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Manipulation of electroosmotic flow in capillary electrophoresis

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ABSTRACT

This paper reports the use of surfactant and polymer-C,, coated capillaries that allow manipulation of electroosmotic flow (EOF). Although this approach to the control of EOF involves the preparation and use of multiple capillaries, all the coatings were prepared by a single procedure. It is shown that the ability to control EOF allows optimization of both separation time and resolution. In the case of proteins, low EOF maximizes resolution whereas high flow gives the shortest analysis time. It should be. noted that proteins are a special case and this conclusion may not be true with other molecular species. Through selection of a specific coating, it is possible to complete a separation in the shortest time while maintaining sufficient resolution to give baseline resolution of proteins. The various coated capillaries were examined in capillary zone electrophoresis (CZE) and capillary isoelectric focusing (cIEF) separations of native protein standards and hemoglobin variants. Separation of glycosylated hemoglobin A, variants was achieved by cIEF within 10 min, including the focusing time. Good run-to-run reproducibility was obtained by flushing the capillary with the coating solution between analyses.

INTRODUCTION

The surface of fused-silica capillaries has approximately 8 μmol/m^2 of silanol groups with a broad pK ranging from pH 3.5 to 8 [1]. These weakly acidic groups impact electrophoretic separations by creating a surface double layer that triggers electroosmotic flow (EOF) [2]. In the case of proteins, analyte transport velocity in fused-silica capillaries from EOF, is almost always greater than by electrophoretic mobility alone at physiological pH [3]. This has several important implications. The first is that all protein species will be swept to the cathode where they can be detected by a single detector, without regard to their charge. A second is that electroosmotic transport decreases separation time of proteins by reducing the effective length of the column [4]. In addition, surface silanols contribute to the adsorption of cationic analytes. Adsorption of basic proteins at the capillary inlet has been shown to diminish recovery, cause bandspreading, and produce complex flow patterns within the column that compromise efficiency [5].

Control of EOF and adsorption has been approached in several ways. One is to use an applied external field at the capillary wall [6]. Although external fields can control EOF below pH 5-6, this technique does not eliminate protein adsorption. A second approach to the control of protein adsorption has been to use organic surface coatings. Simple organosilanes [7–9] and polymeric coatings [1 O-l 2] have shown varying degrees of efficacy in controlling protein adsorption and EOF. Protein adsorption seems to be controlled by high density, hydrophilic coatings. A glycerylpropyl silane bonded-phase column can be as effective in protein separations as an epoxy polymer coating [13,14]. The difference seems to be in column longevity and EOF. Columns derivatized with water soluble silane monomers are less stable than polymeric and octadecylsilane coatings.

Organic coatings also reduce EOF. It has been reported [10] that EOF is controlled by the viscosity of the coating in the surface double layer. Thick polymer coatings have been shown to reduce EOF

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more than thin organosilane monomer coatings [15]. There can be a thirty-fold difference in EOF between uncoated and coated columns. Controlling analysis time by systematically varying the chemical and physical properties of the organic coating in capillary electrophoresis is a new possibility. The objectives of this study were to develop techniques for preparing capillaries with organic coatings that would; (i) allow EOF to be selected within narrow limits, (ii) minimize protein adsorption, (iii) require minimal organic syntheses and, (iv) use a similar approach to prepare all capillaries.

EXPERIMENTAL

Chemicals

Isoelectric focusing markers (hemoglobin C, S, F and A) were gifts from Isolab (Akron, OH, USA). Methyl celluloses, poly(vinyl alcohol), polyvinyl pyrrolidone, Brij 35, mesityl oxide, toluene, glycine and octadecyl-trichlorosilane were purchased from Aldrich (Milwaukee, WI, USA), PF-108 was a gift from BASF (Parsippany, NJ, USA). Pharmalyte (pH 3-10) was purchased from Pharmacia Fine Chemicals (Piscataway, NJ, USA). Protein samples and glycosylated hemoglobins were purchased from Sigma (St. Louis, MO, USA). All of the buffer solutions were prepared by using double distilled water that was passed through a 0.45-μm nylon filter. All the samples were stored at 0°C.

Instrumentation

Isoelectric focusing. Capillary isoelectric focusing (cIEF) was performed on a component system. A Spellman Model FHR 30P 60/EI (Spellman High Voltage Electronics, Plainview, NY, USA) power supply was used to apply the electric field across the capillary. On-line detection was performed by mounting the surfactant-coated octadecyl silane derivatized capillary (either 50 μm I.D. or 25 μm I.D.) on a 254-nm UV absorbance detector (Bio-Analytical Systems, West Lafayette, IN, USA). The detector signal was recorded with an OmniScribe recorder (Houston Instrument, Austin, TX, USA). High-voltage components of the system were placed in a Lucite cabinet with a safety interlock.

Capillary zone electrophoresis. The instrumentation used has been previously described [16]. Briefly, fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) of 375 μm O.D., 75 μm I.D. and 45 cm length with a detector window at 20 cm were used for the separations. A Glassman PS/EL30POl.5 (Glassman High Voltage, Whitehouse Station, NJ, USA) power supply was used to apply the electric field across the capillary. On-column detection was performed with an Isco CV^4 (Lincoln, NE, USA) variable-wavelength UV absorbance detector. Protein samples were detected at a wavelength of 230 nm.

Capillary coating

C_{18} coated capillaries were prepared by a modification of the procedure described by Towns and Regnier [4]. Fused-silica capillaries were first treated with 1 .O *M* NaOH for 30 min and then washed with deionized water and methanol, respectively, for 30 min. The capillaries (25 μm I.D. or 50 μm I.D.) were placed in a GC oven at 90°C for 2 h with a nitrogen carrier stream at 400 kPa to evaporate the residual methanol. Octadecyl-trichlorosilane with 50% toluene was then pushed through the capillaries under pressure. The capillaries were placed in the oven at 90°C for 6 h with new solution continuously being pushed through the capillaries. After 6 h of silylation, the residual octadecyl-trichlorosilane and toluene were removed from the capillaries by pressure. The capillaries were washed with methanol for 20 min and then with deionized water for 30 min. Surfactant solutions were pushed continuously through the capillaries for 6 h to complete the coating process.

The capillary isoelectric focusing process

The protein sample was prepared by mixing a protein solution and Pharmalyte (pH 3-10) to produce a final ampholyte concentration of 1–2%. In the loading step, the capillary was filled with the sample mixture by positive pressure. The two ends of the capillary were then placed into 10 m*M* phosphoric acid anolyte and 20 m*M* sodium hydroxide catholyte, respectively. Focusing ws started by applying approximately 500 volts per centimeter to the loaded capillary. The current dropped gradually to a constant "residual" value. This residual current, which is not zero because of EOF in the capillary, indicates the end of focusing.

Cathodic [17] and EOF mobilization [18] were both used to drive the formed pH gradient and

focused protein zones to the detector window. EOF mobilization utilizes EOF in the capillary as the driving force. It combines the focusing and mobilization steps to simplify the operation of cIEF. After each run, the capillary was flushed with the surfactant solution for a few minutes.

Capillaries of 25 μm I.D. were found to have an advantage over larger I.D. capillaries. The larger surface area-to-volume ratio made heat dissipation more efficient and allowed higher voltage to be applied. This produced more rapid separations.

RESULTS AND DISCUSSION

The literature [19–21] and experience with adsorbed coatings have taught us that variations in surface preparation, differences in the molecular mass of the species that are adsorbed, and increases in the concentration of the coating solution allow a broad array of surface properties to be produced at a silica surface.

Hydrophobically adsorbed coatings

Adsorption is a general phenomenon in which any of a variety of forces can be used to create polymeric surface layers. Both electrostatic and hydrophobic interactions have been used in the preparation of chromatography sorbents and capillary electrophoresis columns [21,22]. The example which has the highest probability of fitting the criteria set forth in the introduction is the adsorption of surfactants to an alkylsilane derivatized capillary to create a hydrophilic coating. It has been proposed that when a surfactant containing both hydrophobic and hydrophilic residues is dissolved in a polar solvent and exposed to a hydrophobic surface that hydrophobic residues would turn inward and drive hydrophobic adsorption while hydrophilic residues would turn outward toward the solution [4]. This concept will be extended here to amphiphobic copolymers and hydrophilic homopolymers with a hydrocarbon backbone. It is anticipated that these polymers will also be hydrophobically adsorbed and undergo the functional group orientation described above.

The surfactant Brij 35 has been shown with alkylsilane derivatized capillaries to both prevent protein adsorption and reduced EOF [4]. This surfactant has the general formula $CH_3(CH_2)_{11}$-$(OCH_2CH_2)_{23}OH$. In addition to Brij 35, Pluronic PF-108 [H-$(OCH(CH_3)CH_2)_m(OCH_2CH_2)_nOH$], methylcellulose (MC), poly(vinyl alcohol) (PVA), Tween and polyvinylpyrrolidone (PVP) were examined as coating materials. MC and PVA were used in several different molecular masses to extend the number of coating options and explore the influence of molecular mass on EOF.

Preparation of coatings

The advantage of adsorbed coatings is their ease of preparation. The surfactant or polymer solutions were pushed or pulled continuously through a C_{18} capillary for several hours to establish the equilibrium between the adsorbed and free forms. Experiments were not conducted to determine the minimum time required to establish this equilibrium. The minimum is probably much less than an hour. Because C_{18} capillaries and all the surfactants and polymers are commercially available, organic syntheses are not required in this coating process. All of the coating agents noted above were applied by the same coating procedure. Since adsorbed surfactants and polymers can slowly leach from columns during use, it is recommended that a small amount of surfactant or polymer be used in the buffers either during electrophoretic separations or between analyses to replace desorbed material.

Electroosmotic flow

The difference in electroosmotic mobility (EOM) between the various surfactant and polymer coated octadecylsilane derivatized capillaries is seen in Table I. The PF-108–C_{18} coated capillary has the lowest EOF and Tween 20 has the largest among the surfactants and polymers surveyed. In the MC and PVA coated series of capillaries, EOF decreased with increasing molecular mass. It has already been established that an octadecylsilane layer covalently bonded to the capillary surface masks the negatively charged silanol groups on the capillary surface and reduces EOF [4]. The results in Table I suggest that adsorbed polymer and surfactants provide further masking and control of EOF and that the degree of control is related to the molecular mass of the adsorbed species. This fact is rationalized in the following way. The hydrophobic backbone of surfactants or polymers interact with the hydrophobic octadecylsilane layer, orienting hydrophilic functional groups towards the bulk solution. This both

TABLE I

ELECTROOSMOTIC MOBILITY (EOM) OF THE VARIOUS MODIFIED C_{18} CAPILLARIES

Conditions: 50 μm I.D. coated capillaries (20 cm separation length and 30 cm total length) were employed to measure the migration time of mesityl oxide at pH 6 under 9 kV. The EOM of uncoated silica under these conditions was 7.1 (10-s $m^2/V \cdot s$).

Coating material	EOM (10^{-8} $m^2/V \cdot s$)
MC-15	0.722
MC-25	0.586
MC-400	0.574
MC-1500	0.428
MC-4000	0.320
PVA-2000	0.645
PVA-124000	0.363
Brij 35	1.650
Tween-20	2.030
PVP	0.672
PF-108	0.200

shields the surface and increases the viscosity in the double layer region at the capillary surface. Higher viscosity in the double layer reduces EOF. The fact that higher-molecular-mass polymers provide better control of EOF would suggest that polymers do not lay flat on the surface. The most likely conformation of polymers at the surface would be by a loop-and-train orientation [23]. Layer thickness tends to increase with molecular mass in this model. These results clearly establish that the magnitude of EOF in open tubular capillaries can be modulated by the selection of specific organic coatings.

Zone electrophoresis

Protein separations were examined by capillary zoneelectrophoresis (CZE) on uncoatedand PF-108–C_{18} coated columns. The four proteins utilized in the study had isoelectric points (p*I*) of less than 6.2 (Table II). At the pH (8.3) of the running buffer, these proteins are negatively charged and migrate against the EOF. Elution is achieved in less than 3 min on uncoated capillaries with the elution order inversely related to protein p*I* (Fig. 1). Carbonic anhydrase (p*I* 6.2) eluted first and trypsin inhibitor (p*I* 4.6) eluted last. In addition, the first two peaks were not resolved. When this same separation was performed in the C_{18}–PF-108 modified capillaries (Fig. 2), elution time increased to 20 min and elution order was reversed.

TABLE II

MOLECULAR MASS AND p*I* OF THE STANDARD PROTEINS

Proteins	Rel. mol. mass	p*I*
Trypsin inhibitor from soy bean	20 000	4.6
Ovalbumin	47 000	4.7
a-Amylase from bacillus species	45 000	5.3
Carbonic anhydrase from bovine	29 000	6.2

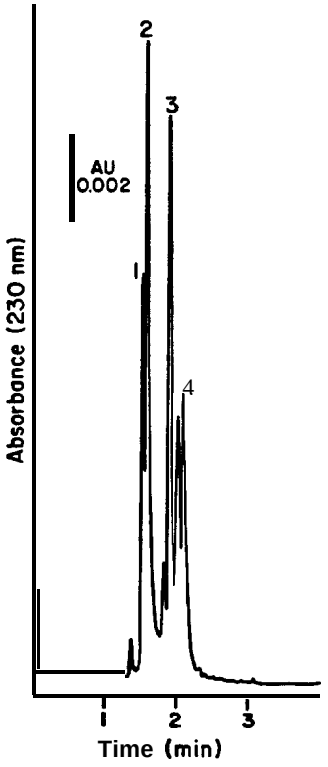

Fig. 1. CZE separation of native proteins on uncoated capillaries. Conditions: capillary length 45 cm; separation length 25 cm; 75 μm I.D.; field strength: 333 V/cm; Tris-glycine buffer, pH 8.30. Carbonic anhydrase, 2 = a-amylase, 3 = ovalbumin, 4 = trypsin inhibitor.

The very large difference between these two capillaries is due totally to differences in EOM. Unmodified capillaries have an average EOM of 7.1 · 10^{-8} $m^2/V \cdot s$. C_{18}–PF-108 modified capillaries, in contrast, produce an EOM of only 2.0 · 10^{-9}

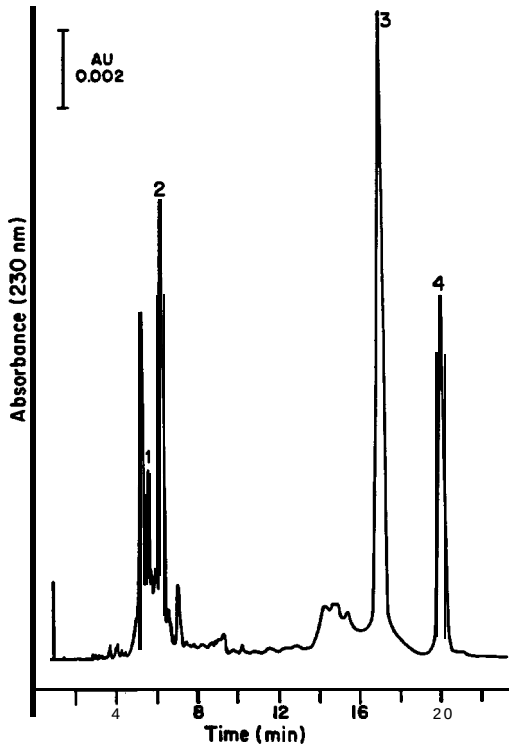

Fig. 2. CZE separation of native proteins on PF-108–C_{18} coated capillary. 1 = Trypsin inhibitor, 2 = ovalbumin, 3 = a-amylase, 4 = carbonic anhydrase. Separation conditions are given in Fig. 1.

$m^2/V \cdot s$, 35 times lower than the EOM in unmodified capillaries. The electrophoretic mobility (EM) of the four proteins is identical in both columns. Because the magnitude of EOM is far greater than EM in uncoated capillaries, proteins were swept to the detector window before they were well resolved. The proteins with higher p*I* values and lower charges, therefore, migrate faster. The same phenomenon occurs in C_{18}–PF-108 modified capillaries, but electrophoretic mobility is greater than EOM in this case. This means that elution order will be reversed. The fact that EOM is so much lower in the coated capillary also increases the separation time and resolution.

This data allows several conclusions to be made relative to the regulation of EOF in CZE. In unmodified capillaries the separation was finished in 2.5 min, whereas in C_{18}–PF-108 it took eight times longer. However, proteins are transported through uncoated capillaries so quickly that resolution is poor. One of the primary goals of CZE is to achieve the separation of components in a mixture in the shortest period of time. When a mixture contains a number of components that vary significantly in charge, large EOF can be used to limit the time of analysis. At the other extreme, minimal EOF is necessary in samples containing species of similar electrophoretic mobility. Through manipulation EOF, both of these extremes may be tolerated. It should be noted that proteins are a special case. The observations and conclusions reached for proteins might not apply to other molecular species.

Isoelectric focusing

Optimization by control of EOF. cIEF may be divided into three operations; loading, focusing, and mobilization. Sample and ampholyte are generally introduced into the capillary simultaneously in the loading step. The time required for this mechanical operation is either dependent on the dexterity of the operator in manual systems or the design of the electrophoretic instrument in automated systems. In both cases, it is not possible to further manipulate time.

Greater opportunity for manipulation of analysis time and resolution exists in the cases of focusing and mobilization. Formation of a pH gradient within the capillary and analyte focusing are generally achieved simultaneously. EOF must be sufficiently low that focusing can be completed before the gradient is transported past the detector. However, when EOF is zero a separate mobilization step is necessary. Focusing and mobilization were combined in these studies to further reduce analysis time. It is in this context that the control of EOF through surface chemistry is important. A typical separation using hemoglobin (Hb) variants is seen in Fig. 3. The influence of various surface coatings on resolution in cIEF is presented in Table III. Coatings which allow longer analysis times are seen to enhance resolution as measured by the difference in p*I* values between adjacent peaks [24]. The sample used in this case was sufficiently simple that baseline separation of all the sample components could be achieved in 9 min. Although very high resolution was not needed in this case, higher efficiency will be need in more complex samples. When this is necessary, the data indicate that a coating must be chosen that allows longer focusing time.

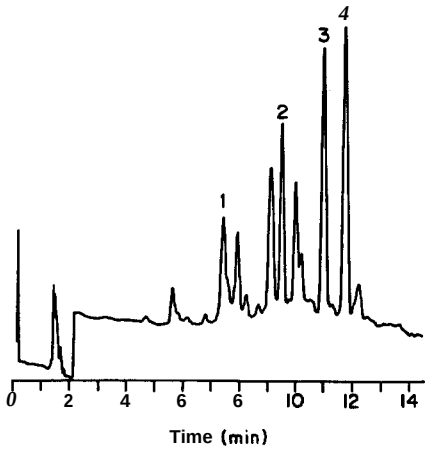

Fig. 3. cIEF separation of the commercial Hb variants. Conditions: MC-4000–C_{18} coated capillary with 10 cm separation length, 15 cm total length and 25 μm I.D.; sample solution: 1 mg/ml Hb in 1–2% ampholyte with a 0.2% polymer additive; field strength: 650 V/cm; a linear UVIS-205 detector was emloyed; detection wavelength: 280 nm; AUFS: 0.05; EOF mobilizations were employed without interrupting the experiments. 1 = HbC, 2 = HbS, 3 = HbF, 4 = HbA.

Fig. 4a and b are electropherograms of Hb variants using the same coating. For comparison, separation time was varied with 15 and 16 cm separation length, respectively. Resolution is seen to be greater in the capillary with the longer analysis time, increasing from 0.06 pH unit when the elution time of the first peak was 8 min to 0.03 pH unit when the elution time was 11 min.

It may be concluded that as in the case of CZE, resolution and analysis time may be varied in cIEF by manipulation of EOF. When the separation is simple and low resolution will suffice, analysis time may be diminished by increasing EOF.

Separation of HbA_1. Clinical studies [25–29] have shown that the level of the HbA_{1c} is a reliable index of long-term blood glucose control in diabetes which can be monitored by either chromatographic or electrophoretic methods [30–33]. In addition to HbA_{1c}, other glycosylated hemoglobin components such as HbA_{1a}, HbA_{1b} and pre-HbA_{1c}, are also formed from hemoglobin A_1. Glycosylation is a non-enzymatic process triggered by the reaction of the aldehyde group of glucose with the N-terminus of the B-chain of hemoglobin to form a reversible, labile aldimine intermediate (Schiff base). This intermediate (pre-HbA_{1c}) subsequently undergoes an Amadori rearrangement to form the stable ketoamine ($A_{,,}$) [34].

TABLE III

THE RELATIONSHIP BETWEEN ANALYSIS TIME AND RESOLUTION AMONG DIFFERENT COATINGS

Conditions: 25 μm I.D. coated capillary; sample solution: 1 mg/ml Hb in 1–2% ampholyte with a 0.2% polymer additive; field strength: 500 V/cm; AUFS: 0.05. EOF mobilization was employed without interrupting the experiments. The length of the capillaries were the same.

C_{18} capillaries	Migration time (min)		m.r.ΔpH[a]
	First peak	Last peak	
MC-15	5.9	8.7	0.041
MC-25	7.4	10.7	0.033
PVA-124K	9.6	12.0	0.032
MC-4000	11.2	16.3	0.030

[a] m.r.ΔpH = for minimum resolvable ΔpH [24].

Two consecutive cIEF separations of HbA_{1c} at normal (Fig. 5) and elevated levels (Fig. 6) of HbA_{1c}, respectively, were examined. Although not confirmed by independent means, we believe the fused peaks indicated by the arrow are HbA_{1c} and pre-HbA_{1c} based on migration orders reported in the literature [33]. For this reason these peaks may be summed in the quantitation of glycoforms of HbA_1. Separations were reproducibly achieved within 10 min; 6 min were required for focusing and 4 min for mobilization. These cIEF separations were obtained with a MC-25-alkyl silane coated capillary using salt mobilization. A large difference can be seen between the magnitude of the fused HbA_{1c} and pre-HbA_{1c} peaks in the samples with normal and elevated levels. Peak area from the elevated level sample is about twice that of the normal level. Reproducibility of the cIEF system, as assessed from the peak height of the fused HbA_{1c} and pre-HbA_{1c} peaks in the "normal" sample, is evaluated in Table IV. Precision by cIEF appears to be equivalent to that with CZE. cIEF might be a potential alternative for routine analysis in clinical studies or monitoring, where speed and reproducibility are desired.

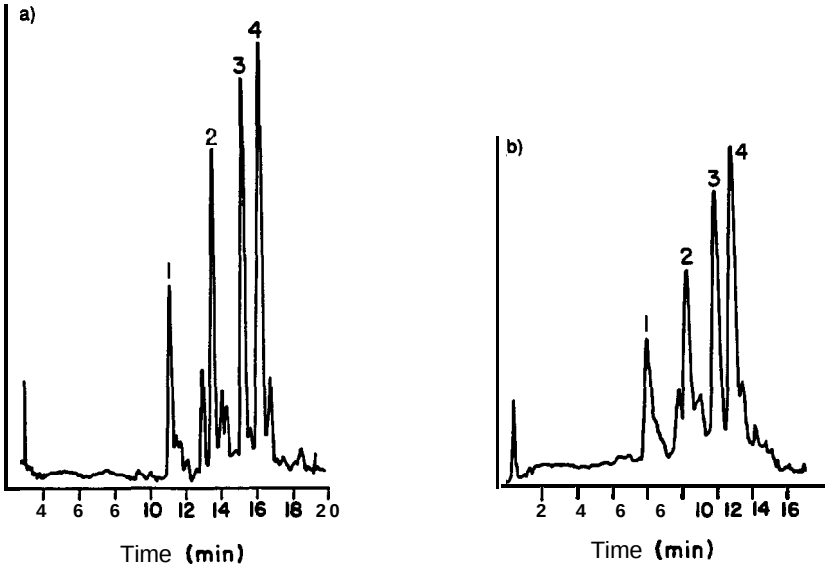

Fig. 4. (a) cIEF separation of the commercial Hb variants (C, S, F and A) with longer analysis time. Conditions: MC-4000–C_{18} coated capillary with 16 cm separation length, 2 1 cm total length and 25 μm I.D.; sample solutions: 1 mg/ml Hb variants in 1–2% ampholyte with 0.2% MC-4000 additive; field strength: 500 V/cm; AUFS: 0.02; EOF mobilization was employed. 1 = HbC, 2 = HbS, 3 = HbF, 4 = HbA. (b) cIEF separation of the commercial Hb variants (C, S, F and A) with shorter analysis time. Conditions: same as (a) except for 15 cm separation length and 20 cm total length of the coated capillary. Peaks as in (a).

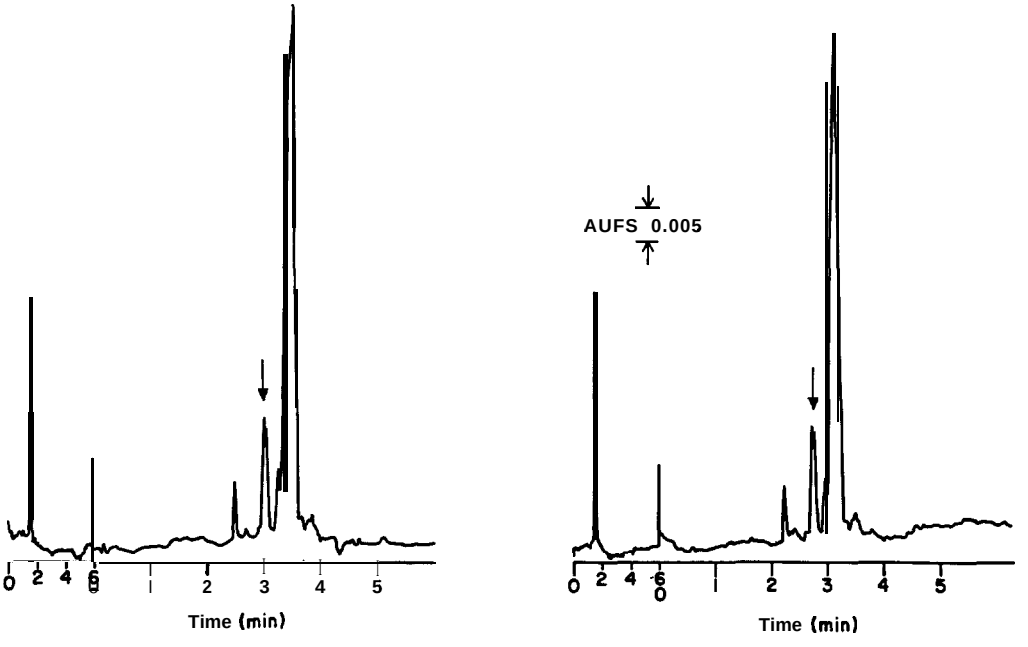

Fig. 5. Electropherogram of separations of hemoglobin Al at normal level for two consecutive runs. Conditions: MC-25–C_{18} coated capillary with 15 cm separation length, 20 cm total length and 25 μm I.D.; sample solutions: 1 mg/ml glycosylated Hb in 1–2% ampholyte with 0.2% MC-25 additive; field strength: 500 V/cm; AUFS: 0.05. Salt mobilization was employed.

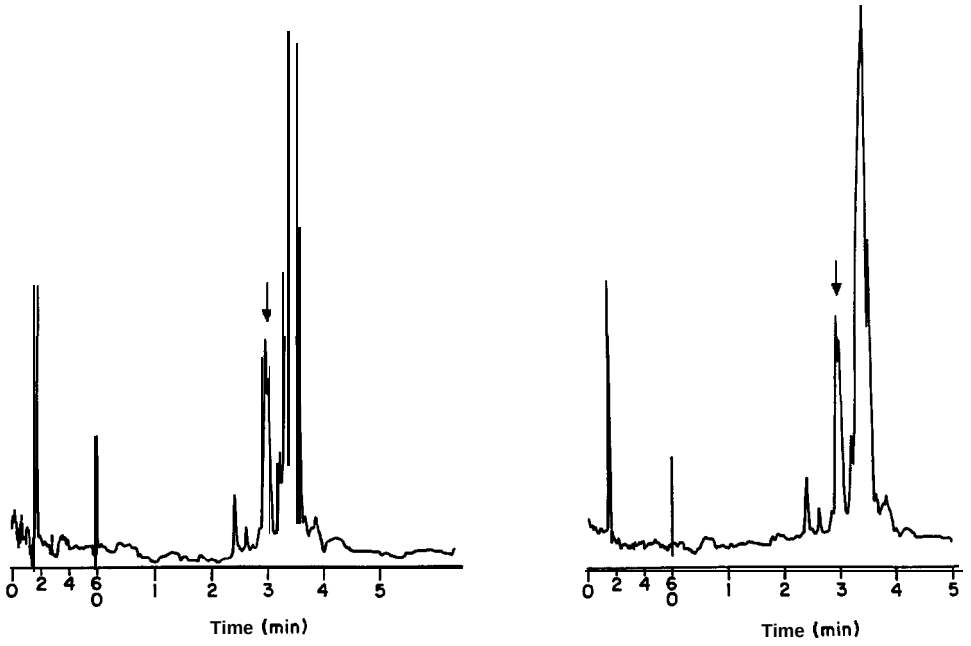

Fig. 6. Electropherogram of separations of hemoglobin Al at elevated level for two consecutive runs. Conditions: MC-25–C_{18} coated capillary with 15 cm separation length, 20 cm total length and 25 μm I.D.; sample solutions: 1 mg/ml glycosylated Hb in 1–2% ampholyte with 0.2% MC-25 additive; field strength: 500 V/cm; AUFS: 0.05. Salt mobilization was employed.

CONCLUSIONS

Analysis time and resolution of electrophoretic separations can be varied by manipulating EOF of the coated capillaries. Optimized separations could be achieved by CZE and cIEF in the shortest analysis time. It is concluded that the ability to control EOF is a valuable asset in optimizing capillary electrophoretic separations.

TABLE IV

THE REPRODUCIBILITY OF PEAK HEIGHT FOR HbA_1 NORMAL LEVEL

Number of runs	Peak height of the major peaks (cm)			
	1	2	3	4
1	13.5	4	28.5	86
2	13.5	3.5	29	88
3	12	3	28.5	87
R.S.D. (%) (n = 3)	6.7	14.3	1.0	1.1

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