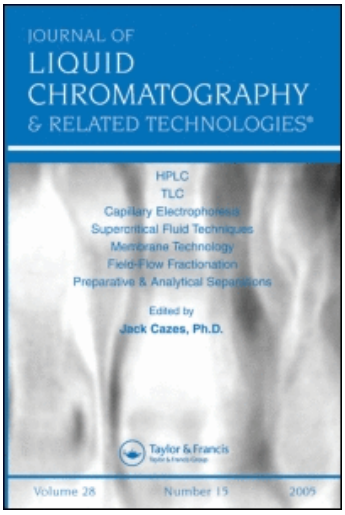

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Use of Native and Chemically Modified Cyclodextrins for the Capillary Electrophoretic Separation of Enantiomers

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USE OF NATIVE AND CHEMICALLY MODIFIED CYCLODEXTRINS FOR THE CAPILLARY ELECTROPHORETIC SEPARATION OF ENANTIOMERS

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Abstract

Capillary zone electrophoresis is shown to be useful for the separation of select enantiomers via the use of mobile phases containing cyclodextrins (CDs). Both native and chemically modified CDs are utilized herein. Parameters important in achieving enantiomeric separations are CD type, concentration, and mobile phase pH. In addition, rapid (1 minute) enantiomeric separations are demonstrated. Experimental parameters important in attaining short analysis times are discussed.

Introduction

Capillary zone electrophoresis (CZE) is a highly efficient separation technique capable of separating solutes based on differences in electrophoretic mobility. CZE is usually performed in narrow-bore open capillaries (typically 25 -100 μm i. d.) filled with aqueous buffer solutions. Solutes are transported

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through the capillary toward the detector via electroosmosis (1) and electrophoretic migration (2). High efficiency in CZE is attained due to efficient heat dissipation of the capillary and the plug-like profile characteristic of electroosmotic flow. Hence, plate numbers are generally limited by longitudinal diffusion (2) and often exceed 500,000 per meter (2, 3).

Resolution in CZE is given by the expression

$$R_s = \frac{\sqrt{N}}{4}\left[\frac{\mu_1-\mu_2}{\mu_{avg}-\mu_{osm}}\right] \qquad (1)$$

where N is the number of theoretical plates, μ_1 and μ_2 the electrophoretic mobilities of the solutes of interest, μ_{ave} the mean of μ_1 and μ_2, and μ_{osm} the electroosmotic flow mobility. It has been shown that maximum resolution is attained when μ_{osm} is very close to $-\mu_{ave}$, albeit at the expense of analysis time (2). This is often achieved by reduction of mobile phase pH; $Si\text{-}O^-$ groups at the capillary walls are neutralized resulting in a lower zeta potential at the capillary surface. Modification of the capillary walls using silanation reagents can have a similar effect on zeta potential (2). In these situations, μ_{osm} is very small and the tendency of bulk electroosmotic flow to "swamp out" electrophoretic mobility differences is minimal. Nevertheless, for solutes with very small differences in mobility, $(\mu_1-\mu_2)$, this manipulation of μ_{osm} may not provide adequate resolution even when high efficiency is observed.

An elegant feature of CZE lies in the ability to alter electrophoretic performance (e.g., improve selectivity) by addition of reagents to the mobile phase. Specific interactions of solutes with reagents in the mobile phase often lead to separations not possible with CZE alone. Approaches to date include the addition of surfactants (4), organic solvents (5), and chelating agents (6), to name a few. Ideally, all chemical or physical interactions of injected solutes with system components occur exclusively with species in solution, so novel

reagents can be investigated without complications resulting from stationary phase bonding chemistries. In addition, the low volume of mobile phase required in CZE facilitates the evaluation of rare and expensive substances for both applied and fundamental studies.

CZE has been routinely applied to the separation of charged solutes such as amino acids (7), proteins (8) , peptides (9), and metal complexes (6, 10). A particularly challenging area of separation science for which the unique characteristics of CZE merit consideration is that of enantiomeric separations. Enantiomers, since they possess nearly identical physical properties, do not usually exhibit appreciable differences in electrophoretic mobility and are not separated in a conventional CZE experiment. Nevertheless, CZE is particularly well-suited to the task due to inherent high efficiency and the possibility of enantio-selective interactions with mobile phase components. To date, CZE has been employed in the chiral separation of amino acids (11), metal complexes (10), and pharmaceuticals (12, 13). These separations were all accomplished due to a preferred formation of a complex between a mobile phase reagent and one of the enantiomers. The CZE work described herein deals with enantio-selective complexation afforded with cyclodextrins (CDs).

Cyclodextrins (CDs) are formed by the enzymatic digestion of starch by cyclodextrin transglycosylase. Consisting of cyclic glucopyranose units, CDs are shaped much like a teacup. The monomeric units are arranged in such a way as to place the hydroxyl groups on the exterior of the structure, thus creating a polar exterior and a hydrophobic cavity. The most commonly occuring CDs consist of 6, 7, and 8 glucopyranose units (14). Designated as α–, β–,and γ–CD, respectively, these substances are able to form "inclusion complexes" with many molecules. Inclusion complex formation is determined by solute molecule hydrophobicity and, more interestingly, size. Solute

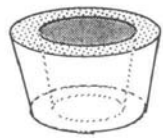
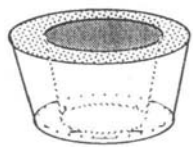
molecules having dimensions larger than the CD cavity are excluded from the cavity while molecules of the proper dimensions may enter and form complexes. Inclusion complex formation has proven useful in separations of closely related molecules such as optical and positional ring isomers (15). Figure 1 shows schematic representations and cavity dimensions for these CDs. In general terms, the CD cavity for the α form will accommodate a single substituted aromatic ring, the β a naphthalene ring, and the γ form can accommodate molecules as large the four-fused-benzene-ring polyaromatic hydrocarbon, pyrene. These guidelines, provide an estimation of the steric limitations of the various CD cavities .

The use of cyclodextrins and cyclodextrin derivatives in CZE is a logical extension of existing separation technology. Recent reports of CD use in CZE have presented successful separations of drug enantiomers (12, 13). In this work, we investigate the use of native CD's as stereospecific complexation reagents in CZE for the separation of binaphthyl and fluorescently derivatized amino acid enantiomers. In addition, certain CD derivatives that have not been previously used in connection with CZE are investigated. The importance of parameters such as CD concentration, type, and mobile phase pH in optimizing resolution are investigated. In addition, factors leading to rapid separation are considered and applied to the determination of enantiomers in a commercial dietary formulation.

Experimental

Apparatus

Capillary columns of 25 and 50 μm internal diameter were obtained from Polymicro Technologies (Phoenix, AZ), lengths specified where relevant. All experiments were performed under constant applied voltage, provided by a

Cyclodextrin	#Glucopyranose units in structure	Cavity Size, nm
 α-CD	6	0.57
 β-CD	7	0.78
 γ-CD	8	0.95

1. Cyclodextrin characteristics

regulated high voltage supply (Hipotronics, Inc.). Detection was achieved via uv absorbance with a Linear Model 204 (Reno, NV) spectrophotometer operated at 210 nm or by laser-excited fluorescence. The latter mode of detection employed the 325 nm output of a HeCd laser for excitation. Fluorescence emission was collected at 90°.and isolated with a bandpass filter centered at 580 nm (FWHM 20 nm). Further details concerning this detector can be found in reference 6.

Reagents

Native cyclodextrins, dansyl chloride (DNS-Cl), and DNS-phenylalanine were obtained from Sigma Chemical Company (St. Louis, MO). Modified cyclodextrins were obtained from Pharmatec (Alachua, FL). 1,1' binaphthyl diyl hydrogen phosphate ($BNPO_4$) was purchased from Aldrich Chemical Company and 1,1' binaphthyl 2,2' dicarboxylic acid ($BN(COOH)_2$) was supplied by Dr. S. Toda, Ehime University, Japan. Dietary supplement capsules of DL-phenyalanine were obtained locally. Buffer salts and other conventional chemicals were obtained from Baxter Chemical Co. All solutions were made in HPLC grade water, also obtained from Baxter.

Procedures

Prior to use, columns were rinsed for approximately 10 minutes with 0.01 M NaOH. The capillaries were then filled with the mobile phase of choice and allowed to equilibrate by applying the running voltage for approximately ten minutes. Injections were made by hydrostatically syphoning at an elevation of 10 cm for 10-12 seconds. All separations were performed using an applied voltage, V,of 15-20 kV, with resulting currents ranging from 20-40 μA.

Resolution was calculated via the relation

$$R_s = \frac{2\Delta t_R}{W_1 + W_2} \qquad (2)$$

where Δt_R is the difference in retention time between the solutes in question and w_1 and w_2 the widths of each peak.

Electrophoretic mobilities of anions were calculated via the relationship

$$\mu_{obs} = \frac{LI}{Vt_o} - \frac{LI}{Vt_r} \qquad (3)$$

where μ_{obs} is the experimentally observed mobility, L the total capillary length, l,

the length to the detector, and t_o the column dead time, estimated by a solvent baseline disturbance.

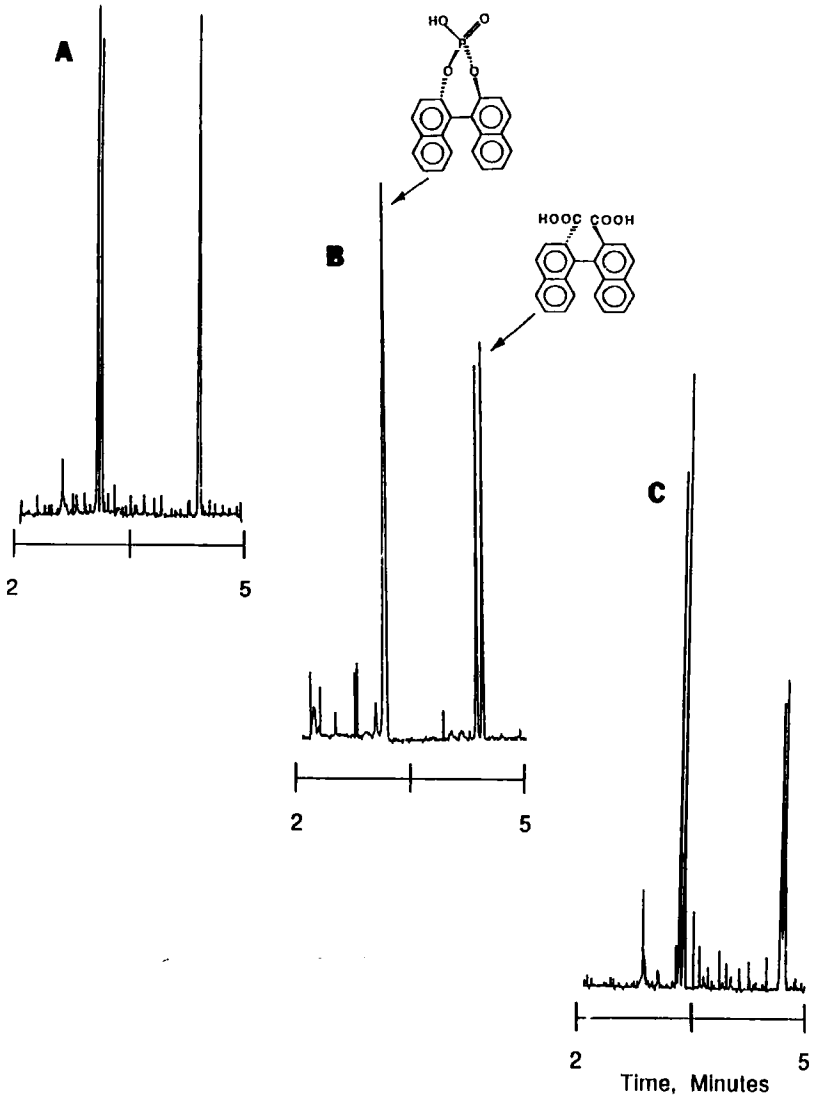
Derivatization of the DL-phenylalanine dose form was performed by first dissolving 1.0 mg of the compound in 5.0 mL 0.01 M disodium phosphate/0.006 M disodium borate buffer, pH 8.5. A 100 μL aliquot of this solution was added to 100 μL of 1 mg/mL dansyl chloride in acetone and diluted to 1 mL with acetone. This mixture was allowed to react for 5 hours at room temperature in the dark. The mixture was diluted to 10.0 ml with 0.01 M disodium phosphate -0.006 M disodium borate buffer (pH 9) and allowed to stand for approximately 1 hour prior to injection.

Results and Discussion

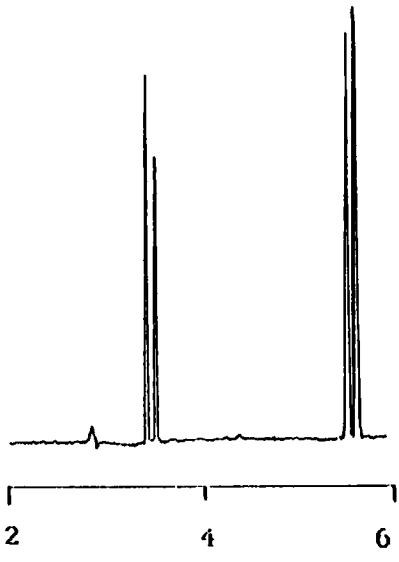
Enantiomeric separations can be accomplished with charged compounds in CZE when solutes differentially complex with a mobile phase additive. In this case, differential CD inclusion complex formation is employed to alter the velocity of one enantiomer relative to the other. Mobile phase pH, and thus the magnitude of electroosmotic flow, determines the instrumental configuration of the experiment. At low pH (*ca.* pH 2), electroosmotic flow is small, and charged solutes generally migrate toward the electrode of opposite sign. In this situation, the enantiomer with the highest affinity for the CD migrates more slowly and elutes last (12). Detection is performed at the appropriate end of the capillary based on solute charge. At high pH (*ca.* pH 9), electroosmotic flow is strong and negatively charged solutes attempt ,generally unsuccessfully, to "swim upstream" in opposition to electroosmotic flow. Detection is performed at the cathode end of the capillary. In this case, the anionic enantiomer forming the strongest CD complex has a lower effective electrophoretic mobility and elutes from the capillary first. Separations reported herein all fall into the latter category.

Effect of Cyclodextrin type on Enantiomeric Separation

Enantiomeric separations with CDs often hinge on favorable interactions between solutes and the CD cavity. Strong interaction with the CD does not necessarily result in chiral separation. In some cases, solutes form strong CD complexes without chiral discrimination. The work reported herein illustrates the importance of the type of CD employed. Figure 2 shows the separation of two sets of charged binaphthyl enantiomers by CZE in the presence of different types of CDs. In the case of the $BNPO_4$ derivative, both enantiomers were available in pure form, leading to the determination that the S enantiomer eluted prior to the R form. While the binaphthyl skeleton is identical between the two derivatives, differences in CD complex formation are evident. Interaction of the binaphthyl compounds with β-CD in the mobile phase allows separation of the $BNPO_4$ enantiomers, but not the diacid ($BN(COOH)_2$) enantiomers . Conversely, addition of the smaller α-CD to the mobile phase has the opposite effect (Fig. 2B). This is contrary to the general observation that β-CD is more successful in enantiomeric separations of molecules possessing a naphthyl group (16). This apparent contradiction may be related to differences ($BNPO_4$ *vs.* $BN(COOH)_2$) in free rotation about the 1,1' bond joining the naphthyl rings and differences in the angle between them (17). The $BNPO_4$ enantiomers, due to the bridging phosphate group, are more rigid and thus less able to rotate than the diacid forms. This may result in the $BNPO_4$ being less able to access the smaller α-CD cavity. The $BN(COOH)_2$ enantiomers, on the other hand, are able to change conformation more freely in such a way as to interact with the α-CD cavity. However, interactions of $BN(COOH)_2$ with β-CD may not result in a sufficiently "snug fit" for enantiomeric separation. The results presented in Figure 2 A and B indicate the potential utility of using mobile phases containing mixed CDs when separating mixtures of enantiomeric compounds. Figure 2C shows the partial separation of both sets



2. Separation of charged binaphthyl enantiomers. Capillary: 50 μm i.d. x 50 cm long (40 cm to detector). Buffer: 0.01 M disodium phosphate, 0.006 M disodium borate (pH 9) with [A] 0.01 M β-CD, [B] 0.01 M α-CD, and [C] 0.01 M of each CD. The applied voltage was 20 kV and absorbance detection at 210 nm employed



3. Separation of the binaphthyl enantiomers from Fig. 2 using a buffer containing 0.01 M G1α-CD. Other conditions as in Fig. 2

of enantiomers employing a mobile phase containing equal concentrations of α– and β-CD. Resolution is slightly poorer for each set of enantiomers in this case, most likely due to unfavorable interactions between solute and the CD possessing the improper cavity size. This approach shows potential for the use of mixed CD mobile phases in CZE. The fact that solutes are excluded from interaction with the CD cavity on the basis of size allows more than one type of selectivity mechanism to be operative, since interferences between mechanisms may not be detrimental in some cases.

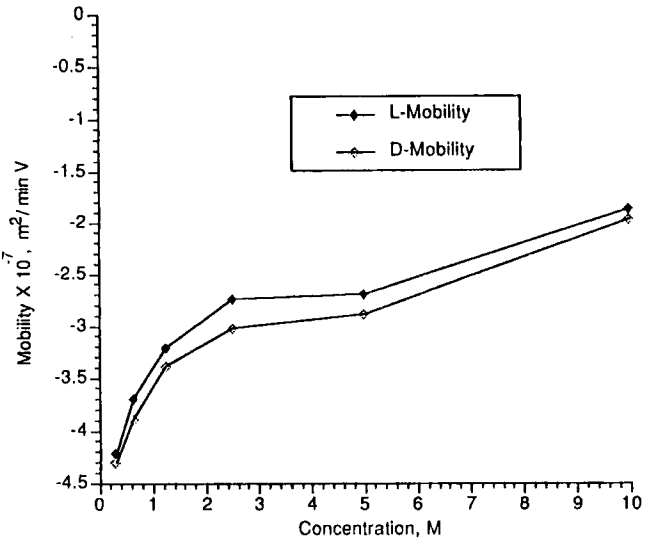
Chemical modification of the CD exterior can affect the enantio-selectivity of guest-host complex formation. Figure 3 shows the separation of both charged binaphthyl compound enantiomers from the previous figure using a

glycosylated α-CD derivative (G_1α-CD). In this case, interaction between the phosphoralated binaphthyl compound and G_1α-CD allowed enantiomeric recognition to occur. Chemical modification of the CD has been shown to "stretch" the cavity mouth and can change the steric qualities of the host/guest interaction (18). The cavity expansion afforded by the substitution of the CD primary hydroxyls may explain the separation of the $BNPO_4$ enantiomers demonstrated in Figure 3. These separations add validity to statements concerning the importance of the structure of the CD cavity mouth in chiral complexation (16, 18). Other examples of partially successful enantiomeric separations with G_1α-CD in our laboratory include DNS-phenyalanine and DNS-tryptophan, but only at high (0.1 M) concentrations of G_1α-CD. Underivatized α–and β-CD were unsuccessful in achieving chiral separation of these amino acids.

An alternative form of β-CD, hydroxypropyl β-CD(HPβ-CD), was also examined for utility in chiral separations of the binaphthyl compounds. Similar results were obtained for this derivative as for G_1α-CD. In addition, the HPβ–CD afforded baseline separation of the enantiomers of DNS-phenlyalanine and DNS-tryptophan. While the cavity of the HPβ-CD is most likely larger than the G_1α-CD, specific interactions at the mouth of the CD cavity can also be important in the enantiomeric recognition of binaphthyl and DNS-amino acid compounds.

Effect of Cyclodextrin Concentration

Enantiomeric resolution depends on the concentration of the CD in the mobile phase. Figure 4 is a best fit plot of the experimentally determined mobilities of DNS-phenylalanine enantiomers as a function of concentration of HPβ-CD in the mobile phase. At low (0.0003 M) concentrations, mobilities are similar and enantiomeric resolution is low. As the concentration is increased, the L- form increasingly finds enantiomerically favorable interaction with the



4. Plot of DNS-phenylalanine observed mobility as a function of HPβ-CD concentration. Other conditions as in Fig. 2 except that a 25 μm i.d. capillary, 25 kV, and laser excited fluorescence detection employed

neutral CDs, thereby decreasing its negative mobility. Mobility differences, and thus resolution, is optimum at about 0.0025 M HPβ-CD. Further increases in the concentration of HP-β-CD result in more similar mobilities and resolution is degraded. The guest-host interaction is driven by the tendency of solutes to associate with the hydrophobic interior of the CD cavity. Under some conditions, as in the case with DNS-phenylalanine enantiomers, chiral separation can occur. However, when CD concentration is high, enantioselective interactions seem to be "swamped out" by non-specific hydrophobic association.

Effect of Mobile Phase pH

The effects of pH on the separation of DNS-phenylalanine enantiomers are summarized in Table 1. The table is arranged to illustrate the pH

TABLE 1

The Effect of pH on the Separation of DNS-phenylalanine Enantiomers

pH	μ_{osm} $cm^2/(s\ kV)$	μ_{ave} $cm^2/(s\ kV)$	$\Delta\mu$ $cm^2/(s\ kV)$	$\mu_{osm}+\mu_{av}$ $cm^2/(s\ kV)$	N $*10^5$	R_s
11	0.690	-0.131	0.013	0.558	1.7	0.874
7	0.671	-0.108	0.012	0.563	1.4	0.832
5	0.527	-0.119	0.011	0.408	1.5	1.05
3	0.392	-0.184	0.003	0.210	0.27	0.732

dependency of R_s, as well as the efficiency and mobility terms that appear in the theoretical expression for resolution (Eq 1). Mobilities where calculated by using Equation 3. The appearance of an injection solvent disturbance was used to estimate t_o. Enantio-selective interactions with the 0.0025 M HP-β-CD that was incorporated in the mobile phase, as reflected in the $\Delta\mu$ values in the table, was essential for resolving these enantiomers.

The pH of the mobile phase influences the ionic state at the capillary wall (see Introduction Section), thereby altering both electroosmotic flow and solute-capillary wall interactions. As pH is reduced from 7 to 3, the value of μ_{osm} (and hence the denominator of the parenthetical expression in Eq. 1) is significantly decreased and improvements in resolution are expected. Unfortunately, a loss in efficiency (tailing of the peaks was observed at pH 3) mitigates this effect. The ionic state of the amphoteric DNS-phenylalanine is also influenced by pH. Thus, changes in pH would be expected to alter the intrinsic mobility of the solute, as well as its observed mobility (represented in the table by μ_{ave}), which can differ from the intrinsic value due to interaction with neutral cyclodextrin. In fact, the assumption that the cyclodextrin has no electrophoretic mobility over the entire pH range may not be valid, further

complicating matters. Despite the complex interplay of factors that influence resolution, it is apparent from this study that at pH 5, (near the isoelectric point, 5.5, of the native amino acid) the value of μ_{osm} is resonably small, efficiency is high, and selective interactions with the HP-β-CD are evident, producing baseline resolution of the enantiomers (i.e. $R_S > 1$).

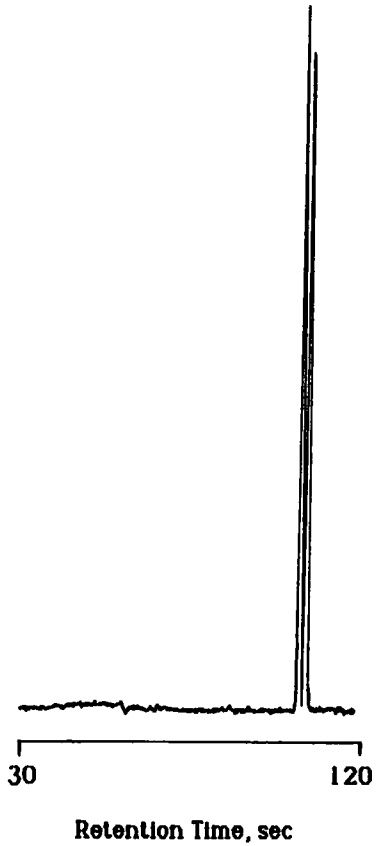
Optimization of CZE Analysis Time

Rapid separations (analysis times on the order of 1-2 minutes) are readily attained with CZE. Parameters such as applied voltage and column dimensions are critical in determining the speed and quality of separation (19). High flow rates are required to quickly transport solutes through the capillary to the detector. In CZE, increases in applied voltage are concomitant with increases in electroosmotic flow velocity.

Critical to the success of rapid separations is high efficiency. Unfortunately, a balance between speed of analysis and efficiency is generally required, since conditions required for speed of analysis often degrade separation performance. Efficiency is degraded at high fields due to Joule heating, and the temperature gradients which result (20). Thus, speed of analysis is ultimately limited by problems associated with capillary heat dissipation.

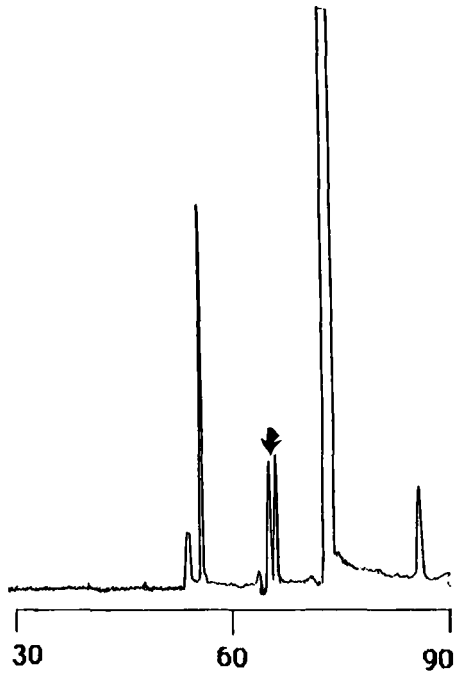
Rapid separations in CZE are favored in capillaries of short length and narrow internal diameter. A 25 μm i.d. capillary was used to generate the data for DNS-phenylalanine presented in Figure 4. An example of a rapid electropherogram is presented in Figure 5 . In this case L and l were 50 cm and 40 cm respectively and the applied field was 25 kV.

While 25 μm i. d. capillaries allow high efficiency to be maintained under high (>500 V/cm) field conditions, sensitive detection can be problematic. The bulk of CZE applications to date have employed 50 μm i. d. columns. This diameter allows reasonably sensitive on-column absorbance detection and



5. Rapid separation of DNS-phenylalanine enantiomers. Conditions as in Fig. 4

efficiency adequate for most applications (21). Larger diameter capillaries, since they result in the passage of more current, reach their "thermal limit" at lower applied fields than for 25 μm or 10 μm i. d. capillaries. Poor efficiency and, in the extreme case, mobile phase boiling, can result at moderate (ca. 500 V/cm) applied fields with larger bore capillaries. Rapid separations in 50 μm i. d. capillaries can only be achieved with short lengths which, of course, exhibit



6. Separation of DNS-phenylalanine enantiomers from components in a commercial formulation. Conditions as in Fig. 5 except that 35 kV applied

low plate counts. Electrical breakdown (arcing) and physical constraints imposed by most detectors (many absorbance detectors require at least 20-30 cm of capillary for operation) must be considered when short capillaries are employed.

A potentially important application of rapid CD-modified CZE separations is that of pharmaceutical analysis. Optical purity assessment is important in situations where one enantiomer elicits undesirable physiological response or has toxic effects. CD-CZE has, in some instances, the ability to provide a rapid

means of analyzing routine samples. Figure 6 shows the separation of a nutritional formulation of DL-phenylalanine after derivitization with dansyl chloride. The total analysis time is roughly 90 seconds and the phenylalanine enantiomers (denoted by arrow) show baseline resolution. The application of high-speed CE to optical purity determination shows significant potential in the pharmaceutical area, since many formulations represent relatively simple matrices. In addition, optical purity determination of amino acids are of interest in geologic dating (22) and in and in the synthesis of synthetic antibiotics (23). CE thus offers greatly increased throughput in this area since separation times can be reduced to 1-2 minutes in many cases. Further applications of this technology to new enantiomeric materials are inevitable.

Acknowledgement

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