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Journal of Chromatography A 817 (1998) 105–111

JOURNAL OF CHROMATOGRAPHY A

Systematic approach to methods development for the capillary electrophoretic analysis of a minor enantiomer using a single-isomer sulfated cyclodextrin
A case study of L-carbidopa analysis

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Abstract

A methods development protocol — based on the charged resolving agent migration model — for the capillary electrophoretic analysis of a minor enantiomer using a single-isomer sulfated cyclodextrin is described here. The minor component of L-carbidopa was successfully assayed by capillary electrophoresis using the single-isomer heptakis-(2,3-diacetyl-6-sulfato)-β-cyclodextrin as chiral resolving agent. Adequate separation selectivity was secured by selecting a pH 2.5 background electrolyte, operating at 16°C and adjusting the concentration of the single-isomer sulfated cyclodextrin to just past the point where the migration direction of both enantiomers of carbidopa changes from cationic to anionic due to complexation with the single-isomer sulfated cyclodextrin. Once the background electrolyte composition which leads to adequate selectivity was identified, peak resolution was adjusted by optimizing the magnitude of the dimensionless normalized electroosmotic flow-rate via the addition of poly(ethylene glycol)900 which does not change separation selectivity. Rugged separation conditions permitting the fast, reproducible quantitation of the minor enantiomer in L-carbidopa preparations could be readily established.

Keywords: Method development; Enantiomer separation; Background electrolyte composition; Cyclodextrins; Heptakis(2,3-diacetyl-6-sulfato)-β-cyclodextrin; Carbidopa

1. Introduction

Since enantiomers of a drug substance often have different pharmacological properties, there is a strong interest in minor enantiomer analysis in the biomedical sciences and the pharmaceutical industries [1]. Due to its high resolving power, capillary electrophoresis (CE), has been used for the separation of a variety of compounds, including enantiomers [2,3]. Currently, cyclodextrins (CDs) are the most commonly used chiral resolving agents in CE [4,5]. Although neutral CDs have been used successfully to separate a number of ionic chiral drugs, they cannot be used to separate neutral enantiomers [6]. Therefore, charged CDs, both strong and weak electrolytes, have been introduced to analyze both neutral and ionic enantiomers [4–10].

Although good enantiomer separations were reported with the commercially available, randomly substituted charged CDs [4–10], the possibility of batch-to-batch variation in their composition is a severe liability when the materials are to be used for validated, critical assays where composition-related separation selectivity changes cannot be tolerated

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0021-9673/98/$19.00
PII: S0021-9673(98)00385-9

[11]. In order to eliminate this problem, single-isomer sulfated cyclodextrins were recently synthesized [12–14]. These materials have a sulfate group on the 6-carbon atom of each of the glucopyranose subunits of the CDs (on the nonchiral face of the CDs) and offer the same functional groups (intermolecular interactions) on the 2- and 3-carbon atoms of the glucopyranose subunits (on the chiral face of the CDs) as their neutral CD counterparts.

The objective of this paper is to show that the charged resolving agent migration model (CHARM model) of CE enantiomer separations [15] offers a straightforward, rational approach that one can use with the single-isomer sulfated CDs to develop rugged CE methods for the quantitation of a minor enantiomer. The compound selected for this work is L-carbidopa which contains less than 5% D-carbidopa as impurity in the final product. The resolving agent selected is the single-isomer heptakis-(2,3-diacetyl-6-sulfato)-β-cyclodextrin, HDAS-β-CD [12]. L-Carbidopa (structure shown in Fig. 1) is used to treat Parkinsonism. Since carbidopa is oxidized at high pH, the CE separations have to be carried out in low-pH background electrolytes (BGEs), where carbidopa is fully protonated.

When the general mobility and selectivity equations of the CHARM model (Eqs. 23 and 24 in Ref. [15]) are applied for the separation of a cationic enantiomer pair (such as carbidopa at low pH) with a single-isomer seven-sulfated cyclodextrin as resolving agent (such as HDAS-β-CD), two effective mobility curves and a separation selectivity curve similar to those shown in Figs. 2 and 3 can be calculated. The calculations were made with binding constants and ionic mobilities that are similar to those we observed with single isomer sulfated CDs [16], and they assume only a conservative, 10%

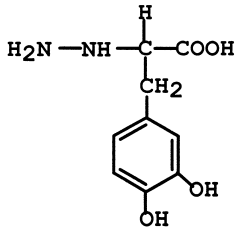

Fig. 1. Structure of carbidopa.

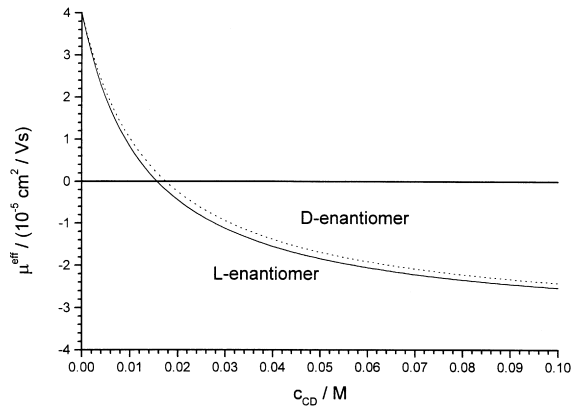

Fig. 2. Effective mobility curves calculated with Eq. 23 of [15] for a cationic enantiomer pair as a function of the single-isomer sulfated cyclodextrin concentration of the BGE. Constants used for the calculation: $\mu_D^0=\mu_L^0=4\cdot10^{-5}$ cm^2 V^{-1} s^{-1}, $\mu_{DCD}^0=-3.36\cdot10^{-5}$ cm^2 V^{-1} s^{-1}, $\mu_{LCD}^0=-3.4\cdot10^{-5}$ cm^2 V^{-1} s^{-1}, $K_{DCD}=68$, $K_{LCD}=75$.

difference between the binding constants of the enantiomers. As the concentration of the charged CD is increased, the initially cationic effective mobilities of the carbidopa enantiomers, μ_L^{eff} and μ_D^{eff} (where subscripts L and D refer to the two enantiomers of carbidopa), begin to decrease, then become zero, then become anionic (Fig. 2). Simultaneously, the

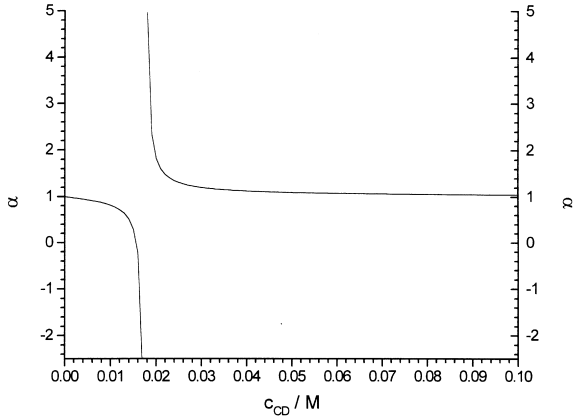

Fig. 3. Separation selectivity curve calculated with Eq. 24 of [15] for the cationic enantiomer pair of Fig. 2 as a function of the single-isomer sulfated cyclodextrin concentration of the BGE. Constants used for the calculation: $\mu_D^0=\mu_L^0=4\cdot10^{-5}$ cm^2 V^{-1} s^{-1}, $\mu_{DCD}^0=-3.36\cdot10^{-5}$ cm^2 V^{-1} s^{-1}, $\mu_{LCD}^0=-3.4\cdot10^{-5}$ cm^2 V^{-1} s^{-1}, $K_{DCD}=68$, $K_{LCD}=75$.

μ_L^{eff}/μ_D^{eff} ratio (the separation selectivity, α [17]), goes from $\alpha=1$ at zero HDAS-β-CD concentration through $\alpha=0$ (where the effective mobility of L-carbidopa becomes zero while the effective mobility of D-carbidopa is still cationic), to $\alpha\rightarrow-\infty$, then to $\alpha\rightarrow\infty$ (where the effective mobility of L-carbidopa is anionic while the effective mobility of D-carbidopa changes from cationic to anionic), and begins to level off towards the limiting $\alpha>1$ value at high charged CD concentrations. Though α is very high in the vicinity of the mobility cross-over point, the separations are very slow because the μ^{eff} values are close to zero. Therefore, for practically useful separations, one has to sacrifice some α to gain in μ^{eff}.

The peak resolution equation of Friedl and Kenndler [17] has been extended [18] to include the effect of the dimensionless normalized electroosmotic flow mobility, β (defined as $\beta=\mu_{EO}/\mu_{slow}^{eff}$ where μ_{slow}^{eff} is the effective mobility of the slower enantiomer). The peak resolution surface is shown in Fig. 4 as a function of α and β. This figure indicates that once an adequate α value is secured (by varying the concentration of the charged CD), one can change the R_s value at will by manipulating the magnitude of β. This is best done by changing the magnitude of the electroosmotic flow-rate without compromising separation selectivity.

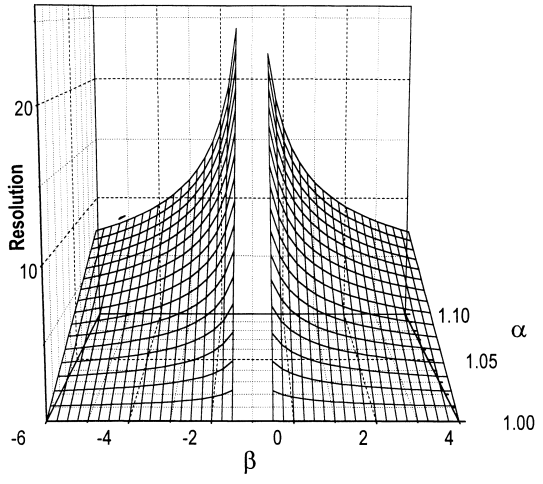

Fig. 4. Peak resolution surface calculated with Eq. 3 of Ref. [15] for the cationic enantiomer pair of Fig. 2 as a function of α and β. Constants used for the calculation: $z_{DCD}^{eff}=z_{LCD}^{eff}=-3$, $(e_0/8k)^{1/2}=38.0868$, $E=217$ V cm^{-1}, $l=39$ cm, $T=289$ K.

2. Experimental

All separations were carried out on a UV detector-equipped P/ACE 2100 CE unit (Beckman Instruments, Fullerton, CA, USA). The detection wavelength was set at 214 nm (for benzyl alcohol, the electroosmotic flow marker) and 280 nm (for carbidopa, the analyte). The cartridge coolant was thermostated at 16°C. The separations were carried out in 25 μm I.D. untreated fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, USA). The injection pressure was set at 5 p.s.i, the injection time was 1 s. The applied potential was set at +10 kV [for the 45 cm (effective length 39 cm) capillary] and +30 kV [for the 23 cm (effective length 17 cm) capillary].

All chemicals used in the BE preparation were obtained from Aldrich (Milwaukee, WI, USA). HDAS-β-CD (Cat. No. 733401, Regis, Morton Grove, IL, USA), was synthesized as described in Ref. [12]. The carbidopa samples (Merck, Princeton, NJ, USA) were kindly donated by A. Dougherty.

The BGEs were prepared by adding 0.0250 mol of concentrated (85%, w/w) phosphoric acid to enough deionized water (Milli-Q, Millipore, Milford, MA, USA) to obtain a solution of approximately 0.95 l. This solution was titrated to pH=2.5 with triethylamine using a combination glass electrode and a precision pH meter (both of them from Corning, Corning, NY, USA). The solution was transferred to a 1 l volumetric flask, the volume was brought to mark with deionized water and the pH was remeasured. The HDAS-β-CD BEs were prepared by weighing out the required amounts of the sodium salt of HDAS-β-CD into 25-ml volumetric flasks, bringing the volumes to mark with the required BGE stock solutions and rechecking their final pH.

3. Results and discussion

Recently, the pressure-mediated capillary electrophoretic method (PreMCE method [19]) was modified to permit the determination of the accurate electroosmotic flow (EOF) mobilities in charged cyclodextrin-containing BGEs which form electrophoretically migrating, charged complexes with the traditional, noncharged EOF markers [20]. Using a

capillary partially filled with both the charged CD-containing BGE and the charged CD-free BGE, the new method (external mobility marker method) permits the direct measurement of the effective mobility (μ^{eff}_{marker}) of any compound that subsequently can serve as external mobility marker. Once the μ^{eff}_{marker} is known in a given HDAS-β-CD containing BGE, the marker can be coinjected with any analyte of interest, its observed mobility can be measured, and the EOF mobility, μ_{EO}, can be calculated from the observed and effective mobilities of the external mobility marker as $\mu_{EO}=\mu^{obs}_{marker}-\mu^{eff}_{marker}$. Then, the effective mobility of the analyte can be obtained as usual: $\mu^{eff}_i=\mu^{obs}_i-\mu_{EO}$ [20]. From the effective mobilities of the enantiomers, separation selectivity, α, is calculated as $\alpha=\mu^{eff}_L/\mu^{eff}_D$ where subscript L refers to the L-carbidopa enantiomer. The dimensionless normalized EOF mobility value, β, is calculated then as $\beta=\mu_{EO}/\mu^{eff}_D$, where D refers to the slower moving enantiomer [18]. The peak resolution values, R_s, are calculated as usual, by dividing the migration time difference of the two enantiomers by one half of the sum of their peak widths.

The measured μ_{EO} curve and μ^{eff}_L curve of L-carbidopa are shown in Fig. 5 as a function of the HDAS-β-CD concentration. At $c_{HDAS\text{-}\beta\text{-}CD}=0$ m*M* $\mu_{EO}=-3.8\cdot10^{-5}$ cm^2 V^{-1} s^{-1}, indicating that the triethylammonium ions of the BGE adsorb onto the wall of the fused-silica capillary and cause a weak anionic EOF. As the concentration of HDAS-β-CD is increased, μ_{EO} becomes positive and levels off around the $18\cdot10^{-5}$ cm^2 V^{-1} s^{-1} value at $c_{HDAS\text{-}\beta\text{-}CD}=40$ m*M*. This high EOF mobility (unexpected at pH 2.5) is thought to be caused by the adsorption of HDAS-β-CD on the wall of the capillary. Meanwhile, μ^{eff}_L of L-carbidopa decreases from the $4\cdot10^{-5}$ cm^2 V^{-1} s^{-1} (cationic) value to zero, then to negative values as the migration direction of L-carbidopa changes towards the anode. μ^{eff}_L of L-carbidopa begins to level off at around $c_{HDAS\text{-}\beta\text{-}CD}=100$ m*M*.

The separation selectivity curve of carbidopa is shown in Fig. 6 as a function of the HDAS-β-CD concentration. At $c_{HDAS\text{-}\beta\text{-}CD}=15$ m*M*, α is negative, indicating that the two carbidopa enantiomers migrate in the opposite direction. At $c_{HDAS\text{-}\beta\text{-}CD}=20$ m*M*, α becomes a positive number ($\alpha=2.76$), then it begins to decrease as the HDAS-β-CD concentration is increased further. This behavior exactly follows the theoretical predictions of the CHARM model [16] for an oppositely charged analyte and resolving agent combination as shown by Figs. 2 and 3. As the effective mobility of one of the carbidopa enantiomers approaches zero and the effective mobility of the other enantiomer remains a finite (though small) value, α becomes large. Unfortunately, under these conditions, separation time becomes excessive. Therefore, a compromise must be found offering a still large enough selectivity coupled with an already large enough effective mobility so that separation

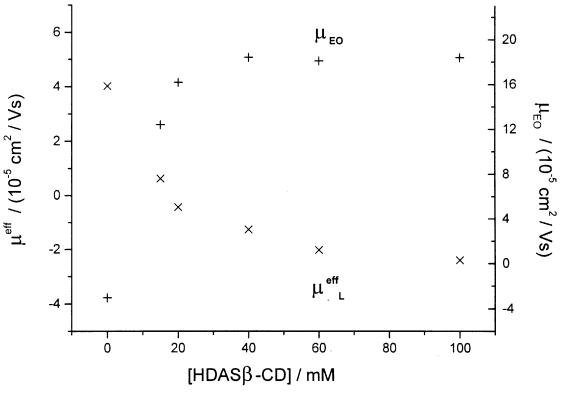

Fig. 5. The electroosmotic flow mobility, μ_{EO} and the effective mobility, μ^{eff}_L of L-carbidopa as a function of the HDAS-β-CD concentration. Applied potential: 10 kV, capillary: 45 cm (effective length 39 cm)×25 μm I.D., uncoated fused-silica thermostated at 16°C. Other conditions are listed in Experimental.

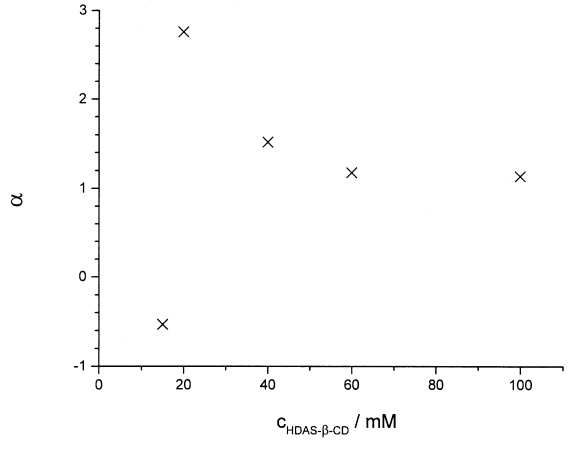

Fig. 6. Separation selectivity, α, for D,L-carbidopa as a function of the HDAS-β-CD concentration. Conditions as in Fig. 5.

times around 10 min, suitable for routine quantitative analysis, can be obtained.

In the case of carbidopa, in a 45 cm (effective length 39 cm) capillary, the 20 m*M* HDAS-β-CD BGE offers $\alpha=2.76$ with a separation time of 21 min at 10 kV applied potential. When the capillary was shortened to 23 cm (effective length 17 cm) and the applied potential was increased to 30 kV to reduce separation time, separation selectivity decreased to $\alpha=1.82$ as a result of the much higher BGE temperature brought about by increased Joule heating (field strength at this point is 1200 V cm^{-1} and power dissipation is 9.4 W m^{-1}). Simultaneously, adequate R_s was lost due to a tremendous change in the β value (from $\beta=-9$ on the long capillary at low thermal load to $\beta=-54$ on the new short capillary at high thermal load). Therefore, β had to be reduced and a compromise had to be found which offered high enough peak resolution ($R_s>2$ to permit quantitation of the minor enantiomer) and acceptable short separation time ($t_{sep}<10$ min).

Poly(ethylene glycol)s (PEGs) and poly(propylene glycol)s have been used to reduce the magnitude of the EOF [21–25]. Therefore, a low-molecular-mass polyethylene glycol, PEG 900 was added to the 20 m*M* HDAS-β-CD BGE to see if it could control the β value without seriously compromising separation selectivity. In Fig. 7 the measured α, β and R_s values as a function of the PEG 900 concentration of the BGE are shown; Fig. 8 shows the corresponding electropherograms. As little as 0.1% PEG 900 added to the BGE already halves the β value and produces partial peak resolution without loss of separation selectivity (see Fig. 7). As the PEG 900 concentration is increased to 8%, β is decreased to −11 while α remains almost unchanged ($\alpha=1.86$ at 8% PEG 900 vs. $\alpha=1.82$ at 0.1% PEG 900). This favorable β change results in an increase of R_s to the desired $R_s>2$ value. Simultaneously, separation time becomes shorter than the desired 10 min (see Fig. 8), and its reproducibility improves significantly. Therefore, the 8% PEG 20 m*M* HDAS-β-CD BGE was used for the minor enantiomer analysis.

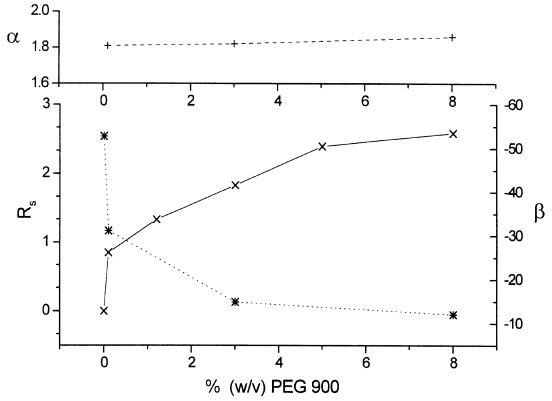

Fig. 7. Separation selectivity, α (symbol +), dimensionless normalized EOF mobility, β (symbol *), and peak resolution, R_s (symbol ×) as a function of the PEG 900 concentration (w/v) in 20 m*M* HDAS-β-CD BGE. Applied potential: 30 kV, thermostat temperature: 16°C, capillary: 23 cm (effective length 17 cm)×25 μm I.D., uncoated fused-silica, detection wavelength: 280 nm.

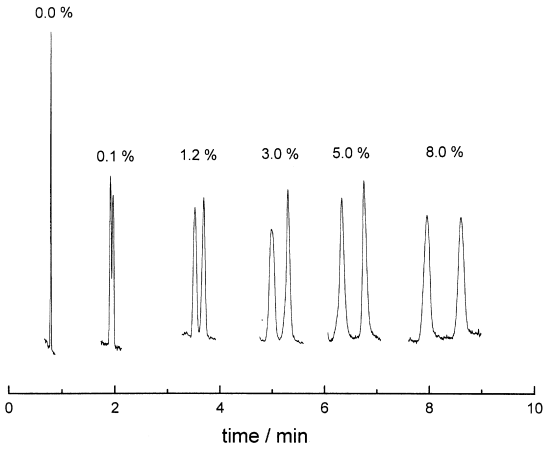

Fig. 8. Electropherograms showing the separation of racemic carbidopa with 0–8% PEG 900. Conditions as in Fig. 7.

A calibration curve was obtained by dissolving 49.7 mg of racemic carbidopa in 10 ml of the 8% PEG 20 m*M* HDAS-β-CD BGE. Five serial dilutions were performed resulting in calibrating solutions containing 2.49, 1.24, 0.621, 0.311, 0.155, and 0.0777 mg of L-carbidopa and D-carbidopa/ml BGE. Peak areas were then measured as a function of the carbidopa concentration. Linear calibration curves were obtained across the entire concentration range studied, as listed in Table 1. The actual L-carbidopa sample to be analyzed was prepared by dissolving 23.7 mg of L-carbidopa in 10 ml of the 8% PEG 20 m*M* HDAS-β-CD BGE. Five replicate analyses were performed: the quantitation results are shown in Table 1. A typical separation of L-carbidopa is shown

Table 1
Quantitative analysis results for the L-carbidopa sample

Calibration curve ($y=a+bx$) *parameters:*

D-Enantiomer			L-Enantiomer		
Parameter	Value	S.D.	Parameters	Value	S.D.
a	−0.0053	0.00356	a	−0.0115	0.00717
b	1.26775	0.00304	b	1.30133	0.00612
$R=0.99999$			$R=0.99996$		
S.D.=0.00625, $n=6$			S.D.=0.0126, $n=6$		
$P=1.9754\cdot10^{-10}$			$P=2.9301\cdot10^{-9}$		

Quantitation results:

Replicate	D-Carbidopa (mg/ml)	L-Carbidopa (mg/ml)	% Purity	Average	S.D.
1	0.082	2.35	3.3		
2	0.079	2.45	3.1		
3	0.076	2.46	3.0	3.2	0.1
4	0.077	2.35	3.2		
5	0.078	2.32	3.2		

Conditions as in Fig. 8, concentration of PEG 900 in the BGE: 8%.

in Fig. 9. The concentration of the D-carbidopa impurity was found to be 3.2±0.1%. Since we did not have access to L-carbidopa samples with lower levels of D-carbidopa contamination, we could not experimentally test the possibility of quantitation down to lower minor enantiomer concentration levels.

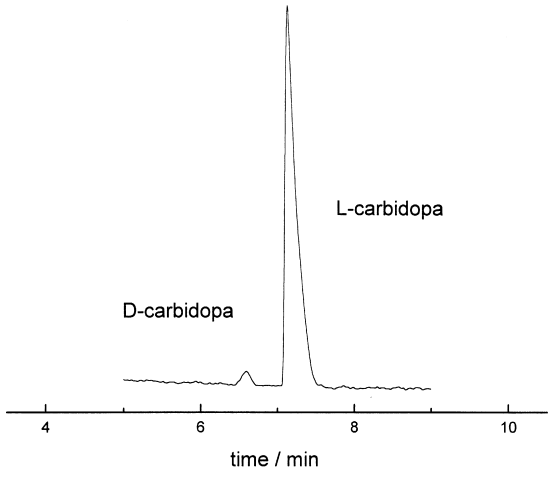

Fig. 9. Typical electropherogram of the L-carbidopa sample. Conditions as in Fig. 8, concentration of PEG 900 in the BGE: 8%.

4. Conclusions

The theoretical predictions of the CHARM model [15] were used successfully to develop a CE method suitable for the quantitation of the minor enantiomer contamination in L-carbidopa. First, the effective mobilities of the enantiomers and the separation selectivity were mapped out by varying the concentration of the single-isomer, fully sulfated cyclodextrin, HDAS-β-CD to identify the point where the cationic migration of carbidopa changed to anionic migration due to complexation with HDAS-β-CD. Though separation selectivity is highest in the vicinity of this point, separation times are very long forcing us to trade some separation selectivity for reduced separation time. Once adequate selectivity is established, both peak resolution and separation time are tuned to the desired values ($R_s>2$ and $t_{migr}<10$ min) by adjusting the dimensionless normalized EOF mobility value via the addition of up to 8% PEG 900 whose presence did not compromise separation selectivity. Under these conditions, linear calibration curves were obtained between peak area and analyte concentration and the minor enantiomer, D-carbidopa was successfully quantitated in L-carbidopa. Since the chiral resolving agent used (HDAS-β-CD) is a

well characterized, pure, single-isomer material, the threat of batch-to-batch variation in the composition of the resolving agent, and subsequently, in the separation selectivity, is eliminated. This allows for the development of a reproducible, easily validated assay for the analysis of the minor enantiomer in L-carbidopa.

Acknowledgements

The authors are grateful for partial financial support by the Texas Coordination Board of Higher Education ARP program (Project Number 010366-016) and Beckman Instruments (Fullerton, CA, USA), as well as acknowledge A. Dougherty for the donation of the carbidopa samples (Merck Pharmaceutical Co., Princeton, NJ).

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