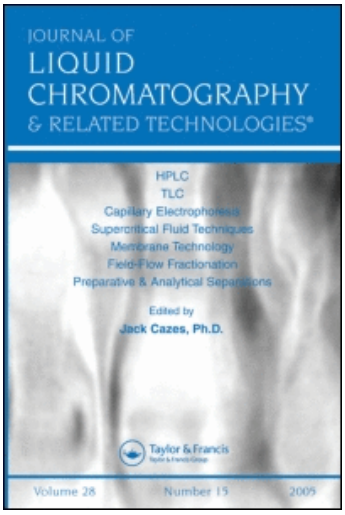

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Method Development and Selectivity Control for Small Molecule Pharmaceutical Separations by Capillary Electrophoresis

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METHOD DEVELOPMENT AND SELECTIVITY CONTROL FOR SMALL MOLECULE PHARMACEUTICAL SEPARATIONS BY CAPILLARY ELECTROPHORESIS

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ABSTRACT

This manuscript outlines an approach for capillary electrophoresis (CE) method development. It is applied to small molecule pharmaceutical separations using analgesics as examples. The effect of various parameters on selectivity are evaluated, and some recommendations are made regarding successful quantitation. Such parameters as linearity, sensitivity, and reproducibility are also examined. This knowledge is extended to other application areas, and examples of CE separations for such compounds as penicillins, water soluble vitamins, and enantiomeric compounds are presented. It is shown that the selectivity, sensitivity, and reproducibility of CE are adequate for routine use in the pharmaceutical laboratory.

INTRODUCTION

Capillary Electrophoresis (CE) is a new instrument-based separations technique first described by J.W. Jorgensen and K.D. Lukacs in the early 1980's (1-3). Since that time, hundreds of articles have appeared in the literature describing the theory, instrumentation, and to some extent the applicability of the technique (4,5, and references therein). However, much of what has been presented to date has not been of much practical use, or demonstrative of how CE can be used as an analytical tool.

Given the inherent nature of the increased efficiency it can be used as an orthogonal technique which complements other analytical methods and thereby has a great potential as an analytical tool for today's more complex pharmaceutical molecules and formulations.

There are many parameters that can be used to effect separations and manipulate selectivity in CE. These include the capillary dimension, buffer composition, ionic strength and pH, applied voltage, sample matrix, and any buffer additives such as organic solvents, surfactants, or ion-pairing reagents. For any method to be successful in terms of ruggedness, each of these parameters should be investigated and exploited to full advantage in a stepwise logical fashion. The method developed should not only provide the necessary selectivity and resolution, but quantitative reproducibility and sensitivity for all components in a reasonable analysis time.

In this work, the effects of buffer pH, ionic strength, applied voltage, and sodium dodecyl sulphate (SDS) concentration were systematically evaluated for their effects on migration time and selectivity in a separation of some common analgesic standards. The resulting conditions were then used to examine linearity, sensitivity, and reproducibility for these same types of compounds. This work was then extended to more complex samples to examine the applicability of CE to additional compound classes. These data prove the usefulness of CE as an analytical tool to provide practical information .

METHODS AND MATERIALS

Capillary Electrophoresis System

A Waters Quanta™ 4000 Capillary Electrophoresis System was used throughout (Waters Division of Millipore, Milford, MA, USA). Separations were performed on capillaries of various dimensions (Waters) and are documented in the appropriate figure or table caption. All analyses were performed with UV detection at 214 nanometers, and hydrostatic injections (10 cm height). Data was collected on an 845 Chromatography Data Workstation (Waters) at 10 points/second.

Chemicals And Reagents

Chemicals and standards were obtained from Aldrich Chemical Co. (Milwaukee, WI), and Sigma Chemical Co. (St. Louis, MO). All were obtained in the highest purity

available and used without further purification. Background electrolytes were prepared with Milli-Q water (Millipore, Bedford, MA), and HPLC grade methanol (Baker, Phillipsburgh, NJ). Phosphate buffers were prepared using monobasic sodium phosphate and adjusted to the desired pH with sodium hydroxide. Phosphate/borate buffers were prepared using monobasic sodium phosphate and adjusted to the desired pH with sodium tetraborate. Tris-phosphate buffers were prepared from Tris-HCl and adjusted to the desired pH with phosphoric acid. Samples identified in Figures as three letter codes are defined as folows: caffeine (CAF), acetominophen (AMP), acetylsalicylic acid (ACE), salicylic acid (SAL), and salicylamide (SAM).

RESULTS AND DISCUSSION

Analgesic Separation Optimization, Free Zone

Analgesic separations by CE have been reported in the literature (6), however since the compounds of interest were somewhat different, and a more thorough understanding of parameter interdependancy was desired, these conditions were used as a starting point for further method development. Figure 1 shows the effect of pH on the migration time for five common analgesic active ingredients. The best separation occurs at pH's in excess of 10.0, and due to the proximity of a phosphate pK, a pH of 11.0 was chosen for subsequent optimization. There are, however, two drawbacks with the separation obtained at this point in the optimization. First, the analysis time is in excess of twenty minutes, and second, and more importantly, caffeine, and any other neutral compounds or excipients that would be present in a typical sample, migrate with the electroosmotic flow. Accurate quantitation of caffeine is not possible under these conditions. Therefore further optimization was neccessary to reduce analysis time, and to selectivly migrate caffeine away from any other neutral species.

By choosing pH 11.0, and increasing the applied voltage, a decrease in analysis time was obtained (Fig. 2). All other conditions being identical, increasing the applied voltage resulted in a 50% decrease in analysis time. Increasing the voltage also has the added advantage of increasing efficiency, however at the sacrifice of additional joule heating in the capillary, and decreased resolution. In the case of this separation,

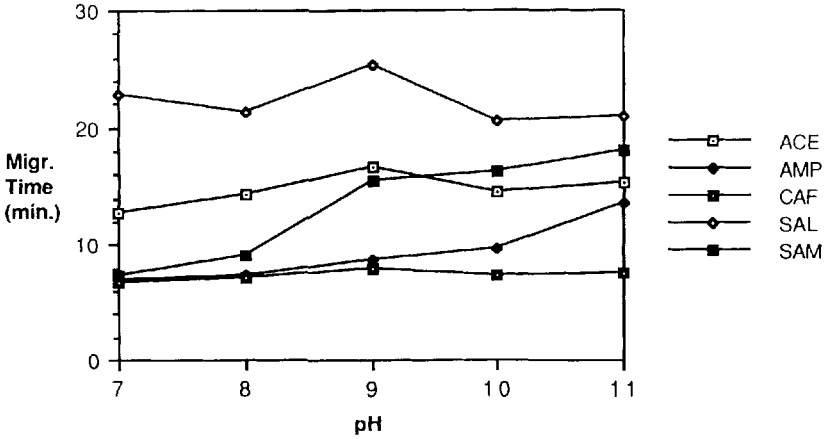


FIGURE 1: Effects of pH on migration time. A buffer of 0.05 M sodium phosphate at the specified pH and a 50 micron by 60 centimeter capillary operated at 15 KV was used. The samples were 0.1 mg/mL in water, with a 15 second injection time. Other conditions as outlined in the text (Methods and Materials).

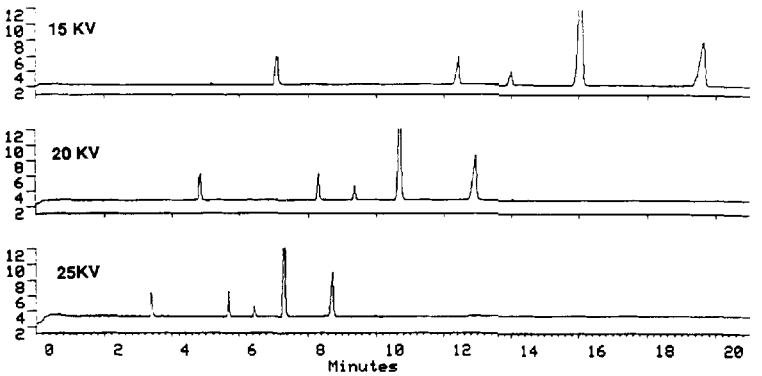


FIGURE 2: Effects of applied voltage on migration time. A pH of 11.0 was used, with voltage as specified; all other conditions are identical to those in Figure 1.

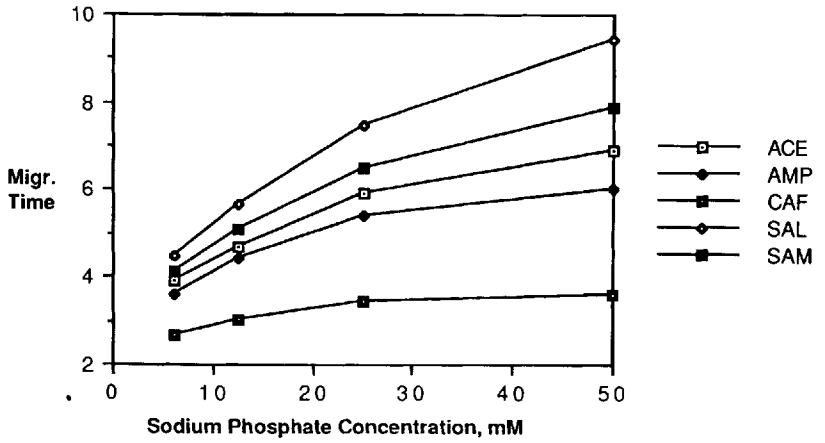


FIGURE 3: Effects of phosphate concentration on migration time. A pH of 11.0 and an applied voltage of 25 KV was used; all other conditions are identical to those presented in Figure 1.

resolution was not a limiting factor, and joule heating could be adequately controlled through heat dissapation and judicious selection of buffer ionic strength. As presented in Figure 3, decreasing the ionic strength at pH 11.0, eventually collapsed the resolution at lower ionic strengths, although run time was again shortened. For this work, an applied voltage of 25 KV was chosen so that the entire range of ionic strenght could be studied without experiencing problems with the joule heating phenomena. For the additional free zone data reported below, a phosphate concentration of 0.015 M and an applied voltage of up to 30 KV was used as these conditions presented the best compromise between resolution and analysis time.

Linearity and Reproducibility, Free Zone

In order to perform quantitatively, a technique needs to be both linear and reproducible. By providing linearity over a wide dynamic range, the need for multiple level calibration curves is eliminated. Using salicylamide as a representative sample, calibration curves were generated with serial dilutions from 1 mg/ml to 0.5 ug/mL that exhibited excellent linearity (Fig. 4).

Reproducibility was evaluated under similar conditions, and as reported in Table 1 are well within requirements for accurate quantitation.

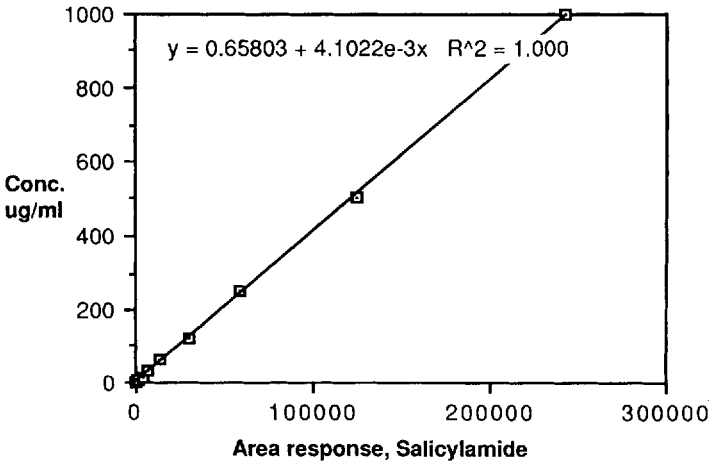


FIGURE 4: Linearity of response of CE detection. A buffer of 0.02 M sodium phosphate pH 11.0, and a 50 micron by 60 cm capillary operated at 20 KV was used. Sample is salicylamide, with an injection time of 10 seconds.

Table 1: CE Reproducibility

75 micron by 60 cm Capillary

	Migr. Time	Peak Area	Peak Height
Avg	4.568	142073	34601
SDV	0.035	2770	714
%RSD	0.773	1.95	2.06

50 micron by 60 cm Capillary

	Migr. Time	Peak Area	Peak Height
AVG	5.894	31616	8361
SDV	0.051	504	193
%RSD	0.865	1.59	2.31

Conditions: Sample was a 0.1 mg/mL solution of Salicylamide, N = 9. A 15 second Hydrostatic injection was used, and a buffer of 0.02 M sodium phosphate, pH = 11.0, with an applied voltage of 25 KV.

Analgesic Separation Optimization, MECC

Changes in applied voltage and ionic strength do not effect the selectivity, however, and to successfully separate and quantitate neutral species, it is necessary to investigate CE separation modes other than free zone. When sodium dodecylsulphate (SDS) is added to the buffer, a separation mode referred to as micellar electrokinetic capillary chromatography (MECC) dictates the separation mechanism (6). In MECC, nonionic species partition between the free solution and any micelles formed which are moving at different velocities in the capillary. Anionic species, on the other hand, are separated by the combination of electroosmotic flow and electrophoresis. The result is a separation of both ionic and nonionic species in the same run. In order to evaluate selective migration of nonionic compounds, a marker such as methanol or formamide can be used. These compounds can be used as electroosmotic flow markers because they do not partition into the micelles, migrating strictly according to electroosmotic flow. The effect of changing the SDS concentration on the migration time of the analgesics is shown in Figure 5. The addition of SDS retards migration and can also effect selectivity; salicylamide elutes fourth in free zone, but fifth in MECC as illustrated by the crossed lines in the graph. However, what is more significant, is that caffeine can now be separated from other neutral compounds as confirmed by separate injections of formamide used as an electrosmotic flow marker. An optimized separation that takes into account pH, applied voltage, ionic strength, and SDS concentration that can be used for quantitation within a realistic time frame can now be obtained (Fig. 6). At pH 11.0, with decreased ionic strength and SDS concentration higher voltages are possible and the analysis is complete in under four minutes. It is interesting to note that an HPLC analysis of these same components, while providing completely different selectivity, must be performed by two separate methods, while CE accomplishes the separation of all components in a single method/run with a much faster analysis time.

Sensitivity in MECC Mode

Detection limits for salicylamide were evaluated and found to be 16 ng/mL (signal to noise ratio of three), obtained under the same conditions as those outlined in Figure 6. One of the specifications often used in the pharmaceutical industry for impurity profiles is the ability to perform quantitatively at 0.1% levels. Figure 7 shows a separation of salicylamide where the scale has been expanded to show that impurities at the 0.1%

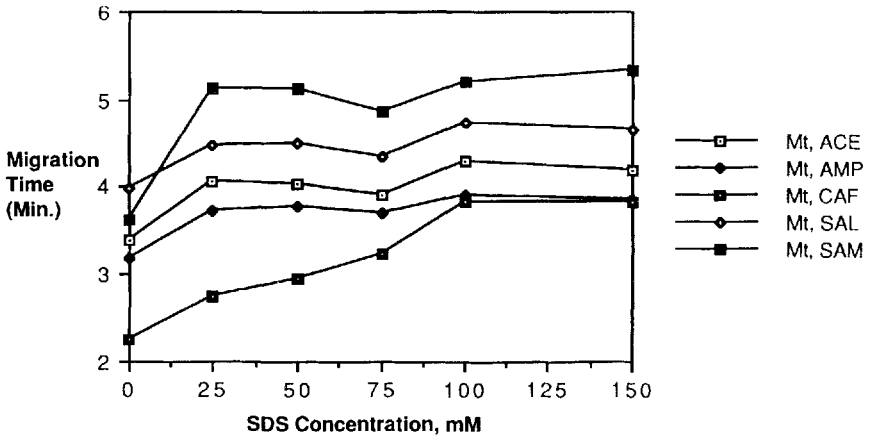


FIGURE 5: Effects of SDS concentration on migration time. A buffer of 0.02 M sodium phosphate, pH 11.0 (SDS concentration as specified), and a 50 micron by 60 cm capillary operated at 20 KV was used. The samples were 0.1 mg/mL in water, with a 15 second injection time. Other conditions as outlined in the text (Methods and Materials).

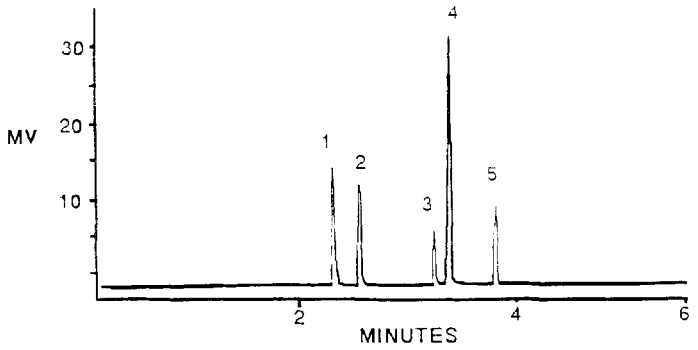


FIGURE 6: Optimized CE separation of the analgesics of interest. A buffer of 0.015 M sodium phosphate, pH 11.0, 0.025 M SDS, and a 50 micron by 60 cm capillary operated at 30 KVwas used. The samples were 0.2 mg/mL in water, with a 15 second injection time. Peak identifications are: 1) caffeine, 2) acetaminophen, 3) acetylsalicylic acid, 4) salicylamide, and 5) salicylic acid.

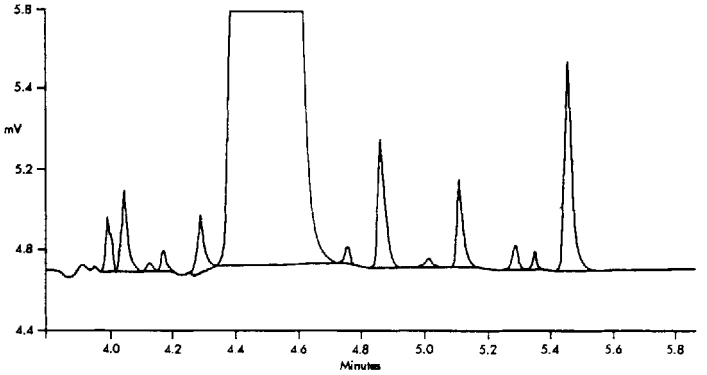


FIGURE 7: Salicylamide CE impurity profile. A buffer of 0.02 M sodium phosphate, pH 11.0, 0.075 M SDS and a 50 micron by 60 cm capillary operated at 20 KV was used. Sample is 0.1 mg/mL salicylamide in water, with an injection time of 10 seconds. Peaks are identified below as integrated from left to right.

Peak No.	Migr. Time	Area	Area %
1	3.99	377	0.13
2	4.04	515	0.18
3	4.12	29	0.01
4	4.16	140	0.05
5	4.23	390	0.14
6	4.52	283443	98.32
7	4.73	87	0.03
8	4.82	870	0.30
9	4.97	46	0.02
10	5.06	576	0.02
11	5.23	184	0.06
12	5.30	84	0.03
13	5.39	1532	0.53

level can be detected. Table 2 summarizes the result of a separate quantitation of a salicylamide sample that was spiked with caffeine at the 0.1% level that satisfies the goal of less than 10% RSD. To increase the sensitivity of the technique, it is possible, as in HPLC, to inject more sample, however while linearity is still preserved, efficiency suffers significantly (Fig. 8,9). This tradeoff between sensitivity and efficiency could play a significant role in separation of complex multi-ingredient formulations or in the case of any closely migrating pair of peaks.

Matrix Effects

Matrix effects can play a significant factor in CE separations due to the fact that the capillary to injection volume ratio is lower than the equivalent ratio in HPLC.

Table 2: Reproducibility of Trace Level Quantitation

	Area, Caffeine	Area, Salicylamide
	450	75969
	509	74628
	565	74673
	488	74274
	575	75113
	546	74441
AVG	522	74850
%RSD	9.3	0.8

Conditions: The buffer consisted of 0.015 M phosphate, pH 11.0, 0.025 M SDS. A 75 micron by 60 cm capillary operated at a voltage of 25 KV, with a hydrostatic injection time of 10 seconds was used.

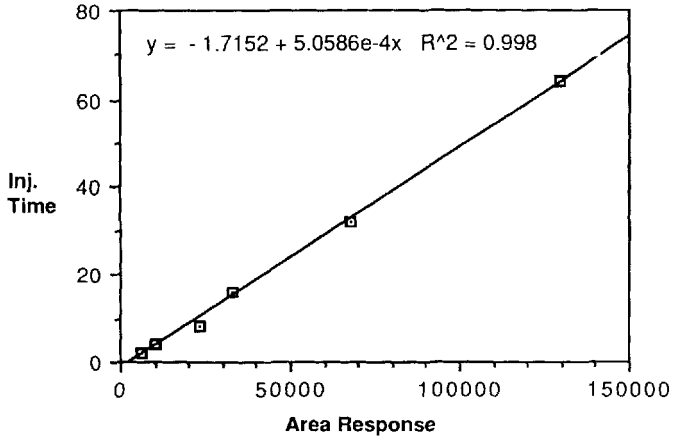


FIGURE 8: Linearity of injection time (volume) versus peak area. Conditions identical to those in Fig. 6. Injection time is in seconds.

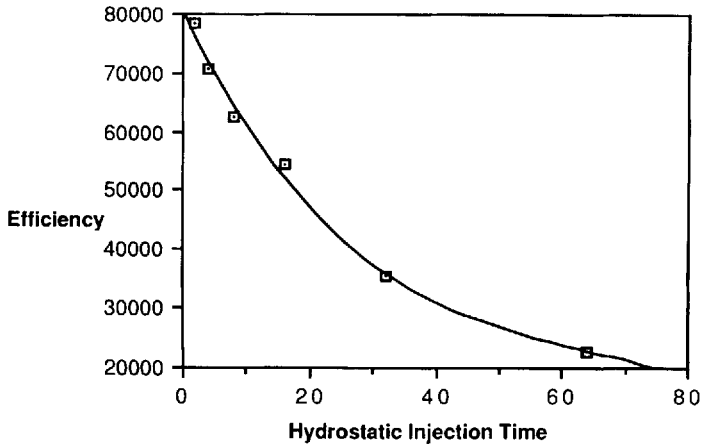


FIGURE 9: Effect of injection time (volume) on efficiency. Conditions identical to those in Figure 6. Injection time is in seconds.

Figure 10 illustrates the effect the sample matrix can have on the separation in the MECC mode. If the sample is prepared in 100% methanol (a typical extraction solvent), the localized micelle environment is disrupted, and the migration and peak shape of caffeine is drastically affected. This would create problems with the reproducibility of integration, resulting in less accurate quantitative results. When the buffer and sample matrix are more evenly matched, better peak shape and migration characteristics are obtained.

Additional Applications

Using the knowledge gained from the analgesic separation method development, and relying on recent literature reports for additional information (7,8), the separation of several additional samples were attempted to further extend the applicability of this technique. Successful separations of penicillin antibiotics (Fig. 11) and some water soluble vitamins (Fig. 12) were obtained. The separation of the penicillins is of particular interest because it is possible to screen synthesis precursers and intermediates and all possible final products in a single run. A combination phosphate/borate buffer was found to offer more buffer capacity for better reproducibility and peak shape for these particular compounds.

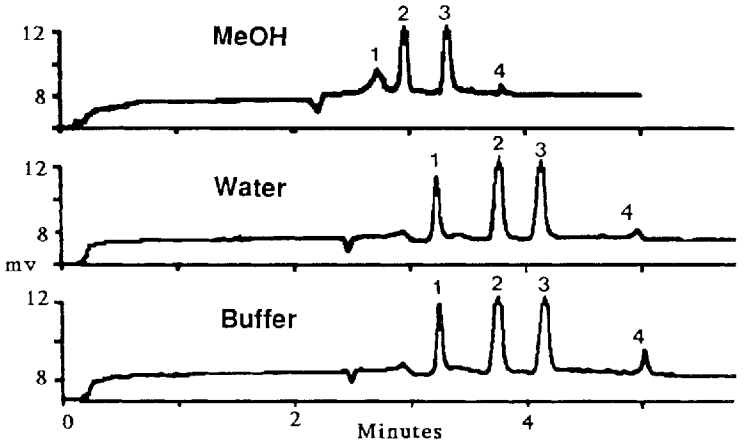


FIGURE 10: Effect of sample matrix effect on peak shape. A buffer of 0.015 M sodium phosphate 0.05 M SDS, and a 75 micron by 60 cm capillary operated at 18 KV was used. Sample was an acidic methanol extracted generic analgesic tablet (prepared by adding 10 mL/L 0.1 M HCl), with an injection time of 5 seconds. Peak identification is: 1) caffeine, 2) acetylsalicylic acid, 3) acetaminophen, and 4) salicylic acid (degradation product from peak 2).

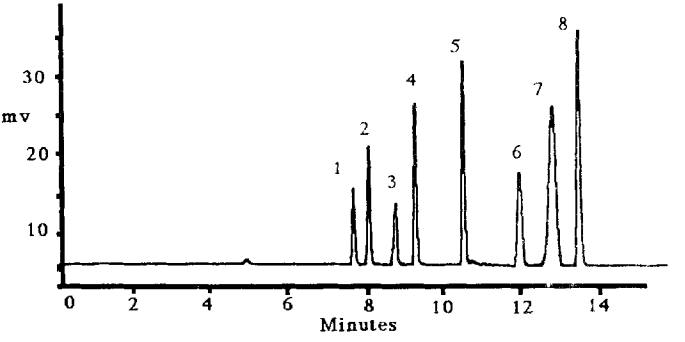


FIGURE 11: Separation of various penicillins by CE. A buffer of 0.02 M phosphate/borate pH 9.0, 0.05 M SDS, and a capillary 75 micron by 60 cm operated at 18 KV was used. The sample was 0.12 mg/mL each component in water, with a 5 second injection time. Peak identification is: 1) amoxicillin, 2) ampicillin, 3) 6-amino penicillanic acid, 4) oxacillin, 5) cloxicillin, 6) ticarcillin, 7) nafcillin, and 8) dicloxicillin.

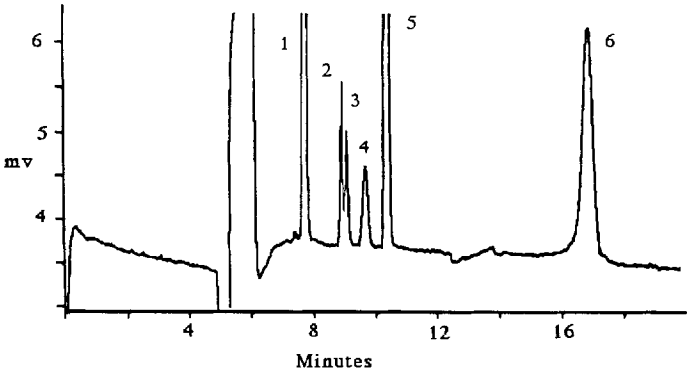


FIGURE 12: Separation of water soluble vitamins by CE. Conditions are identical to those in Figure 11. Sample is 0.1 mg/mL of each component in 50/50 water/methanol. Peak identification is: 1) vitamin B-6, 2) vitamin C, 3) pantothenic acid, 4) vitamin B-2, 5) niacin, and 6) vitamin B-1.

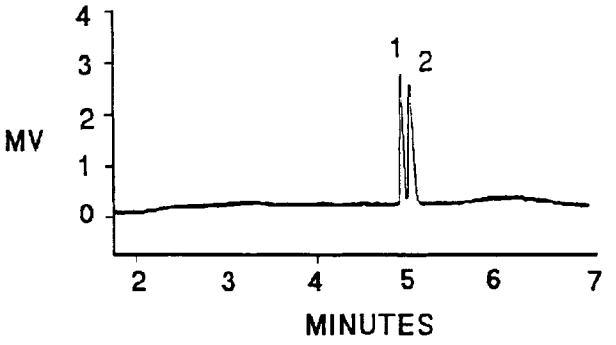
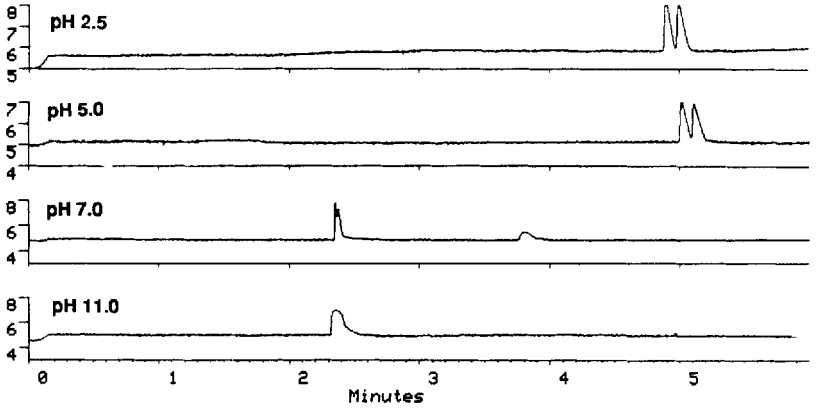


FIGURE 13: Separation of the enantiomers of ephedrine by CE. A buffer of 0.025 M Tris-phosphoric acid , pH 2.5, 0.015 M heptakis(di-O-methyl)-B-cyclodextrin/methanol, 80/20, ans a capillary 50 micron by 35 cm operated at 18 KV was used. Sample was 0.1 mg/ML in water/methanol 50/50, with a 10 second injection time. Peaks 1 and 2 are (-) and (+) ephedrine respectively.



F IGURE 14: Effects of pH on the resolution of ephedrine enantiomers. Conditions are identical to those presented in Figure 13, with the exception that the tris buffer system was used at pH 2.5 and 5.0, while the phosphate buffer system was used at pH 7.0 and 11.0.

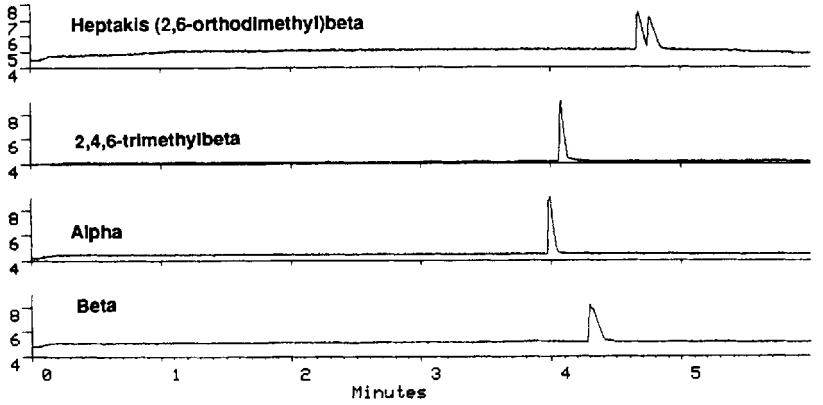


FIGURE 15: Effects of the cyclodextrin composition on the resolution of ephedrine enantiomers. Conditions are identical to those in Figure 13, with the appropriate cyclodextrin as noted in each electropherogram above.

Enantiomeric Separations

Another area of potential CE applicability is in the area of enantiomeric separations. Due to the inherent high efficiency of this technique, direct separations of enantiomers with buffer additives are possible by a number of different mechanisms (9). Figure 13 illustrates one such separation of (+/-)-ephedrine using a derivatized beta-cyclodextrin as a buffer additive to accomplish the chiral discrimination (separation conditions adapted from reference 7). The separation is performed under conditions of no electroosmotic flow, hence the migration of the compound of interest is governed strictly by electrophoresis of the charged analyte-cyclodextrin complex. It can be shown that pH (Fig. 14), and cyclodextrin type (Fig. 15) , play an important role in the separation. The enantiomers of other chiral compounds including norephedrine, psuedoephedrine, and phenylpropanolamine have been successfully separated under these conditions. The enantiomers of some additional chiral compounds, for example propranolol, are not separated under these conditions and work continues in this area.

CONCLUSION

CE has been shown to be a viable tool for small molecule pharmaceutical separations. It possesses the required sensitivity, reproducibility, and selectivity to accomplish separations of widely diverse compound classes. While in most cases, CE provides complimentary information, it may be possible to dramatically decrease analysis times, and manipulate selectivity in ways that may provide information unobtainable by other techniques. It is possible, in an analogous fashion to HPLC, to systematically evaluate all of the parameters that can affect a separation in CE, and to arrive at a set of conditions that would provide rugged analyses for applications of this type.

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