Capillary Electrokinetic Separations: Influence of Mobile Phase Composition on Performance

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Summary

The composition of the mobile phase employed in capillary zone electrophoresis and the related technique, micellar electrokinetic capillary chromatography, is an important factor in determining separation performance. The influences of ionic salt, surfactant, and organic solvent mobile phase additives on separation efficiency, retention, and elution range are discussed and demonstrated.

1 Introduction

Capillary zone electrophoresis (CZE) has developed over the past decade into an electrophoretic separation mode that is capable of rapid, very high efficiency separations of charged solutes[1]. As with other forms of electrophoresis, separation is based on differences in electrophoretic mobility. The use of microcapillaries permits the application of relatively large electric fields without the problem of thermal peak dispersion[2]. Under the proper conditions, plate counts in excess of 10^6 can be achieved.

A major limitation of CZE is its inability to separate neutral compounds. One approach to extend the applications of CZE to include neutrals involves the addition of surfactants to the mobile phase at concentrations sufficient to form charged micelles[3]. With this type of mobile phase, neutral compounds can be separated based on differential chromatographic partitioning between the aqueous and micellar components of the mobile phase, which are transported through the capillary at different velocities due to electrophoretic effects[4]. We refer to this technique as micellar electrokinetic capillary chromatography (MECC) and have applied it in the separation of a variety of mixtures of compounds, including many of biological interest[4-8].

Instrumental and operational aspects of these techniques, except for detection which is complicated by diminutive peak volumes, are straightforward and amenable to automation. In contrast, critical adjustments of the composition of the mobile phase, with a variety of additives, can produce both dramatic and subtle effects on separation performance. Separation performance is ultimately measured in resolution, which depends largely on efficiency and selectivity. Additionally, elution range is important with the MECC technique[9]. This paper will first focus on the effects that pH and salt concentration in the mobile phase have on the efficiency obtained in CZE for a selected protein. The objective in this case is the minimization of adsorption on the surface of the capillary column. Second, the influences of surfactant concentration and the use of mixed aqueous-organic mobile phases will be presented for the MECC separation of fluorescently-derivatized alkylamines. Efficiency, capacity factor, and elution range are all influenced by these mobile phase additives. Finally, significant changes in elution profiles will be demonstrated for the same chemical system when organic solvent gradients are employed.

2 Experimental

2.1 Materials and Chemicals

Separations were performed using untreated fused-silica capillary tubing supplied by either Scientific Glass Engineering (Austin, TX) or Polymicro Technologies (Phoenix, AR) and octadecylsilane (ODS) modified capillary tubing supplied by Supelco, Inc. (Bellefonte, PA). Capillary diameters ranged from 25 μm to 75 μm with lengths up to 1.5 m. Sodium dodecyl sulfate (SDS), conalbumin, 2-(N-cyclohexylamine)-ethanesulfonic acid (CHES), and 7-chloro-4-nitrobenz-2-oxa-1,3-diazole (NBD-Cl) were obtained from Sigma Chemical Co. The laser dye Coumarin 343 was purchased from Exciton Corp. Amines and buffer components were reagent graded and obtained from Fischer Scientific. The NBD-Cl fluorescent derivatives of the alkyl amines used in this work were prepared using a previously reported procedure[10].

2.2 Apparatus and Procedures

The apparatus and procedures for the work reported herein varied depending on the study and, consequently, only a cursory description will be provided herein. More detailed descriptions can be found in the appropriate cited references. The basic CZE/MECC apparatus, including Hipotronics Model 840A power supply (Brewster, NY) and arrangement of column and buffer reservoirs, is the same as described in previous reports (e.g., 4). Samples were injected by both hydrostatic and electromigration techniques. Gradient

elution was performed using an inlet reservoir chamber than included a means to pump-in new solvent and drain-out the contents of the reservoir[11]. Linear, concave, and convex shaped solvent gradients were possible via suitable adjustments of the reservoir volume and the pump-in and drain-out rates.

Several modes of on-column optical detection were employed. Investigations involving the effect of pH on the efficiency of conalbumin in CZE were performed using a Laboratory Data Control UV III monitor absorbance detector that was modified with fiber optics, a cadmium pen lamp (229 nm) light source, and an unique laser-etched flow cell[11]. The effect of mobile phase salt concentration on efficiency was studied using the natural fluorescence of the protein. Excitation was provided using a Coherent Radiation Corp. (Palo Alto, CA) Model Innova 100 argon ion laser (515 nm, 9 W) frequency double to 257 nm (9 mW) using an Inrad Corp. (Northdale, NJ) Model 527-003 harmonic generator. The emission was isolated with a monochromator/filter combination centered at 330 nm. More details concerning the optical detection of proteins can be found elsewhere[12]. The NBD-amines were also detected via laser fluorimetry using either a Cyonics (San Jose, CA) Model 2001-20BL argon ion laser (488 nm, 20 mW) or a Liconix (Sunnyvale, CA) Model 4230 NB He-Cd laser (422 nm, 30 mW). The emission was isolated with a monochromator/filter combination centered at 525 nm[11].

3 Results and Discussion

3.1 Effects of pH and Salt Concentration on CZE Efficiency for Proteins

High separation performance is generally associated with the ability of a separation technique to rapidly resolve structurally similar compounds or to resolve the many components in complex samples. In CZE resolution depends on differences in electrophoretic mobility (selectivity) and efficiency. Improvements in resolution can be achieved, at the expense of time, by modifying the capillary walls or the composition of the mobile phase such that electroosmotic mobility opposes and is of similar magnitude to the electrophoretic mobilities of the solutes to be separated[2]. Although the mobile phase can be adjusted to alter solute electrophoretic mobility, the effects are not dramatic unless highly specific interactions are involved (e.g., the formation of charged complexes[13]). Thus, efficiency is generally the most important factor in determining separation performance. Ignoring injection and detection related band dispersion, the major factors that reduce plate counts in CZE are longitudinal diffusion, thermal dispersion, and wall-solute interactions[14]. The latter two factors are particularly important in the application of CZE to protein/peptide analysis.

Proteins can "stick" to the surfaces of the untreated or treated silica capillaries that are usually employed in CZE via coulombic, induced dipole, and dispersive interactions. Moreover, the large size of the protein can facilitiate multiple interactions for a single protein molecule. The coulombic interactions are generally the strongest and are of primary concern. It is also clear that these interactions vary greatly depending on the protein and the interested reader should not overextrapolate the usefullness of CZE or specific operating conditions to the separation of proteins that have not been previously investigated.

Approaches to minimize protein "sticking" include modifying the surface of the capillary[15-17], adding large concentrations of salts, including zwitterionic salts, to the mobile phase to compete for interaction sites on the capillary wall[18], and utilizing mobile phases buffered to low pH[17] or high pH[19,20]. The effect of pH adjustment and surface modification are demonstrated in Figure 1 with elution profiles of conalbumin obtained in our laboratory.

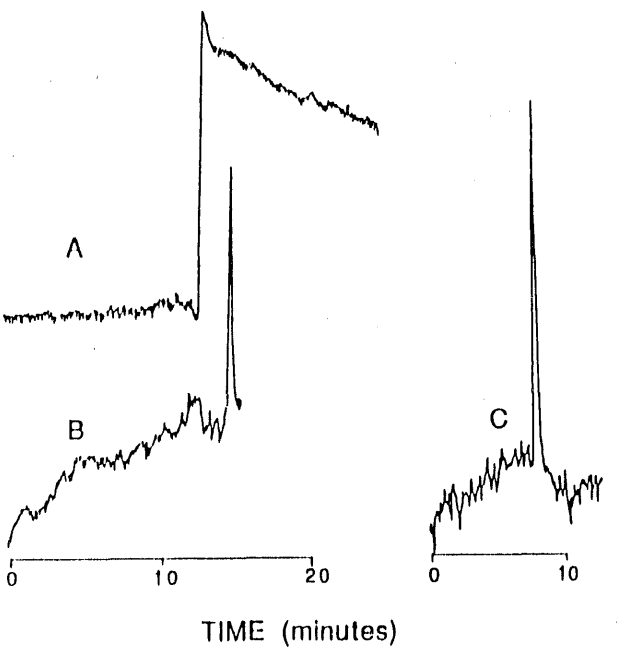

Figure 1

Elution profiles of Conalbumin using (A) 50μm i.d. x 75 μm untreated capillary, mobile phase, 0.01 M CHES, 0.015 M KCl, pH adjusted to 8.3, (B) Same capillary and mobile phase, pH adjusted to 10.1, (C) ODS modified capillary, 50μm i.d. x 50 cm; mobile phase, 0.01 M Na_2HPO_4, 006 M $Na_2B_4O_7$, pH adjusted to 8.0

By increasing the pH significantly above the isoelectric point of the protein (the pI of conalbumin is 6.7) both the protein and the silica surface of the capillary acquire a net negative charge. Because of the complex nature of the protein-surface interaction, this pH adjustment does not totally eliminate the "sticking" problem. If limited only by longitudinal diffusion, the plate count for the conalbumin should be greater than 10^6[18]. The observed efficiency at pH 10.1 in Figure 1B is only approximately 12,000 plates. Nevertheless, comparison of Figures 1A and B illustrates the effect of increasing the pH to increase the coulombic repulsion between the protein and the capillary wall. The strong wall adsorption at pH 8.1 is evident. It is surprising that the leading edge of the conalbumin peak at pH 8.1 is sharp and the peak height is roughly the same as for the symmetric peak at pH 10.1. This may be partly explained by differences in detector response factors at the two pH values. However, the elution profiles in this experiment were reproducible and intermediate effects were observed at pH values between 8.1 and 10.1. Figure 1C is the elution profile obtained with a capillary modified with ODS. A short "break-in" period , involving a few protein injections, was required with this column. These preliminary studies indicate that surface interactions were significantly reduced by the ODS modification. The ability to operate at a moderate pH can be important in the separation of protein mixtures as at extreme pH values differences in protein mobility are reduced and selectivity suffers. The silica surface is also unstable at very high pH.

The absorption of proteins on the capillary wall can also be reduced by employing mobile phases with relatively high salt concentrations[21]. Our studies of the influence of KCl concentration on efficiency for 25 μm and 75 μm i.d. capillaries is summarized in Table 1. On-column absorbance detection is commonly employed in CZE but is difficult to perform with very small i.d. capillaries. This study was conducted employing laser-based fluorescence detection using the frequency double output of an argon ion laser for excitation[12]. The ability to focus laser beams to extremely small spot sizes facilitates the use of very small diameter capillaries. This is important since electrophoretic current, hence thermal load, decreases and the ability to dissipate heat increases as the diameter of the capillary is reduced. Thus, the advantages of high salt concentration can be exploited with very small diameter capillaries. As seen from the table the 25 μm i.d. capillary provided the higher efficiency. However, at 45 mM KCl the effects of thermal dispersion are evident even with the small diameter capillary.

Table 1

Influence of salt (KCl) concentration and column diameter on efficiency for conalbumin

Capillary diameter (μm)	N(plates x 1000/m)/Power Dissipated (W)		
	KCl Concentration (mM)		
	15	30	45
25	78/0.10	390/0.20	280/0.40
75	80/0.24	12/0.42	

3.2 The Influence of Mobile Phase Composition on Separation Performance in MECC

Resolution, R_S, in MECC is given by equation 1[3]

$$R_S = \left(\frac{N^{1/2}}{4}\right)\left(\frac{\alpha-1}{\alpha}\right)\left(\frac{k'}{1+k'}\right)\left(\frac{1 - to/tm}{1 + (to/tm)k'}\right) \quad (1)$$

where performance is dependent on plate count, N, selectivity factor, α, capacity factor, k', and the elution range as reflected in t_o/t_m. The most common surfactant employed in MECC is SDS. However, other surfactants have been employed to provide unique selectivities and improve performance (e.g., consider our accompanying report involving the use of chiral-order bile salts for the separation of binapthyl enantiomers). Likewise, mobile phase additives such as organic modifiers and complexing reagents have been shown to influence selectivity. It is, however, the general influences of surfactant concentration and organic solvent additives on efficiency, capacity, and elution range that are considered in this report.

Table 2 presents the results of our experiments involving manipulation of mobile phase composition in MECC. NBD derivatives of alkylamines were used as test solutes in the experiments. The data was obtained using 50 μm i.d. x 1.5 m columns, an applied voltage of 20 kV, and a mobile phase that contained 0.01 M $NaHPO_4$, 0.005 M $Na_2B_4O_7$ (pH 7.5). NBD-ethanolamine and the fluorescent dye coumaxin 343 were used to mark t_o and t_m, respectively. Increasing SDS concentration from 0.015 M (slighly above the SDS critical micelle concentration) to 0.10 M resulted in a dramatic increase in plate number and an increase in capacity factor for NBD-n-butylamine. The latter effect is a result of an increase in micellar phase volume. The optimum k'

in MECC depends on elution range, but is generally in the range of 1-5[3]. The increase in plate count with SDS concentration is probably attributable to an increase in the rate of monomer-micelle exchange, which minimizes the band dispersion caused by micelle polydispersity[22,23]. SDS micelle systems exist with a range in aggregation number that produces a concomitant range in micelle electrophoretic mobility. Increasing the dynamics of micelle rearrangement via increases in surfactant concentration and temperature has an averaging effect in solute band velocity that improves efficiency[22]. Unfortunately, large increases in surfactant concentration can produce the thermal band dispersion discussed in the previous section and excessive k' values. Optimization of surfactant concentration in MECC is straightforward but critical to achieving high separation performance.

Table 2

Effects of [SDS] and organic additive on the electrophoretic behavior of NBD-n butylamine in MECC

[SDS]	%2-propanal	k'	N(plates/m)	t_o/t_m
0.015	0	1.20	8,000	
0.10	0	3.26	350,000	
0.075	0	1.02	310,000	0.65
0.075	10	0.41	250,000	0.14

MECC generally exhibits large k' values for hydrophobic compounds and a limited elution range. As can be seen by inspection of equation 1 this degrades resolution for solutes with large k' values. Organic solvents can be added to MECC mobile phases to provide some solution to this problem. The bottom two rows in Table 2 demonstrate the effects of 2-propanol on the elution characteristics of NBD-n-butylamine. An intermediate SDS concentration was employed. The addition of 2-propanol has several effects. Critical micelle concentration is increased, resulting in a decrease in the micellar phase volume. The partition coefficient for the test solute is also decreased (elution characteristics with this MECC system resemble reversed phase LC). These effects result in a significant reduction in k'. It should be noted that this experiment was performed separately from the SDS concentration experiment described above. Differences in operating temperature are

likely responsible for the disparity of k' values between the two experiments.

A slight reduction in plate count is observed with addition of 2-propanol. This is persumed to be a result of a reduction in micelle concentration that influences the problem of polydispersity discussed above. Nevertheless, significant reductions in k' are possible without excessive reductions in plate count. Organic solvents that interact with the capillary wall such as alcohols reduce electroosmotic flow and extend the elution range. This is seen in the t_o/t_m values in the table and the chromatograms in the next section. The extended elution range and reduction in k' improves the resoltuion of moderately hydrophobic compounds

3.3 Gradient Elution in MECC

The discussion and data presented in the previous section illustrates the importance of manipulating retention in MECC to optimize separation performance. As in conventional LC, solvent gradients offer promise in MECC for separations of complex mixtures. The addition of organic solvents during the course of a MECC separation causes partition coefficients and micellar phase volume to reduce, both of which reduce solute capacity factor. The organic solvent also tends to increase the MECC elution range. The implimentation and optimization of solvent gradients represents a significant advance in MECC methodolgy. The effects discussed above are demonstrated in Figure 2 for the separation of NBD-derivatized n-alkylamines using the gradient apparatus described in the experimental section and discussed in reference 11.

The isocratic chromatogram in Figure 2A clearly demonstrates the aforementioned problem of resolving moderately hydrophobic compounds. Compounds with large k' values tend to "bunch-up" with retention times near that of the micelle (t_m). The acetonitrile gradients that produced the chromatograms in Figure 2B & C clearly improved the separation by lowering the k' values for the late eluting components and extending the elution range. Acetonitrile was used to generate the gradient because it alters retention while causing only moderate changes in electroosmotic flow and t_o/t_m. It can be further seen from the figure that the shape and extent of the gradient influences the elution pattern for the text mixture. Current efforts in our laboratory involve using elution parameters (i.e., k' and electroosmotic and micellar flow velocities) obtained for isocratic runs of standard compounds, and computer simulations, to optimize gradients for the efficient separation of mixtures of the compounds.

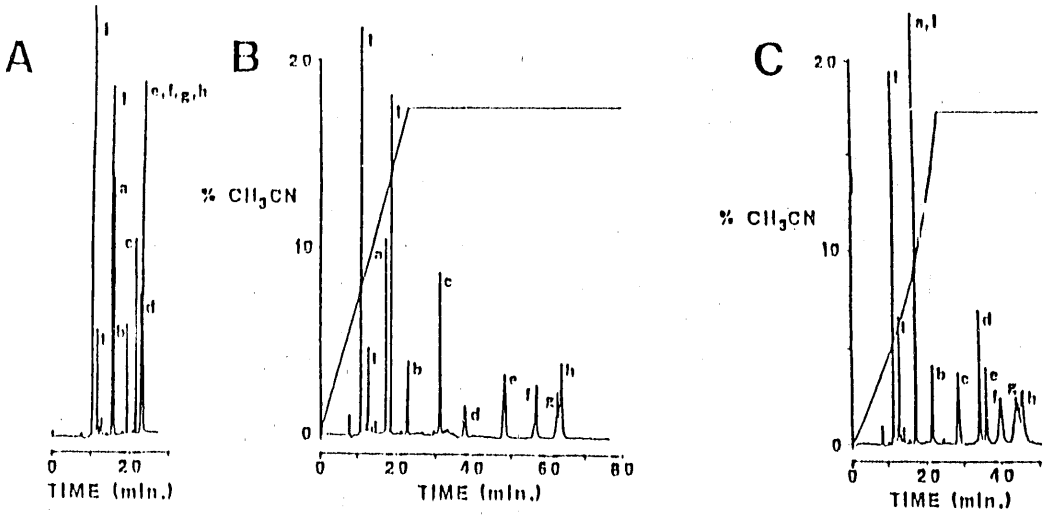

Figure 2

MECC chromatograms for separations of a mixture of (a) NBD-n-propylamine, (b) NBD-n-butylamine, (c) NBD-n-pentylamine, (d) NBD-n-hexylamine, (e) NBD-n-heptylamine, (f) NBD-n-octylamine, (g) NBD-n-decylamine, (h) NBD-n-dodecylamine, and (I) impurities using a mobile phase consisting of 0.01 M Na_2HPO_4, 0.006 M $Na_2B_4O_7$, 0.05 M SDS with (A) no solvent gradient, (B) a linear acetonitrile gradient, and (C) a concave acetonitrile gradient.

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