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Capillary electrophoresis of glutamate and aspartate in rat brain dialysate
Improvements in detection and analysis time using cyclodextrins

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Abstract

Addition of cyclodextrins (CDs) to the electrolyte buffer in the capillary zone electrophoresis (CZE) separation of derivatized amino acids was evaluated in terms of fluorescence signal enhancement, resolution, and migration time effects. Maximum fluorescence signal enhancement was observed with separation buffers containing 4 m*M* β-cyclodextrin or 10 m*M* hydroxypropyl β-cyclodextrin. Resolution values decreased as the CD concentrations increased. Migration times were dependent on CD concentration. Inclusion complex formation constants calculated using changes in migration time showed slight agreement with those calculated by the steady-state fluorescence enhancement technique. Analysis of 20 μl of rat brain microdialysate by CZE using 4 m*M* β-cyclodextrin in borate buffer resulted in baseline resolution of glutamate and aspartate in 3.6 min. The results of this work indicate that, when used as separation buffer additives, cyclodextrins are capable of increasing the fluorescence signal and decreasing the migration times of NDA-derivatized acidic amino acids. © 1997 Elsevier Science B.V.

Keywords: Glutamate; Aspartate

1. Introduction

An important characteristic of cyclodextrins (CDs) is their ability to form inclusion complexes with a wide variety of guest molecules. Two factors which impact on complex formation are the size and hydrophobicity of the internal cavity. The size of the internal cavity available for complexation is determined by the number of D-(+)-glucopyranose units bonded together with α-1,4-glycosidic bonds. The three most common CDs are α-, β-, and γ-, which contain six, seven, and eight glucose units, respectively. The inclusion complexation between the host CD and guest molecules has been employed for drug-delivery, controlled-release processes, and fluorescence signal enhancement [1–4]. The complexation of fluorescent molecules with cyclodextrins results in a higher fluorescence quantum yield and resultant signal enhancement [5,6].

Glutamate and aspartate are the neurotransmitters utilized by the major excitatory neuropathways in the brain. Monitoring changes in the extracellular brain levels of these amino acids is useful in determining their function in various neurological processes (i.e.

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epilepsy and learning). Derivatization of endogenous amino acids to generate fluorescent compounds is a common technique used for quantitative determinations of these compounds. Enhancement factors ranging from 1.08 to 2.91 have been reported when γ-CD was added to the mobile phase in the microcolumn liquid chromatography determination of dansyl-amino acids [7]. The use of CDs in capillary electrophoresis has focused on enantiomeric separations [8–10]. To our knowledge, fluorescence signal enhancement by cyclodextrins in capillary electrophoresis separations has not been investigated.

The naphthalene-2,3-dicarboxaldehyde/cyanide (NDA–CN) derivatization of amino acids (to form the 1-cyano-2-substituted benz[*f*]isoindole (CBI) derivatives) is widely used in conjunction with column chromatographic analysis [11–15]. Laser-induced fluorescence (LIF) has been the detection method of choice for the determination of derivatized amino acids following separation by CE due to its inherent improved fluorescence intensities. Nickerson and Jorgenson used laser-induced fluorescence detection with capillary electrophoresis for amino acid determinations following NDA–CN derivatization [16]. Robert et al. reported the use of NDA–CN derivatization to analyze brain extracellular fluid from the medial frontal cortex of the rat for noradrenaline (NA) and dopamine (DA) using LIF–CE [17]. By employing cyclodextrins in the separation buffer of CE systems with UV-visible light sources, an improvement in the fluorescence signal from NDA-derivatized amino acids may be possible. In this paper we have investigated the effect of various cyclodextrins on the fluorescence signal, resolution, and migration time of the acidic amino acids, glutamate and aspartate, separated by CZE and detected using a conventional polychromatic light source for excitation.

2. Experimental

2.1. Chemicals

2.1.1. Electrophoresis buffers

Borate buffer (0.02 *M*) was prepared by dissolving sodium borate crystals ($Na_2B_4O_7$, Fisher Scientific, Fair Lawn, NJ, USA) in deionized water. The pH was adjusted to 9.0 with 6.0 *M* HCl. Borate buffer with the addition of CD (American Maize, Hammond, IN, USA) was prepared by diluting appropriate amounts of α-, β-, hydroxypropyl (HP)-β- and γ-CD in 0.02 *M* borate buffer.

2.1.2. Derivatizing reagents

A 5 m*M* NDA solution was prepared by dissolving NDA (Aldrich, Milwaukee, WI, USA) in HPLC-grade acetonitrile (Fisher). A 9 m*M* potassium cyanide (KCN) solution was prepared by dissolving KCN (J.T. Baker, Phillipsburg, NJ, USA) in borate buffer.

2.1.3. Amino acid samples

Stock solutions (1000 μ*M*) of glutamic acid, aspartic acid, and butylamine (Sigma, St. Louis, MO, USA) were prepared in deionized water. These stock solutions were then diluted to provide the concentrations required for individual experiments. Solutions and buffers were filtered with a 0.2-μm Nylon membrane filter and degassed with helium before use. All samples were filtered through a 1-mm syringe-type disposable polypropylene filter (0.45 μm) prior to analysis.

2.1.4. Derivatization procedure

Amino acid samples were prepared as follows: 5.7 μl of an 88 μ*M* glutamate, aspartate, and butylamine mixture, 33.0 μl of 9.0 m*M* KCN in 5 m*M* borate, and 50.0 μl of 5.0 m*M* NDA were mixed in a 500-μl polyethylene centrifuge tube and sonicated for 10 min. The structures of the derivatized compounds are shown in Fig. 1.

2.2. Equipment

All of the experiments were carried out on a Dionex Capillary Electrophoresis System (CES, Dionex Corp., Sunnyvale, CA, USA). Conventional fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA) columns were used for all experiments. The dimensions of the capillary columns were as follows: total length 65 cm, 62 cm from the injection end to the detection window, 75 μm I.D. and 375 μm O.D. Before each set of experiments, a NaOH caustic rinse (0.5 *M* NaOH) was performed. The volume of the capillary was

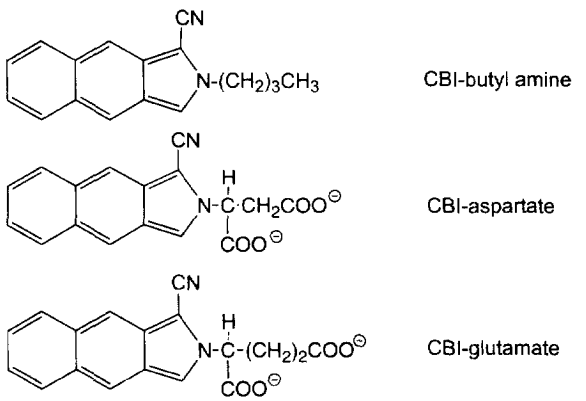

Fig. 1. Structures of the derivatized primary amines.

calculated to be 2.65 μl from source end to destination end. All experiments were carried out under positive polarity at 25 kV. The separations were performed at ambient temperatures.

Samples were introduced into the capillary column by gravity injection (100 mm elevation above the destination vial for 50 s). On-column fluorescence detection was carried out using an excitation wavelength of 420 nm and a 455-nm high-pass optical emission filter. Signals were processed by an Advanced Computer Interface (ACI) (Dionex Corporation) and analyzed using the Dionex AI-450 Chromatography Software Package. The sampling rate used for all CE experiments was 5 Hz.

2.3. Fluorescence signal enhancement by cyclodextrins

In order to keep all procedures of each experiment constant, the trials were done as follows: a sample containing the derivatized amino acids was injected three or four consecutive times using 0.02 *M* borate buffer as the separation buffer. The capillary was then rinsed twice with 0.02 *M* borate containing the individual CDs. Immediately following the rinse, the same samples were injected and analyzed three or four consecutive times using the CD-modified borate buffer as the separation buffer. The typical separation current was 90–130 μA with borate as the separation buffer and 80–120 μA with CD-modified borate. The relative fluorescence signal enhancement was calculated by dividing the average peak height of the CBI-amino acids in the CD-modified buffer by the average peak height of the derivatized amino acids in borate buffer.

2.4. Steady-state fluorescence experiments

The steady-state fluorescence intensity of standard CBI-Glu and CBI-Asp solutions was measured in the presence and absence of α-, β-, hydroxypropyl-β-, and γ-cyclodextrins. A 400-μl volume of borate buffer, 45 μl of either 500 μ*M* glutamate or aspartate solution, 14 μl of NaCN and 12 μl of NDA solutions were pipetted into a 1.5-ml microcentrifuge tube and mixed by inversion. The reaction mixture was then allowed to stand for 30 min. Different CD stock solutions were added to disposable glass test tubes and diluted with borate buffer to the appropriate concentration. A 400-μl volume of the derivatized amino acid solution was added and the solutions mixed by inversion. Fluorescence intensities were recorded from a PE LS-5 (Perkin-Elmer Corp, Norwalk, CT, USA) fluorimeter using an excitation wavelength of 420 nm and emission wavelength of 486 nm.

3. Results and discussion

3.1. Effects of the addition of CD to electrolyte buffer

3.1.1. Fluorescence signal enhancement

Table 1 lists the relative fluorescence enhancement factor (RFEF) that resulted from the addition of cyclodextrins to the CE separation buffer. When α-CD or γ-CD were added to the separation buffer essentially no enhancement of the fluorescence signal was observed (RFEF=0.97–1.20). The α-CD results are in agreement with those of Takeuchi and Miwa who observed no enhancement of fluorescence signal for dansylamino acids when 1 m*M* α-CD was added to the mobile phase in a microcolumn liquid chromatography method [18].

Adding β-CD to the separation buffer resulted in a significant enhancement of the fluorescence signal for the derivatized amino acids. A maximum signal enhancement was observed for separation buffer containing 4 m*M* β-CD (CBI-Glu RFEF=1.73; CBI-

Table 1
Relative fluorescent enhancement by cyclodextrins

Cyclodextrin	Concentration (m*M*)	CBI-Glu	CBI-Asp
α	5	1.04±0.09	1.06±0.07
	10	1.04±0.09	1.17±0.12
	25	1.16±0.13	1.05±0.09
	50	1.07±0.10	1.11±0.12
	100	1.05±0.12	1.16±0.10
β	2	1.55±0.12	1.1±0.10
	4	1.73±0.13	1.72±0.15
	8	1.17±0.11	1.21±0.12
	16	1.28±0.14	1.21±0.10
HP-β	5	1.36±0.08	1.41±0.21
	10	1.69±0.27	1.53±0.15
	25	1.14±0.12	1.12±0.08
γ	1	1.16±0.13	1.12±0.13
	10	0.97±0.08	1.11±0.07
	30	1.00±0.15	1.06±0.16

See Section 2 for details.

Asp RFEF 1.72). Takeuchi and Miwa observed an increase in the fluorescence signal when using β-CD in place of α-CD as a liquid chromatography mobile-phase additive for the detection of dansyl-amino acids [18]. The fluorescence enhancement present in CZE separations employing β-CD but not α-CD suggests that the size of the internal cavity of the α-CD cyclodextrin is too small for inclusion complexation with the CBI-amino acids.

Hydroxypropyl-β-CD (HP-β-CD) was investigated because of its enhanced solubility over that of β-CD. A maximum relative fluorescence signal enhancement of 1.69 was observed with separation buffers containing 10 m*M* HP-β-CD. Increasing the concentration of HP-β-CD to 25 m*M* resulted in the complete loss of enhancement of the fluorescent signal for both CBI-Glu and CBI-Asp. For the determination of derivatized amino acids and naturally fluorescent organic and pharmaceutical compounds, Frankewich et al. reported that the use of 0.20 *M* HP-β-CD produced larger fluorescence enhancement factors compared to 0.014 *M* β-CD [6]. These authors hypothesized that the fluorescence enhancements were due to the fact that a larger fraction of analyte molecules formed inclusion complexes at the higher HP-β-CD concentrations. The present fluorescence enhancement results indicate that HP-β-CD and β-CD exhibit nearly identical complexation tendencies with the CBI-derivatized amino acids.

3.1.2. Migration time

Experiments were conducted to determine the effect of different CD concentrations on the overall migration time of the CBI amino acids. *n*-Butyl amine was chosen as a marker to measure the effect of CD addition on the electroosmotic flow (EOF). Changes in CBI-butyl amine (CBI-BA) migration time were interpreted as reflecting changes in electroosmotic flow.

As seen in Fig. 2A, the migration time for all three compounds increased as a function of α-CD concentration. The increase in migration time seen for the two charged amino acid derivatives paralleled that seen for CBI-BA. This indicates that a decrease in EOF is responsible for the observed increase in migration time and that inclusion complexation is not occurring between the derivatized amino acids and α-CD. This latter conclusion is supported by the lack of fluorescence signal enhancement in α-CD-containing separation buffers (Table 1).

Fig. 2B shows the change in migration time of CBI-Glu, CBI-Asp and CBI-BA as a function of β-CD concentration. The largest relative decrease in migration time was seen for the separation buffer containing 4 m*M* β-CD. A decrease in migration time of 1.3 min (28%) for CBI-Glu and 1.5 min (29%) for CBI-Asp occurred when using this buffer. There was a slight increase in migration time of CBI-BA (indicating a decrease in EOF) over the range of concentrations examined. The decrease in migration time seen for CBI-Glu and CBI-Asp can be attributed to a decrease in electrophoretic mobility caused by inclusion complexation with β-CD. The cyclodextrins migrate at a speed equal to the electroosmotic flow, while the derivatized amino acids' mobility includes an opposing electrophoretic component. Complexation between the amino acids and the cyclodextrins would result in a decrease in the apparent electrophoretic mobility of the amino acids and an overall decrease in migration time. The interpretation of complex formation between the derivatized amino acids and the β-CD is supported

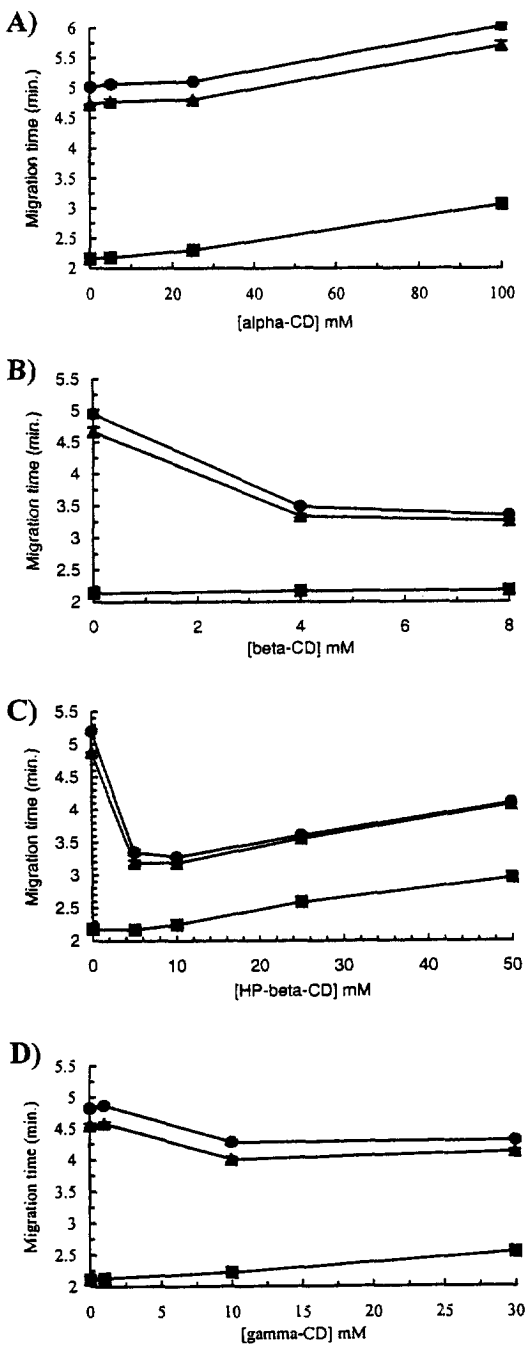

Fig. 2. The effect of (A) α-cyclodextrin, (B) β-cyclodextrin, (C) hydroxypropyl-β-cyclodextrin and (D) γ-cyclodextrin added to the separation buffer on the migration time of CBI-BA (■), CBI-Glu (▲), and CBI-Asp (●). The data represent the average of two experimental trials with triplicate injections. See text for experimental details

by the fluorescence signal enhancement data seen in Table 1.

The effect of HP-β-CD on the migration time of the derivatized amino acids is shown in Fig. 2C. In separation buffers containing 5 m*M* HP-β-CD, a decrease in migration time of 1.7 min (35%) was observed for CBI-Glu and 1.9 min (36%) for CBI-Asp. Increasing the concentration to 10 m*M* produced essentially no further reduction in migration time. CBI-BA migration time increased steadily (0.8 min, 36%) over the range of HP-β-CD concentrations studied. This can be attributed to a decrease in EOF as a result of changes in the viscosity of the CD-containing buffer. This data coupled with the fluorescence enhancement data (Table 1), indicates significant complexation between the derivatized amino acids and HP-β-CD.

Fig. 2D shows the change in migration time of CBI-Glu, CBI-Asp and CBI-BA as a function of γ-CD concentration. A slight increase in migration time was observed in the 1 m*M* γ-CD separation buffer for all three solutions suggesting a decrease in EOF and virtually no complexation. This interpretation is supported by the lack of fluorescence signal enhancement by 1 m*M* γ-CD (Table 1). CBI-Glu migration time decreased 0.5 min (12%) and CBI-Asp migration time decreased by 0.5 min (11%) in separation buffer containing 10 m*M* γ-CD. CBI-BA migration time increased 0.1 min (5%) from 0 to 10 m*M* γ-CD. The decrease in migration time for the amino acids is interpreted as resulting from complexation with γ-CD, however this is not supported by the data in Table 1. It is suggested that the large size of the γ-CD allows for complexation, yet does not prevent the derivatized amino acids from undergoing non-radiative vibrational relaxation.

The data in the present study indicate that the formation of inclusion complexes between the cyclodextrins and the derivatized amino acids can affect the overall migration time of the amino acids. Neutral, uncharged CDs will migrate at a velocity equivalent to the electroosmotic flow. CBI-Glu and CBI-Asp have an electrophoretic mobility (EM) opposite that of the EOF. When inclusion complexation occurs, the EM component is decreased and the dominant component in the migration of the analytes becomes the EOF. The difference in migration time observed for an analyte is strongly depen-

dent on the degree of complexation of the analyte with the CD. Ueda et al. demonstrated this reduction in migration time with the use of β-CD in the determination of 14 NDA-CN-labeled amino acids by LIF–CE [12]. They attempted separations by micellar electrokinetic chromatography (MEKC) and CD-modified MEKC. With the addition of 10 m*M* β-CD by MEKC (50 m*M* SDS), a decrease in the migration time of all CBI-amino acids was observed (as opposed to MEKC alone), along with different selectivities that allowed the separation of all 14 CBI-amino acids. Yik et al. reported that the larger the decrease in migration time observed for polycyclic aromatic hydrocarbons (PAHs), the greater the extent of inclusion complexation [9]. They found that when determining these neutral and non-ionizable PAHs, the migration time was decreased the most with 3.0 m*M* γ-CD when compared to 5.0 m*M* α-CD and 5.00 m*M* β-CD. In the present study, the greatest change in migration time occurred with the 5 m*M* HP-β-CD. A very similar decrease was observed with the 4 m*M* β-CD, while only slight decreases were observed for γ-CD. The discrepancies are most likely due to the different nature of the analytes used in the two studies.

Taken together, the fluorescence enhancement data and the observed changes in migration time provide useful information regarding the relative complexation between the CBI-acidic amino acids and the various CDs investigated. The lack of fluorescence signal enhancement and the increase in migration time observed for separations conducted using separation buffers containing α-CD indicate little or no inclusion complexation between the derivatized amino acids and the cyclodextrins. Alternatively, in separation buffers of β-CD and HP-β-CD, the concentration that produced the maximum fluorescence signal enhancement also produced the largest decreases in migration time. These results tend to suggest maximal complexation at this concentration. The migration time decrease observed with γ-CD suggests complexation is occurring, yet the lack of fluorescence signal enhancement suggests little or no complexation. It may be the case that the hydrophobic internal cavity of the γ-CD is so large that the CBI-amino acids are not bound as tightly as in the β-CD and HP-β-CD complexes and, as a result, non-radiative vibrational relaxation is occurring.

3.1.3. Resolution

The resolution between the derivatized amino acids was only slightly affected by the addition of the various cyclodextrins to the separation buffer. Table 2 lists the calculated resolution factor (R_s) as a function of different CD concentrations. The R_s value for α-CD increased only slightly from 3.1 in borate buffer alone to 3.6 with 100 m*M* α-CD. β-CD addition caused a significant change in resolution at concentrations greater than 2 m*M*. With the addition of 4, 8 and 16 m*M* β-CD, the R_s value decreased from 4.1 (0 m*M* β-CD) to 3.1 (24%), 1.8 (56%) and 1.2 (71%), respectively. With the addition of 10 and 25 m*M* HP-β-CD, the R_s value decreased from 3.2 (borate only) to 1.9 (41%) and 1.4 (56%), respectively. γ-CD produced a slight loss in resolution with 30 m*M* γ-CD, decreasing the R_s value by 27%. Baseline resolution ($R_s > 1.5$) was maintained for all cyclodextrin concentrations investigated except 25 and 50 m*M* HP-β-CD and 16 m*M* β-CD.

CBI-Asp is smaller than CBI-Glu by a CH_2 group, and under the current experimental conditions has a slightly larger electrophoretic mobility. Since both amino acids are negatively charged at pH 9, their

Table 2
Resolution of CE separation of CBI-Glu and CBI-Asp using cyclodextrins as separation buffer additives

CD	Concentration (m*M*)	*R*
α	0	3.1
	5	3.2
	25	3.3
	100	3.6
β	0	4.1
	2	4.1
	4	3.1
	8	1.8
	16	1.2
HP-β	0	3.2
	5	3.2
	10	1.9
	25	1.4
	50	0.5
γ	0	3.3
	1	3.4
	10	3.1
	30	2.4

Resolution values were calculated as $MT_{Asp} - MT_{Glu} / 0.5(W_{Asp} + W_{Glu})$, where W=peak width measured at the baseline.

electrophoretic mobilities are in opposition to the electroosmotic flow and the difference in their mobilities is responsible for the observed resolution. When inclusion complexes are formed with the cyclodextrin molecules in the separation buffer, the difference in the electrophoretic mobilities is decreased, resulting in decreased resolution.

3.2. Comparison of association constants, K_a, calculated by steady-state fluorescence and CE migration times

Since inclusion complexation appeared to play a large role in the overall separation performance of the present study, it was of interest to determine the association constants (K_a) for this behavior. Changes in migration time from the capillary electrophoresis data were used to calculate K_a for the cyclodextrin/CBI-amino acid complex. Since this complexation is taking place in a flowing stream, comparisons to K_a values calculated by steady-state fluorescence enhancement experiments were of interest. For the steady-state experiments, fluorescence intensities of individual derivatized amino acids, in the absence and presence of varying concentrations of the individual CDs, were measured and a double reciprocal plot of fluorescence intensity vs. concentration was generated. From the regression data, K_a can be estimated by dividing the intercept by the slope [19]. Association constants were also calculated based on changes in migration time as described by Heegaard and Robey [20]. Table 3 lists the estimated K_a values as calculated by each method. In general, the two methods produced similar association constants. From the migration time calculations, the α-cyclodextrin K_a values were the smallest and consistent with the previous interpretation of little or no complexation. In general the magnitude of the calculated K_a values were consistent with the extent of interaction indicated by the fluorescence enhancement and migration time data. The differences in the calculated K_a values based on the two methods suggest that the dynamics of electroosmotic flow and electrophoretic mobility can influence the inclusion formation process. This fact may impact on similar studies utilizing changes in CZE migration times in calculations of cation binding sites on proteins and other biomolecules [20–23]. Experiments are currently underway to investigate this phenomenon further.

3.3. Determination of excitatory amino acids in rat brain dialysate

Twenty microliters of dialysate collected from the striatum of an awake rat were analyzed by CZE following NDA/CN derivatization and the resultant electropherograms are shown in Fig. 3. The total volume of the sample following derivatization was 125 μl and a total of six injections were made from this sample using different separation buffers. The injection parameters were the same as the fluorescence enhancement experiments. Separations carried out in 0.02 *M* borate buffer (pH 9.0) of dialysate spiked with 25 μ*M* Glu and Asp (Fig. 3I) and basal dialysate (Fig. 3II) show baseline resolution with an analysis time of approximately 5 min. Incorporation of 4 m*M* β-CD (Fig. 3III), or 10 m*M* HP-β-CD (Fig.

Table 3
Association constants for cyclodextrin–CBI-amino acid complex

Cyclodextrin	Amino acid	Steady-state fluorescence	CE migration time
α	CBI-Glu	1.75×10^{-1}	7.26×10^{-2}
	CBI-Asp	5.75×10^{-2}	8.33×10^{-2}
β	CBI-Glu	4.79×10^{-1}	6.10×10^{-1}
	CBI-Asp	6.07×10^{-1}	9.19×10^{-1}
HP-β	CBI-Glu	4.13×10^{-1}	2.19×10^{-1}
	CBI-Asp	1.46	5.89×10^{-1}
γ	CBI-Glu	9.77×10^{-1}	3.68×10^{-2}
	CBI-Asp	9.61×10^{-1}	5.89×10^{-1}

Association constants were calculated based on the method described in Ref. [19] (steady-state) and Ref. [20] (migration time).

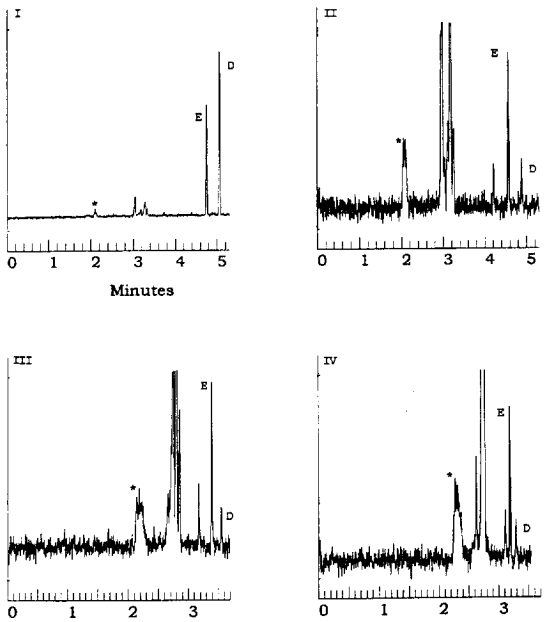

Fig. 3. Capillary zone electrophoresis analysis of an individual dialysate sample (20 μl) using different separation buffers: (I) original sample spiked with a mixture of 25 μ*M* CBI-Glu and CBI-Asp and run in 20 m*M* borate buffer alone; unspiked sample in (II) 20 m*M* borate buffer; (III) 4 m*M* β-cyclodextrin; and (IV) 10 m*M* hydroxypropyl-β-cyclodextrin. The peaks are identified as follows: *, CBI-BA; E, CBI-Glu; D, CBI-Asp. Units on the *X*-axis are time (min). Units on the *Y*-axis are relative fluorescence units (RFU). See text for experimental details.

3IV) resulted in a decrease in analysis time to approx. 3.7 and 3.4 min, respectively. Both CBI-Glu and CBI-Asp are well resolved in each separation. β-CD addition produced a fluorescence signal enhancement of 1.1 and 1.2 for CBI-Glu and CBI-Asp, respectively. No fluorescence enhancement was observed for separations using 5 m*M* HP-β-CD. The relatively minor enhancement of the fluorescent signal may be due to the derivatization of other primary amines in the dialysate sample. Optimization of the derivatization procedure for dialysate samples specifically may improve this result.

4. Conclusion

This paper describes studies carried out to investigate the use of cyclodextrins as fluorescence signal-enhancing agents for the determination of NDA/CN-derivatized amino acids following separation by capillary zone electrophoresis. Addition of CDs to the separation buffer influenced the magnitude of the fluorescence signal, the overall migration time, and the resolution of the separation of derivatized glutamate and aspartate. The changes observed can be explained based on inclusion complex formation between the amino acids and the cyclodextrins. Cyclodextrins can be incorporated into the separation buffer for the CZE determination of glutamate and aspartate in striatal extracellular fluid of rats to both increase the fluorescence signal of the NDA/CN-derivatized acidic amino acids and decrease the overall analysis time while maintaining baseline resolution. The inherent small injection volumes and short analysis time make this method ideal for behavioral and pharmacological studies utilizing microdialysis and CZE to study important central nervous system amino acids and other primary amines.

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