{-1}\ V^{-1}$, 60 cm (effective length 40 cm)×75 μm I.D. IEF: anolyte 10 m*M* H_3PO_4, catholyte 20 m*M* NaOH, 15 kV voltage. Detection: UV, 280 nm. Peaks: (1) myoglobin, p*I* 7.2; (2) myoglobin, p*I* 6.8; (3) carbonic anhydrase, p*I* 6.6; (4) carbonic anhydrase, p*I* 5.9.

Since the point of detection is 40 cm from the anode, proteins with p*I* values above 7.7 will focus past the detection window in a pH gradient of 3 to 10. This is assuming that the pH gradient is evenly extended over the entire length of the capillary, which is not completely true unless the EOF is zero and the ampholytes create a perfect gradient. However, the p*I* value detected at the window is most likely not much less than 7.7 since the EOF used is low. Still, the protein chymotrypsinogen A, with a p*I* value of 9.6, will focus past the detection window and be lost to detection under these conditions.

Table 1
Percent relative standard deviation of migration times for peaks in Fig. 2

Protein	t_M (min)	R.S.D. (%)
Myoglobin, p*I* 7.2	19.68	2.4
Myoglobin, p*I* 6.8	21.92	2.7
Carbonic anhydrase, p*I* 6.6	29.37	2.6
Carbonic anhydrase, p*I* 5.9	34.26	3.2

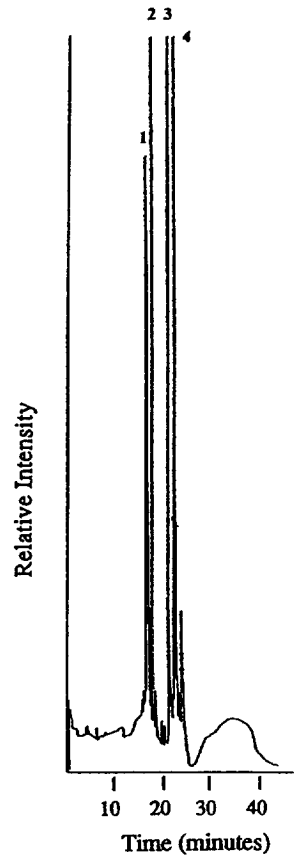

Fig. 3. Linearity of cIEF shown by the separation of chymotrypsinogen A (p*I* 9.6), cytochrome *c* (p*I* 9.1) and myoglobin (p*I* 7.2 and 6.8). Sample: 1 mg/ml each protein, 5% Pharmalyte 3-10, 1% TEMED. Capillary: NaAMPS–acrylamide coated with EOF of $4.52 \cdot 10^{-4}$ $cm^2\ s^{-1}\ V^{-1}$, 60 cm (effective length 40 cm)×75 μm I.D. IEF: anolyte 10 m*M* H_3PO_4, catholyte 20 m*M* NaOH, 15 kV voltage. Detection: UV, 280 nm. Peaks: (1) chymotrypsinogen A, p*I* 9.6; (2) cytochrome *c*, p*I* 9.1; (3) myoglobin, p*I* 7.2; (4) myoglobin, p*I* 6.8.

One way to detect the more basic proteins is to extend the pH gradient by adding 1% TEMED to the sample–ampholyte mixture. It has been reported that the addition of TEMED to the ampholytes will extend the pH gradient to 12 [15]. Thus when TEMED was used, the pH gradient was extended so

that the basic proteins would focus on the anode side of the detection window, and would be mobilized past the window for detection.

A plot of p*I* versus migration time for the proteins in Fig. 3 is shown in Fig. 4, and is linear with a correlation coefficient of 0.993. Therefore, the pH gradient is linear in the range of p*I* values used here even with the use of TEMED. This method could possibly be used to estimate the p*I* of a particular protein.

3.4. Quantitation

The quantitative capability of cIEF on anionic coated capillaries was investigated using the cIEF of myoglobin and carbonic anhydrase on a Spectraphysics SpectraPHORESIS commercial instrument. The sample solutions used were 0.6 mg/ml, 0.2 mg/ml, 20 μg/ml and 6 μg/ml of each protein dissolved in 5% Pharmalyte 3-10. The NaAMPS–acrylamide coated capillary used had an EOF of $2.14 \cdot 10^{-4}$ cm^2 s^{-1} V^{-1}, and a shorter total length of 40 cm, with an effective length of 32 cm. The peak area for the myoglobin I peak was obtained using PC 1000 System Software. The peak area was then plotted against the sample concentration. The relationship was determined to be linear with a correlation coefficient of 0.999, as shown in Fig. 5. Thus, in the concentration range of the samples used here, this method is quantitative in the case of myoglobin.

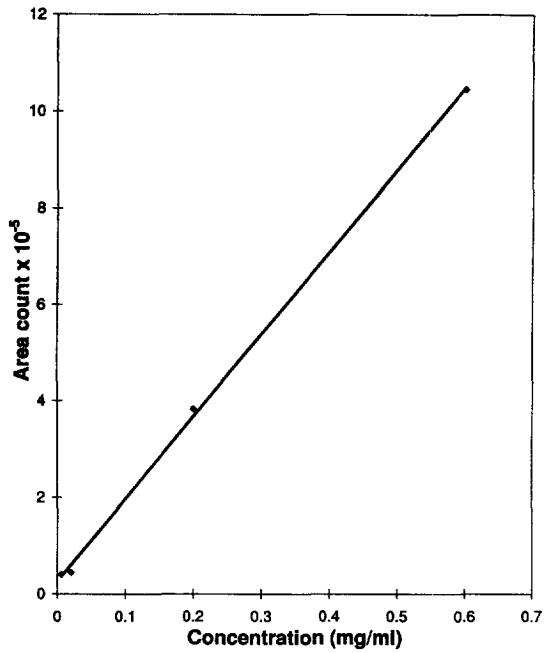

Fig. 5. Quantitation of myoglobin.

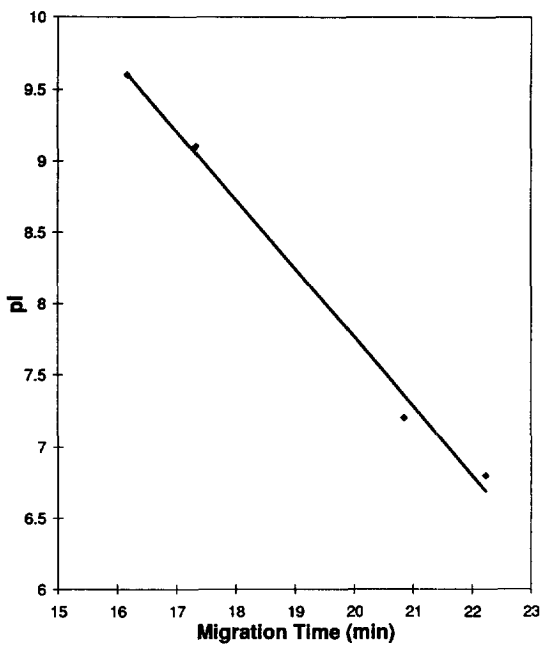

Fig. 4. p*I* versus migration time.

3.5. Effect of capillary length

The next parameter that was investigated was the effect of the capillary length on cIEF. The same separation of chymotrypsinogen A (p*I* 9.6), cytochrome *c* (p*I* 9.1) and myoglobin (p*I* 7.2 and 6.8) was performed on three capillaries of different length, but the same EOF. The capillary lengths were 40, 60 and 80 cm with the detection window 20 cm from the cathode. The migration times of each peak for the different length capillary are shown in Table 2. For these conditions, the resolution increases with capillary length. This is presumably a result of a pH gradient which increases in length along with the

Table 2
Migration times on different length anionic coated capillaries

Capillary length (cm)	Migration Time (min)			
	Chymotrypsinogen A	Cytochrome *c*	Myoglobin I	Myoglobin II
40	5.51	Unresolved	6.14	Unresolved
60	16.62	18.86	19.51	Unresolved
80	28.78	31.87	32.30	33.52

capillary length. Increasing the distance between pH units in the gradient should cause more time between the detection of proteins and higher resolution of the components.

Increasing capillary length can also introduce factors that are undesirable. First, increasing the capillary length also causes a corresponding decrease in field strength, which leads to lower resolution at greater capillary lengths. Secondly, the migration time also increases with capillary length. Fig. 6 shows that the relationship between capillary length and migration time is linear with a correlation coefficient of 0.999. Thus, the length of the capillary used for cIEF is another parameter that should be chosen to optimize a separation.

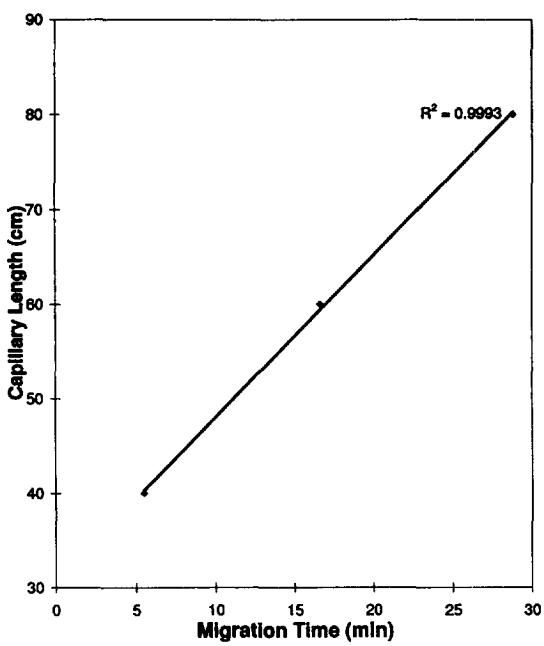

Fig. 6. Capillary length vs. migration time.

3.6. Applications

To further evaluate the viability of this method, its applicability to acidic proteins was studied using the cIEF of myoglobin (p*I* 7.2 and 6.8) and bovine serum albumin (p*I* 4.8). The electropherogram in Fig. 7 shows that there is significant peak broadening for the acidic bovine serum albumin peak. This peak broadening may in part be due to the fact that the acidic proteins focus closer to the anode and migrate last from the capillary. Therefore, they spend more time in the capillary which leads to more peak broadening due to diffusion or interaction with the capillary wall. The problem of peak broadening for acidic proteins is common for cIEF methods with controlled EOF [9]. This laboratory is presently developing a method to possibly improve separations for acidic proteins. By performing cIEF on cationic coated capillaries, the migration order of the proteins would reverse so that acidic proteins would migrate past the detection window first.

Upon completion of an initial evaluation of the viability of this method for cIEF, a commercially available bovine hemoglobin sample was analyzed as shown in Fig. 8. The four major peaks are due to the different oxidation states of the hemes in the hemoglobin.

4. Conclusions

We have demonstrated that cIEF on NaAMPS–acrylamide coated capillaries is a viable and practical method for the analytical separation of proteins.

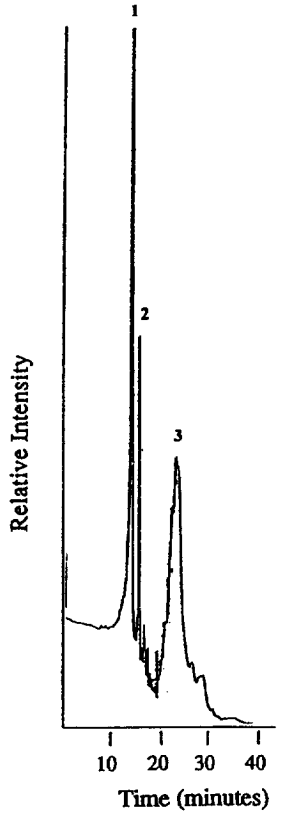

Fig. 7. cIEF of an acidic protein shown by the separation of myoglobin (p*I* 7.2 and 6.8) and bovine serum albumin (p*I* 4.8). Sample: 1 mg/ml each protein with 5% Pharmalyte 3-10. Capillary: NaAMPS–acrylamide coated with EOF of $3.88 \cdot 10^{-4}$ cm^2 s^{-1} V^{-1}, 60 cm (effective length 40 cm)×75 μm I.D. IEF: anolyte 10 m*M* H_3PO_4, catholyte 20 m*M* NaOH, 15 kV voltage. Detection: UV, 280 nm. Peaks: (1) myoglobin, p*I* 7.2; (2) myoglobin, p*I* 6.8; (3) bovine serum albumin, p*I* 4.8.

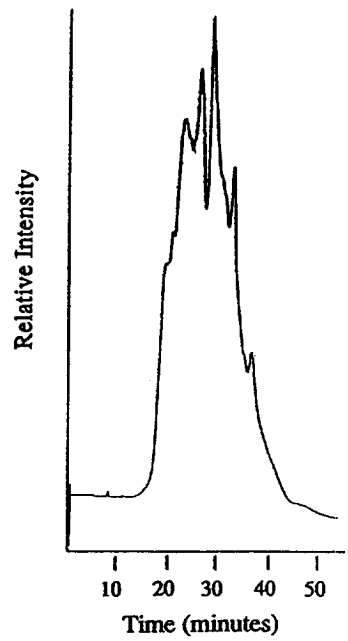

Fig. 8. cIEF of commercial bovine hemoglobin sample. Sample: 2 mg/ml protein with 5% Pharmalyte 3-10. Capillary: NaAMPS–acrylamide coated with EOF of $2.88 \cdot 10^{-4}$ cm^2 s^{-1} V^{-1}, 60 cm (effective length 40 cm)×75 μm I.D. IEF: anolyte 10 m*M* H_3PO_4, catholyte 20 m*M* NaOH, 20 kV voltage. Detection: UV, 280 nm.

Standard criteria of method viability, such as a linear relationship between p*I* and migration time and reproducible migration times, have been met. Protein mixtures over a wide pH range can be resolved using this system, although significant peak broadening occurs with acidic proteins with p*I* values less than 5. When comparing this method to cIEF with polymeric additives in uncoated capillaries, a few advantages can be noted. First, sample preparation in this method is simpler, requiring only the protein–ampholyte mixture. Also, the total analysis time is shortened since only a short water rinse in between analyses is required to achieve high reproducibility, rather than longer washes with harsh solutions. Although this method does involve using a coated capillary, the coating process is quite simple and results in a relatively stable coating.

Acknowledgments

The authors gratefully acknowledge the Clifford D. Clark fellowship for financial support of this work.

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