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Effects of alkylamines on electroosmotic flow and protein migration behaviour in capillary electrophoresis

Danilo Corradini*, Gianfranco Cannarsa, Emanuela Fabbri, Claudio Corradini

Istituto di Cromatografia del CNR, Area della Ricerca di Roma, P.O. Box 10, I-00016 Monterotondo Stazione (Rome), Italy

Abstract

This paper reports the use of four closely related alkylamines as running electrolyte additives in capillary electrophoresis that permit the control of electroosmotic flow and protein migration behaviour in uncoated capillaries. At pH 2.5 the direction of the electroosmotic flow was anodic with all additives and at constant ionic strength its magnitude increased with increasing alkylamine concentration. The observations are in qualitative agreement with a previous reported theoretical model that correlates the electroosmotic mobility with the charge density in the Stern region of the electric double layer, arising from the adsorption of the additive, and the charge density at the capillary wall due to dissociation of silanols.

1. Introduction

The electric double layer at the interfacial region between the inner wall of the capillary tube and the electrolyte solution gives rise to two fundamental phenomena in capillary electrophoresis: the electroosmotic flow, which is the flow of the electrolyte solution along the capillary tube generated by the electric field applied tangentially to the electric double layer, and the attraction of charged analytes by the oppositely charged groups or ions that form the immobilized part of the electric double layer.

When the capillaries are made of fused silica, the inner wall is negatively charged in the pH range where the silanol groups are dissociated. Under these conditions the electroosmotic flow is directed toward the cathode and positively charged analytes, such as basic proteins, can interact strongly with the capillary wall, leading to poor efficiency, low resolution and irreproducibility of the retention times.

Many different approaches to control the electroosmotic flow in capillary electrophoresis have been addressed. These include chemical coating of the capillary wall [1–4], extremes of electrolyte pH [5,6], the application of applied radial voltage to the capillary wall [7,8] and the addition to the running electrolyte solution of organic solvents [9], surfactants [10], neutral salts [11] or other additives. Several of these additives have been reported to attenuate protein–capillary wall interactions [12–18] and a few of them also to reverse the direction of the electroosmotic flow [15–18].

Reversal of the direction of the electroosmotic flow in fused-silica capillaries occurs when specific adsorption of counter ions in the immobilized region of the double layer, the so called Stern layer, takes place. In this case, the potential at the plane of shear between the Stern layer and the diffuse layer, ψ_δ, which can be

* Corresponding author.

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approximated to the zeta potential, ζ, becomes positive and the electroosmotic flow is directed towards the anode.

Alkylamines are generally believed to interact strongly with silanol groups [19] and their effectiveness at masking silanophilic activity in both HPLC [20,21] and capillary electrophoresis [17–18,22] has been reported.

In this work, the effect of cationic additives on the control of the electroosmotic flow and on protein migration behaviour in bare fused-silica capillaries was further investigated by using four closely related alkylamines, differing in the number of ethanol groups replacing the ethyl groups in the molecule of triethylamine. The investigation was performed at pH 2.5 in order to have all alkylamines full protonated and therefore behaving as monovalent cations of similar chemical composition and with gradual differences in their molecular size and hydrophobicity.

2. Experimental

2.1. Samples and chemicals

Cytochrome *c* (from horse heart), lysozyme (from chicken egg white), ribonuclease A (from bovine pancreas) and α-chymotrypsinogen A (from bovine pancreas) were supplied by Sigma (St. Louis, MO, USA) and 5-(hydroxymethyl-2-furaldehyde by Aldrich (Milwaukee, WI, USA). Triethylamine, N,N-diethylethanolamine, N-ethyldiethanolamine and triethanolamine were purchased from Fluka (Buchs, Switzerland) and were used without further purification. Analytical-reagent-grade phosphoric acid, hydrochloric acid, sodium hydroxide, sodium chloride and HPLC-grade water and methanol were obtained from Carlo Erba (Milan, Italy).

2.2. Apparatus

All experiments were performed using a P/ACE Model 2100 capillary electrophoresis unit, operated under System Gold Version 7.11 control, data acquisition and analysis software (Beckman, Fullerton, CA, USA). A fused-silica capillary (Quadrex, New Haven, CT, USA) of 0.075 mm I.D. and 0.375 mm O.D. and with a total length of 37 cm (30 cm to the detector) was mounted in the cartridge. The capillary tube temperature was maintained at 25 ± 1°C by means of a fluorocarbon liquid continuously circulating through the cartridge. A deuterium light source with either a 214- or 280-nm bandpass filter was used. The samples were injected by applying 0.5 p.s.i. (1 p.s.i. = 6894.76 Pa) pressure for 1 s, and the approximate sample volume of 9 nl was calculated according to the literature [23].

2.3. Electrophoresis

All experiments were carried out without any chemical coating of the inner wall of the fused-silica capillaries. Prior to use, the untreated capillary was flushed successively with 0.5 *M* sodium hydroxide (30 min), water (10 min) and 0.1 *M* hydrochloric acid (30 min), followed by a second treatment with sodium hydroxide (0.5 *M* for 30 min) and water (10 min), and then rinsed with the running electrolyte.

The running electrolyte was renewed after five or six runs, and before each run the capillary was rinsed with the running electrolyte for 3 min. For storage the capillary was rinsed with water for 10 min and then dried by flushing nitrogen for 10 min.

The capillary tube was flushed with 0.5 *M* sodium hydroxide (3 min) and water (3 min) each time a running electrolyte of new composition was used. All experiments were carried out by applying a constant voltage of 10 kV. Protein solutions of 1.0–3.0 mg/ml were prepared in HPLC-grade water.

2.4. Electroosmotic mobility measurements

The electroosmotic mobility was determined by measuring the migration time of 5-(hydroxymethyl)-2-furaldehyde, used as an inert tracer, detected at 280 nm at the anodic end of the capillary, on reversing the polarity. The inert tracer was dissolved in methanol–water (1:10, v/v) at a concentration of 1.0 mg/ml. All measurements of electroosmotic mobility were made

in triplicate. Running electrolytes at various additive concentration and constant ionic strength were made by adding the appropriate amount of sodium chloride to the electrolyte solution, containing 50 m*M* phosphate buffer (pH 2.5), for compensation of the changes in the additive concentration. All solutions were filtered through a Type HA 0.22-μm membrane filter (Millipore, Bedford, MA, USA) and degassed by sonication before use.

3. Results and discussion

The dependence of the electroosmotic mobility on the concentration in the running electrolyte of cationic species which adsorb at the interfacial region between the capillary wall and the electrolyte solution was investigated by using four closely related tertiary alkylamines, namely triethylamine (TEA), N,N-diethylethanolamine (DEEOHA), N-ethyldiethanolamine (EDEHOA) and triethanolamine (TEOHA). At pH 2.5 the direction of the electroosmotic flow, measured with 5-(hydroxymethyl)-2-furaldehyde as a neutral marker, was anodic with all additives and at constant ionic strength (123 m*M*) its magnitude increased with increasing the alkylamine concentration.

In a previous paper [18] we presented a theoretical model that correlates the electroosmotic mobility with the charge density in the Stern region of the electric double layer, arising from the adsorption of counter ions, and the charge density at the capillary wall, due to dissociation of silanols. According to this model, we can express the dependence of the electroosmotic mobility on the concentration of the cationic additive in the running electrolyte under otherwise identical conditions as

$$\mu_{eo} = \frac{4\pi}{\kappa\eta}\left\{\frac{zen_0\dfrac{C}{55.6}\exp\left(\dfrac{ze\psi_\delta + \Phi}{kT}\right)}{1+\dfrac{C}{55.6}\exp\left(\dfrac{ze\psi_\delta + \Phi}{kT}\right)} - \left(\frac{\gamma}{1+\dfrac{[H^+]}{K_a}}\right)\right\} \quad (1)$$

where κ is the reciprocal of the Debye length, η is the viscosity of the electrolyte solution, e is the elementary charge, z is the valence of the adsorbing ion, k is the Boltzman constant, T is the absolute temperature, n_0 is the number of accessible sites in the Stern layer, ψ_δ is the potential at the Stern plane, Φ allows for any specific adsorption potential, γ is the sum of the ionized and protonated surface silanol groups, $[H^+]$ is the bulk electrolyte hydrogen ion concentration and K_a is the silanol dissociation constant.

Eq. 1 suggests that at constant ionic strength and pH the electroosmotic mobility would depend mainly on the surface density of adsorbed counter ions in the Stern region of the double layer, which should follow a Langmuirian-type adsorption model. The good agreement between the theoretical prediction of Eq. 1 and the experimental trends in the data is illustrated in Fig. 1 by plots of the electroosmotic mobility against the concentration of the additive in the running electrolyte. These curves, the slopes of which decrease with increasing additive concentration, are Langmuirian or quasi-Langmuirian

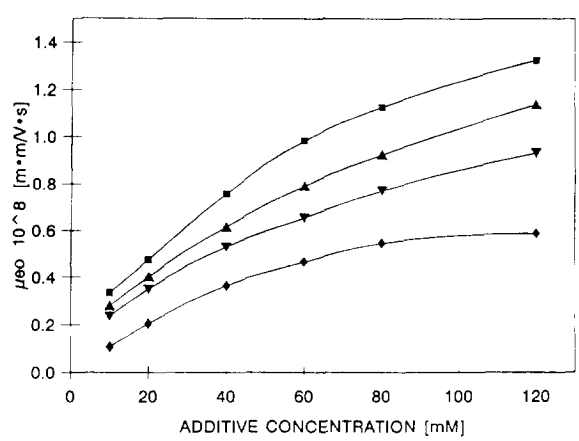

Fig. 1. Dependence of the electroosmotic mobility on the concentration of additive in the running electrolyte at constant ionic strength (123 m*M*) and pH (2.5). Capillary, fused silica, 0.075 mm I.D. × 370 mm total length (300 mm to the detector); applied voltage, 10 kV; temperature, 25°C; neutral marker, 5-(hydroxymethyl)-2-furaldehyde; detection wavelength, 280 nm at the cathodic end. Additives: ■ = triethylamine; ▲ = N,N-diethylethanolamine; ▼ = N-ethyldiethanolamine, ◆ = triethanolamine.

Table 1
Molecular volumes of alkylamines calculated by the Chem-X 1990 Molecular Modelling Program

Additive	Molecular volume (Å^3)
Triethylamine	98.5
N,N-Diethylethanolamine	104.9
N-Ethyldiethanolamine	111.1
Triethanolamine	117.3

in shape [22]. The differences in the slopes of the four plots in Fig. 1 can be accounted for by differences in either the specific adsorption energy (Φ) or the molecular size of the additives, or both.

The four additives are closely related tertiary alkylamines differing only in the number of hydroxyl functions, which vary from one to three according to the number of ethanol groups which replace the ethyl groups in TEA. The replacement of one, two or three ethyl groups by a corresponding number of ethanol groups is expected to lead to alkylamines with proportionally lower hydrophobic character and larger molecular size. Three-dimensional molecular modelling of the four alkylamines was performed with the Chem-X 1990 Molecular Modelling Program (Chemical Design, Oxford, UK) in order to estimate their relative molecular size by the theoretical molecular volume calculated for a single molecule over its Van der Waals surface [24] and the results are reported in Table 1.

The electroosmotic mobilities obtained with the four alkylamines at different concentrations were plotted against the number of hydroxyl groups present in each additive (Fig. 2A) and against the molecular volumes calculated by the Chem-X 1990 Molecular Modelling Program (Fig. 2B). In both cases the plots obtained were almost linear, with negative slopes. These ob-

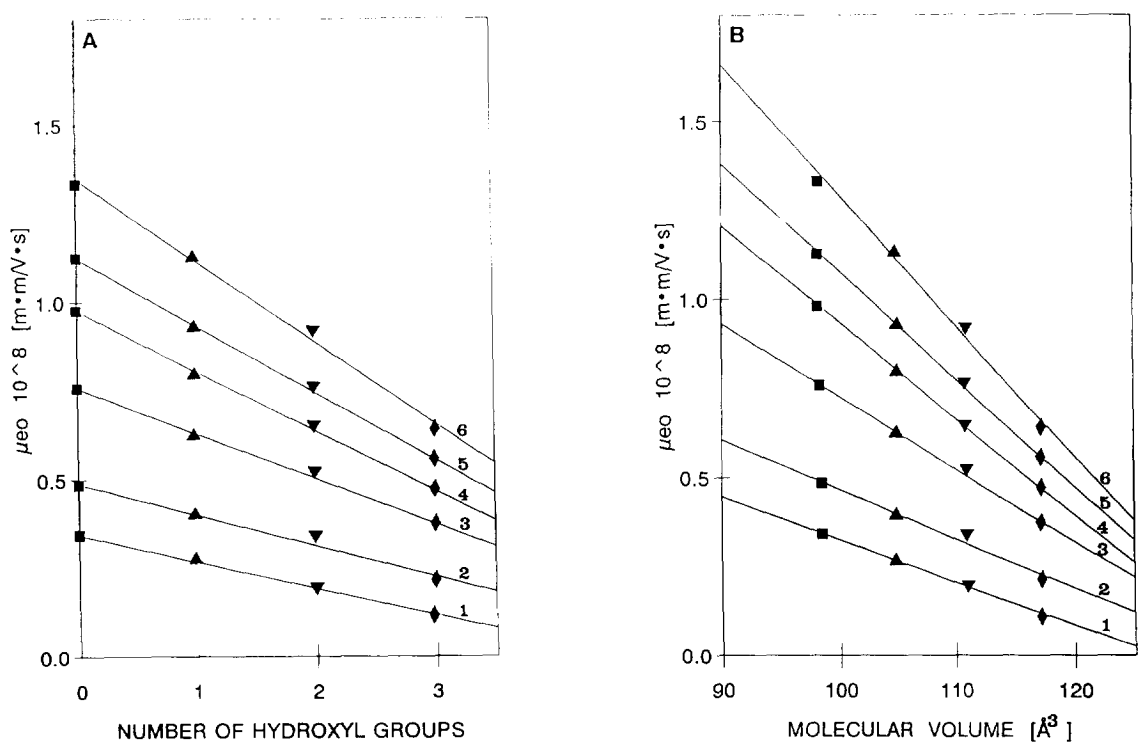

Fig. 2. Dependence of the electroosmotic mobility at different additive concentrations on (A) the number of hydroxyl groups present in each additive and (B) their molecular volumes calculated by the Chem-X Molecular Modelling Program. Additive concentrations (from line 1 to 6): 10, 20, 40, 60, 80 and 120 mM; ionic strength, 123 mM; symbols and other conditions as in Fig. 1.

servations suggest that in addition to the contribution arising from the specific adsorption energy (Φ), which is expected to vary with the different content of hydroxyl groups of the four additives, the electroosmotic mobility may depend on the molecular size of the adsorbing counter ions in such a way that the number of accessible sites (n_0) in the Stern layer can be occupied by a limited number of counter ions, which will be larger for additives of smaller molecular size.

The beneficial effect of controlling the electroosmotic flow by the addition of the four alkylamines to the running electrolyte on the migration behaviour of four basic proteins was also investigated. All experiments were performed under the same conditions as employed to study the effect of these additives on the electroosmotic flow, except that proteins were detected at the cathodic end of the capillary tube.

The four basic proteins cytochrome *c* (Cyt), lysozyme (Lys), ribonuclease A (RNase) and α-chymotrypsinogen A (Chy) have isoelectric points ranging from 9 to 11 and therefore at pH 2.5 they are positively charged and moved towards the cathode. In the absence of the alkylamines in the running electrolyte, the four basic proteins comprising the mixture test were resolved in a very narrow separation space and migrated with poorly reproducible migration times as fairly symmetric peaks. The poorly reproducible migration times may arise either from protein–capillary wall interactions, which would affect the electroosmotic mobility and consequently the migration times, or from the difficulty of maintaining a constant electroosmotic flow in bare fused-silica capillaries, as evidenced by Cohen and Grushka [25], or both. The narrow separation space is a consequence of full protonation of proteins, which diminishes charge differences between species.

In the presence of the additives, the four basic proteins moved towards the cathode against the electroosmotic flow, which travelled in the opposite direction. Consequently, the apparent mobility of proteins, which is the resultant of the oppositely directed vectors of the protein elec-

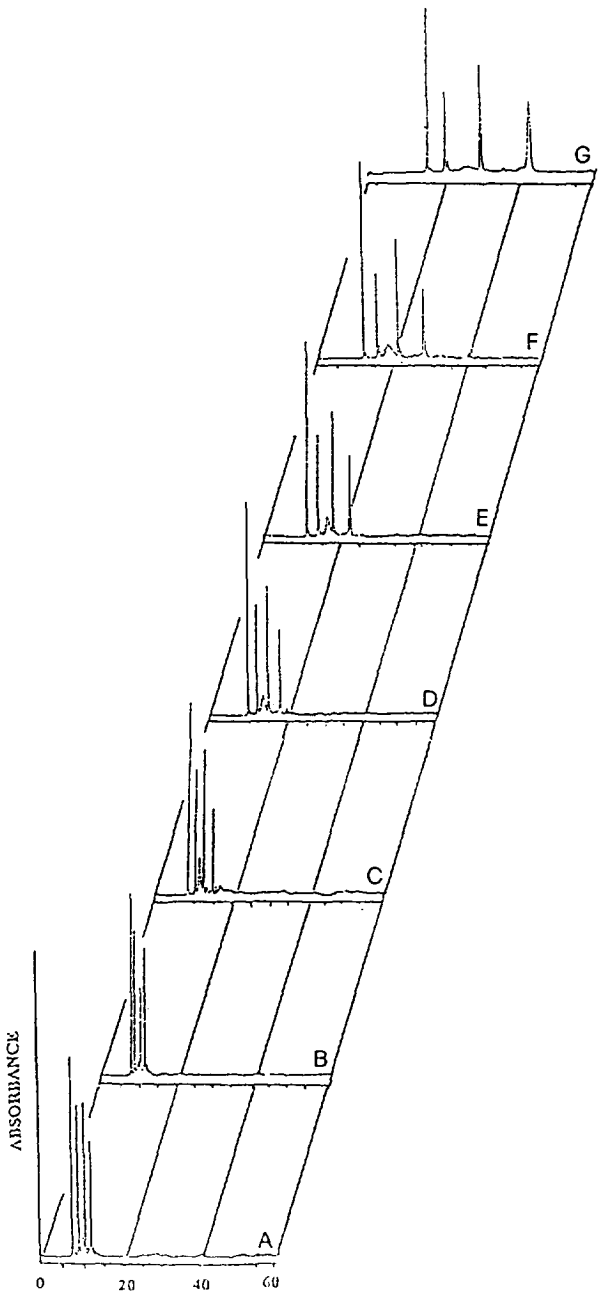

Fig. 3. Electropherograms of standard basic proteins obtained with N,N-diethylethanolamine as the additive at pH 2.5, constant ionic strength (123 m*M*) and concentrations of (A) 4, (B) 10, (C) 20, (D) 40, (E) 60, (F) 80 and (G) 120 m*M*. Migration order of proteins as in Fig. 4. Experimental conditions as in Fig. 1, except detection at 214 nm.

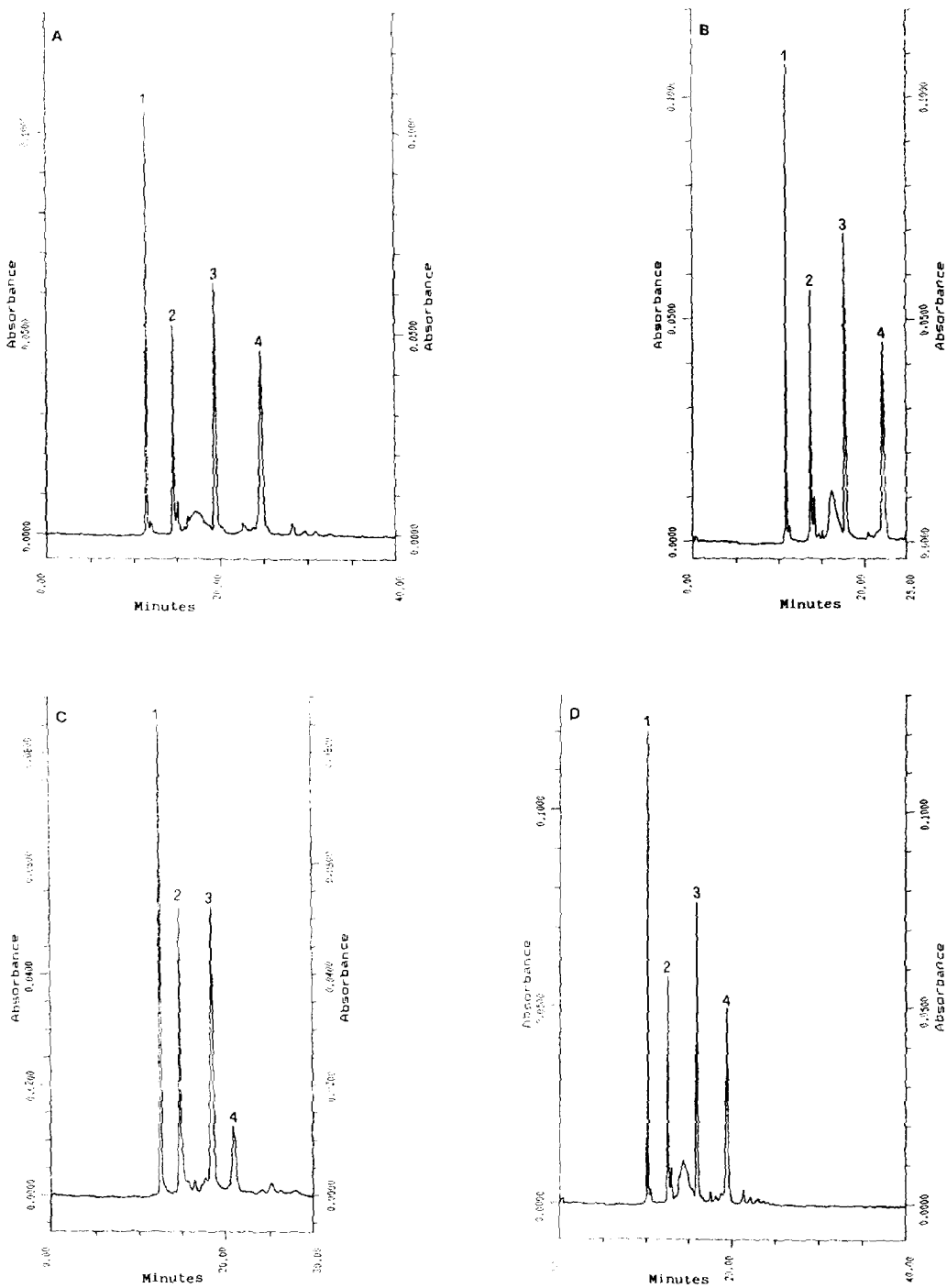

Fig. 4. Electropherograms of standard basic proteins obtained with different additives at the same concentration (60 m*M*), ionic strength (123 m*M*) and pH (2.5). (A) Triethylamine; (B) = N,N-diethylethanolamine; (C) N-ethyldiethanolamine; (D) triethanolamine. Proteins: 1 = cytochrome *c*; 2 = lysozyme; 3 = ribonuclease A; 4 = α-chymotrypsinogen A. Other conditions as in Fig. 3.

trophoretic mobility and of the electroosmotic mobility, varied on changing the additive and for all of them decreased with increasing additive concentration in the running electrolyte. Thus, as shown in Fig. 3 for DEEOHA, an increase in the additive concentration led to an increase in the protein migration times and in the separation space. However, the electrophoretic mobility of proteins was independent from the additive type and concentration, as it varied within 3.0% when measured with each of the four additives over the whole investigated concentration range, and within 5.5% when measured with either additive over the same concentration range. For this the absence of interactions between the investigated proteins and the additives can be inferred.

The effect of using different additives on the separation of a model mixture of the four basic proteins, under otherwise identical conditions, is depicted in Fig. 4. All separations were carried out in the same capillary tube at an applied voltage of 10 kV and with running electrolytes containing the different additives at the same concentration (60 mM), ionic strength (123.5 mM) and pH (2.5). The values of the measured current were 160 ± 5 μA.

Comparison of the four electropherograms shows that the separation of the four basic proteins was improved and the speed of analysis was decreased by using the additive which generated the highest counter electroosmotic flow, i.e., TEA.

The effectiveness of the four alkylamines in preventing protein–capillary wall interactions was investigated by multiple injections ($n = 5$) of this protein mixture into the same capillary using the above running electrolytes and experimental conditions. No washing of the capillary was performed between runs in order to detect irreversible protein adsorption that would affect the electroosmotic flow and consequently the migration times. Under these conditions, the reproducibility of the protein migration times obtained with all additives was better than 2.0% (R.S.D.). These results indicate that the four alkylamines are effective in preventing capillary wall interactions, as can also be inferred from the absence of peak tailing in the electropherograms displayed in Fig. 4.

4. Conclusions

We have further investigated the effect of cationic additives on the electroosmotic flow in bare fused-silica capillaries. Our experimental results showed that the dependence of the anodic electroosmotic flow on the concentration of alkylamines in the electrolyte solution maintained at pH 2.5 and constant ionic strength is described by a Langmuirian-type relationship.

At any given additive concentration, the magnitude of the electroosmotic flow increases according to the order of increasing basicity and decreasing molecular size. This indicates that the observed differences in the electroosmotic flow may reflect differences in the adsorbability of the four closely related alkylamines, which is believed to depend on their specific adsorption energy (Φ) and molecular size [26]. Further, in addition to controlling the electroosmotic flow, the four alkylamines were shown to be effective at preventing protein–capillary wall interactions without affecting the electrophoretic mobilities of these biopolymers.

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