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Enantioseparation of 3,4-dihydroxyphenylalanine and 2-hydrazino-2-methyl-3-(3,4-dihydroxyphenyl)propanoic acid by capillary electrophoresis using cyclodextrins

Selma Saraç[a,*], Bezhan Chankvetadze[b,1], Gottfried Blaschke[b]

[a]*Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Hacettepe University, 06100 Sihhiye, Ankara, Turkey*

[b]*Institute of Pharmaceutical Chemistry, University of Münster, Hittorfstr. 58–62, 48149 Münster, Germany*

Abstract

The enantiomeric separations of 3,4-dihydroxyphenylalanine (dopa) and 2-hydrazino-2-methyl-3-(3,4-dihydroxyphenyl)propanoic acid (carbidopa) by capillary electrophoresis were studied using several native, neutral and anionic cyclodextrins as chiral additives and uncoated fused-silica capillaries. The effect of the type and concentration of the cyclodextrin added to 20 m*M* phosphate buffer (pH 2.5) on enantioseparation and migration times was studied. A high resolution value of 15.63 was obtained for dopa enantiomers with a buffer containing 20 m*M* single isomer, heptakis(2,3-diacetyl-6-sulfato)-β-cyclodextrin. The enantiomers of carbidopa were separated using 20 m*M* carboxymethyl-β-cyclodextrin as a chiral resolving agent. Both methods allowed the determination of 0.1% of the D-enantiomer (second migrating) in the presence of the L-enantiomer (first migrating) of dopa and carbidopa with a good precision. These methods also gave good results in terms of precision for both peak area, migration time, linearity and accuracy.

Keywords: Enantiomer separation; Dihydroxyphenylalanine; DOPA; Hydrazinomethyl(dihydroxyphenyl)propanoic acid monohydrate; CarbiDOPA

1. Introduction

Levodopa or L-dopa [(−)-3,4-dihydroxyphenyl-L-alanine] (Fig. 1a) is one of the major drugs for the treatment of the prominent symptoms of Parkinson's disease [1]. In order to prevent its decarboxylation in the extracerebral tissues and to prolong its anti-Parkinsonial effect, it is formulated with carbidopa [L-2-hydrazino-2-methyl-3-(3,4-dihydroxyphenyl) propanoic acid monohydrate] (Fig. 1b), an inhibitor of dopa-decarboxylase. Several combinations of levodopa and carbidopa are commercially available as different formulations [2,3].

While L-dopa and L-carbidopa are pharmacologically active, the D-forms are inactive [4,5] and D-dopa has also toxic properties [6,7]. The use of racemic mixtures containing L-dopa, D-dopa, L-carbidopa and D-carbidopa may cause serious side effects because of the different metabolisms of the active and inactive components of the racemates [8]. Therefore, it is important to develop a method for the separation and determination of enantiomers of these compounds. Husain et al. [9] reported the simultaneous separation and determination of enantiomers of levodopa and carbidopa by HPLC as used in the treatment of Parkinson's disease.

*Corresponding author. Fax: +90-312-311-4777.

[1]Permanent address: Department of Chemistry, Tbilisi State University, Chavchavadze Ave 1, 380028 Tbilisi, Georgia.

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Fig. 1. Structures of (a) dopa and (b) carbidopa.

Capillary electrophoresis (CE) is becoming a routine analytical technique for the analysis of pharmaceutical samples. Its speed, high efficiency, ease of operation and low consumption of reagents make CE a valuable complementary and even an alternative technique to LC [10].

In this paper, an enantioselective CE method for the enantiomeric purity determination of L-dopa and L-carbidopa was developed using several native, neutral and anionic cyclodextrin derivatives as chiral additives. The effect of the type and concentration of cyclodextrin on the resolution and migration behaviour of dopa and carbidopa enantiomers was studied using a sodium dihydrogen phosphate buffer (pH 2.5) and uncoated fused-silica capillaries thermostated at 20°C. Several criteria such as selectivity, linearity of detector response, reproducibility of migration times and peak area measurements and quantification for the minor enantiomer were investigated.

2. Experimental

2.1. Apparatus

All experiments were performed on a Beckman P/ACE MDQ capillary electrophoresis system (Beckman Instruments, Fullerton, CA, USA) equipped with a UV detector.

2.2. Chemicals and reagents

Sodium dihydrogen phosphate and ortho-phosphoric acid (85%) were of analytical-reagent grade from Merck (Darmstadt, Germany). Water was deionized and doubly distilled. α-, β- and γ-cyclodextrins (α-, β- and γ-CDs), methyl-α-cyclodextrin (Me-α-CD), with an average molecular substitution degree (D.S.) of 10.8; methyl-β-cyclodextrin (Me-β-CD), with D.S. values of 12.6 and 4.2; carboxymethyl-β-cyclodextrin (CM-β-CD), with a D.S. of 3.5; succinyl-β-cyclodextrin (SUC-β-CD), with a D.S. value of 2.8; hydroxypropyl-α-cyclodextrin (HP-α-CD) with a D.S. of 3.6 and hydroxypropyl-γ-cyclodextrin (HP-γ-CD) with a D.S. of 4.8 were gifts from Wacker-Chemie (Munich, Germany). Heptakis(2,3,6-tri-*O*-methyl)-β-cyclodextrin (TM-β-CD), (−)-carbidopa, racemic dopa and its (−)-enantiomer (L-dopa) were purchased from Sigma (Deisenhofen, Germany) and 2,6-di-*O*-methyl-β-cyclodextrin (DM-β-CD) from Fluka (Buchs, Switzerland). Sulfated β-cyclodextrin (SU-β-CD), with a D.S. value of 10.7, and hydroxypropyl-β-cyclodextrin (HP-β-CD), with a D.S. value of 5.6 was from Aldrich (Steinheim, Germany). Sulfobutyl ether-β-cyclodextrin (SBE-β-CD), with a D.S. value of 4 was obtained from Cydex, L.C. (Kansas, USA). Heptakis(2,3-diacetyl-6-sulfato)-β-cyclodextrin (HDAS-β-CD), heptakis-6-sulfato-β-cyclodextrin (HS-β-CD), heptakis(2,3-dimethyl-6-sulfato)-β-cyclodextrin (DMS-β-CD) and racemic carbidopa were kindly provided by Professor Gy. Vigh (Texas A&M University, College Station, Texas, USA).

2.3. Electrophoretic technique

Electrophoretic separations were carried out in 41 cm (effective length 30 cm)× 50 μm I.D., uncoated fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, USA). The running buffer consisted of 20 m*M* sodium dihydrogen phosphate solution adjusted to pH 2.5 with 100 m*M* phosphoric acid. At the beginning of each working day, the capillary was rinsed with running buffer for 5 min. Between each

injection, the capillary was washed with 50 m*M* phosphoric acid solution for 1 min followed by a rinse with running buffer for 2 min and a chiral separation buffer for 2 min. The applied voltage was +20 or +30 kV. The detection wavelength was set at 214 nm. Injections were made by pressure of 0.5 p.s.i. for a period of 3 s during the resolution studies (1 p.s.i.=6894.76 Pa). For the testing of enantiomeric purity, the electrokinetic injection mode (injection voltage 10 kV) was used for a period of 10 or 7 s for dopa and carbidopa, respectively. The temperature of the capillary tube was maintained at 20°C. The standard solutions were prepared by dissolving racemic compounds and their pure D-enantiomers at a concentration of 5 mg/ml in 0.025 *M* hydrochloric acid.

The determination of the migration order was performed by spiking a stock solution of racemic dopa or carbidopa with the pure enantiomers of the two analytes. The separation factor, α, was calculated as t_2/t_1. The resolution (R_s) and plate number (N) were calculated according to the standard expressions based on the peak width at half-height [11].

3. Results and discussion

3.1. Selection of chiral selector

The main goal of this study was to accurately quantify small enantiomeric impurities of dopa and carbidopa by applying several special requirements to the chiral selector. At first, the selectivity of enantioseparation should be sufficient in order to avoid the peak overlap between the major and minor components. Furthermore, a chiral selector is desirable to be well characterized, inexpensive and commercially available. Because of the difficulties in optimization and validation of a chiral separation using randomly substituted CD derivatives, it is better to use, if possible, the native CDs rather than multicomponent mixtures of CD derivatives. If the use of multicomponent CD derivatives is impossible to avoid, these derivatives should be characterized by CE and spectral methods in order to obtain detailed information about their degree of substitution (the number of charges per CD molecule) and location of substituents. These parameters can have a great effect on the chiral selectivity of the system [12].

Enantioseparation of dopa in CE has been studied with various chiral selectors such as $18C6H_4$ crown ether [13–15], anionic β-CD derivatives, SBE-β-CD and SU-β-CD [16–18], single isomer sulfated cyclodextrin HDAS-β-CD [19], ligand exchange type chiral selectors [20,21] and the combination of $18C6H_4$ crown ether and CD [22]. Together with enantioseparations in aqueous CE [13,14] the enantioseparation in nonaqueous CE was also studied [15]. The crown ether, $18C6H_4$, does not meet several of the aforementioned requirements. The selectivity of enantioseparation is insufficient and peaks are broad with this chiral selector. In addition, $18C6H_4$ is not more easily commercially available and quite expensive. SBE-β-CD also suffers with rather low enantioselectivity [16] and it is a multicomponent mixture which is not very desirable for use in validated analysis. The latter limitation applies also for SU-β-CD which otherwise exhibits high enantioselectivity.

Carbidopa was enantioseparated in CE by use of a running buffer containing HDAS-β-CD as chiral resolving agent and poly(ethylene glycol) 900 (PEG 900) as an additive. An adequate separation selectivity has been obtained by selecting 8% PEG 900 20 m*M* HDAS-β-CD in pH 2.5 [23].

In this study, in order to design a separation system for the determination of 0.1% of either enantiomer of dopa (and carbidopa) in the presence of its stereoisomer, several native, neutral and anionic CDs were examined at different concentrations (Tables 1 and 2).

The baseline enantioseparation for dopa was observed only with the single isomer HDAS-β-CD (Fig. 2a) and SBE-β-CD (Fig. 2b). The enantioselectivity was insufficient for the validated analysis with SU-β-CD. It seems interesting to note that the enantiomer migration order was opposite with these chiral selectors. D(+)-Dopa migrated as the first in the case of SBE-β-CD. D(+)-Dopa is expected a minor peak in L(−)-dopa. Therefore, this migration order of the enantiomers seems to be preferable in the case of peak tailing. However, rather low enantioselectivity and wide peaks in the case of SBE-β-CD were the reason why we objected to this chiral selector. Single isomer HDAS-β-CD afforded very

Table 1
Enantioseparation of (±)-dopa with various CDs[a]

Cyclodextrin[b]	Concentration (m*M*)	Migration time		Selectivity (α)	Resolution (R_s)	Theoretical plate number	
		t_1 (min)	t_2 (min)			N_1/m	N_2/m
Without CD	–	14.44	–	–	–	–	–
α-CD	18	16.20	–	–	–[b]	–	–
	100[c]	17.10	17.27	1.00	–	–	–
β-CD	18	16.12	–	–	–	–	–
γ-CD	18	17.00	–	–	–	–	–
	100	20.46	–	–	–	–	–
	150[c]	23.04	23.34	1.01	–	–	–
Me-α-CD	50	18.01	–	–	–	–	–
Me-β-CD (1.8)	50	17.20	–	–	–	–	–
Me-β-CD (0.6)	50	15.27	–	–	–	–	–
DM-β-CD	50	17.45	–	–	–	–	–
TM-β-CD	50	17.46	–	–	–	–	–
	100	23.37	–	–	–	–	–
HP-α-CD	50	20.70	–	–	–	–	–
HP-β-CD	50	22.06	–	–	–	–	–
HP-γ-CD	50	21.39	–	–	–	–	–
CM-β-CD (3.5)	10	24.48	–	–	–	–	–
SUC-β-CD (2.8)	20	33.28	–	–	–	–	–
HS-β-CD	5	28.65	–	–	–	–	–
	10	50.07	–	–	–	–	–
DMS-β-CD	20	11.34	–	–	–	–	–
HDAS-β-CD	10	6.38 (–)	7.00 (+)	1.10	3.66	75 167	90 487
	20	14.48 (–)	20.44 (+)	1.41	15.63	96 797	123 443
SBE-β-CD (4)[d]	5	34.22 (+)	34.88 (–)	1.02	1.20	149 778	105 916
SU-β-CD (10.7)	2.5	21.13	21.28	1.01	–	–	–
	4	21.10 (–)	21.36 (+)	1.01	0.23	17 193	19 460
	6[e]	26.83 (–)	27.23 (+)	1.02	–	–	–

[a] Buffer: 20 m*M* sodium dihydrogen phosphate adjusted to pH: 2.5 with 100 m*M* phosphoric acid; injection mode: hydrodynamic, 0.5 p.s.i., 3 s; capillary: uncoated fused-silica, 41 cm (effective length 30 cm)×50 μm I.D.; applied voltage: +20 kV; λ: 214 nm.
[b] A dash indicates that no resolution was obtained between the enantiomers.
[c] The enantiomers were partially resolved but not sufficiently permit a precise determination of R_s.
[d] Capillary: uncoated fused-silica, 50 μm I.D., 61 cm (effective length 50 cm); applied voltage: +25 kV.
[e] Applied voltage: +15 kV.

high enantioselectivity (α), excellent peak efficiency (N) and high resolution (R_s) (Table 1).

The enantiomers of carbidopa were completely resolved with the use of negatively charged CD derivatives, SBE-β-CD and CM-β-CD (Fig. 3). Although baseline separation of carbidopa was obtained also with SBE-β-CD, enantioselectivity was poor and peaks were broad. The enantiomer separation of carbidopa was successfully achieved by adding 20 m*M* CM-β-CD to the running buffer solution. With the native γ-CD, uncharged DM-β-CD, single-isomer HDAS-β-CD and the anionic CD derivative, SU-β-CD, only partial resolution was obtained. In all cases, except for HDAS-β-CD and SBE-β-CD, the (−)-enantiomer migrated faster than the (+)-enantiomer.

3.2. CD concentration

The effect of the CD concentration on the enantioseparation of dopa was also studied. The continuous increase of the separation selectivity was observed with increasing HDAS-β-CD concentration. However, higher peak dispersion and electric current together with impracticable long analysis time at higher CD concentrations were the reasons why we

Table 2
Enantioseparation of (±)-carbidopa with various CDs[a]

Cyclodextrin	Concentration (m*M*)	Migration time		Selectivity (α)	Resolution (R_s)	Theoretical plate number	
		t_1 (min)	t_2 (min)			N_1/m	N_2/m
Without CD	–	14.62	–	–	–	–	–
α-CD	18	15.29	–	–	–[b]	–	–
	100	17.20	–	–	–	–	–
β-CD	18	14.50	–	–	–	–	–
γ-CD	18	14.40	–	–	–	–	–
	100	24.75 (−)	25.10 (+)	1.01	0.32	–	–
	150	31.62 (−)	32.23 (+)	1.02	0.72	115 397	53 287
Me-α-CD	50	18.66	–	–	–	–	–
Me-β-CD (1.8)	50	15.22	–	–	–	–	–
Me-β-CD (0.6)	50	13.64	–	–	–	–	–
DM-β-CD	50[c]	16.91	17.23	1.02	–	–	–
TM-β-CD	50	17.76	–	–	–	–	–
	100	23.95	–	–	–	–	–
HP-α-CD	50	18.69	–	–	–	–	–
HP-β-CD	50	19.25	–	–	–	–	–
HP-γ-CD	50	21.32	–	–	–	–	–
CM-β-CD (3.5)	10	42.08 (−)	44.75 (+)	1.06	7.88	817 487	924 517
	20[d]	26.46 (−)	28.35 (+)	1.07	11.15	1 292 910	1 484 207
SUC-β-CD (2.8)	10	17.42	–	–	–	–	–
HS-β-CD	10	29.89	–	–	–	–	–
	20	>120.00	–	–	–	–	–
DMS-β-CD	20	11.51	–	–	–	–	–
HDAS-β-CD	10	21.36 (+)	21.83 (−)	1.02	1.85	374 463	391 123
	15	23.89 (+)	24.70 (−)	1.03	3.98	731 910	782 383
SBE-β-CD (4)	0.5	14.86 (+)	15.14 (−)	1.02	0.21	–	–
	1	20.53 (+)	21.47 (−)	1.05	0.85	–	–
	1.5	33.23 (+)	37.23 (−)	1.12	4.97	100 700	102 383
	2	58.60 (+)	77.50 (−)	1.32	13.94	129 417	136 933
SU-β-CD (10.7)	2.5	21.58 (−)	21.98 (+)	1.02	0.59	53 750	55 760
	4	21.02 (−)	21.65 (+)	1.03	2.50	362 637	384 700

[a] Buffer: 20 m*M* sodium dihydrogen phosphate adjusted to pH 2.5 with 100 m*M* phosphoric acid; injection mode: hydrodynamic, 0.5 p.s.i., 3 s; capillary: uncoated fused-silica, 41 cm (effective length 30 cm)×50 μm I.D.; applied voltage: +20 kV; λ: 214 nm.
[b] A dash indicates that no resolution was obtained between the enantiomers.
[c] The enantiomers were partially resolved but not sufficiently permit a precise determination of R_s.
[d] Applied voltage: +30 kV.

selected 20 m*M* HDAS-β-CD as the optimal one. A resolution improvement was obtained for carbidopa by increasing the CM-β-CD concentration from 10 to 20 m*M*. Using higher electrolyte concentrations, higher R_s values were achieved, but the migration times were longer. In order to keep the migration time short, the separation was carried out +30 kV.

The analysis was performed in acidic buffers because dopa and carbidopa are relatively unstable in basic buffers. Some preliminary studies were also performed in order to optimize buffer anions and cations, ionic strength, etc., but the effects were marginal and are not discussed here.

3.3. Application of CE to the optical purity testing of dopa and carbidopa

In order to examine whether these methods could be acceptable for the optical purity testing of dopa and carbidopa, method validations such as the reproducibility of migration times and peak area measurements, selectivity, linearity of detector response,

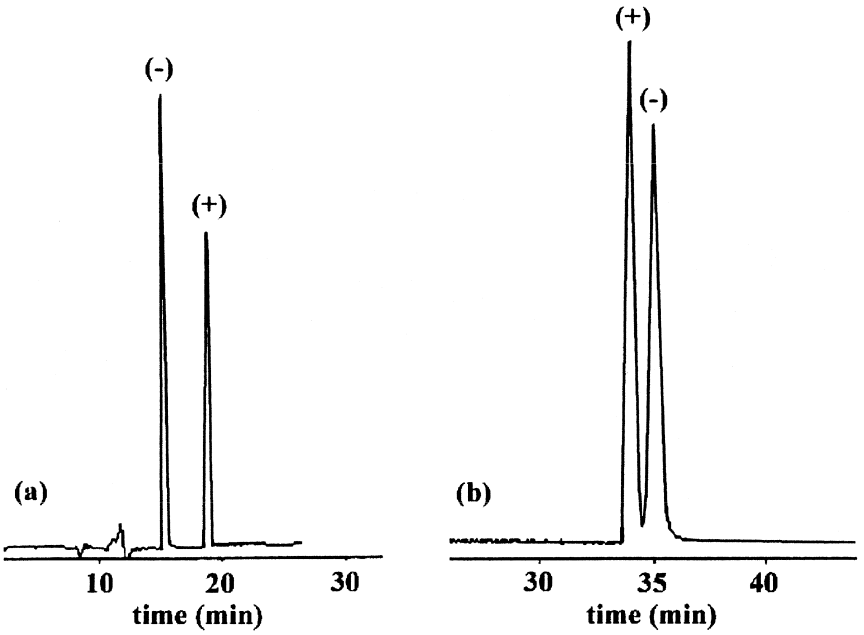

Fig. 2. Enantioseparation of (±)-dopa with 20 m*M* sodium dihydrogen phosphate buffer (pH 2.5) containing (a) 20 m*M* HDAS-β-CD and (b) 5 m*M* SBE-β-CD (4). Conditions as described in Table 1.

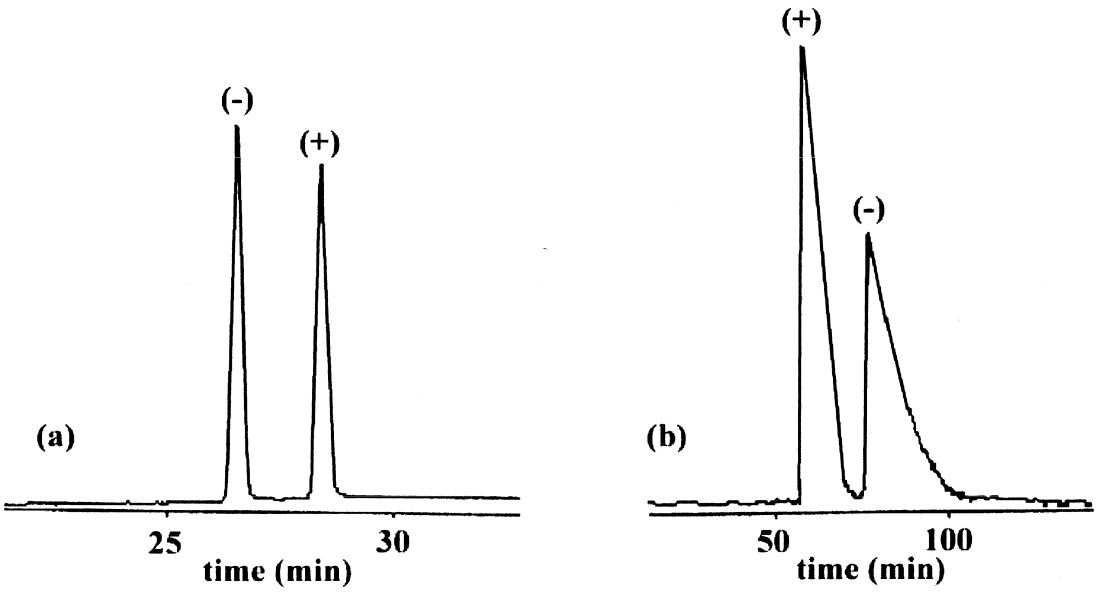

Fig. 3. Enantioseparation of (±)-carbidopa with 20 m*M* sodium dihydrogen phosphate buffer (pH 2.5) containing (a) 20 m*M* CM-β-CD and (b) 2 m*M* SBE-β-CD (4). Conditions as described in Table 2.

limits of detection and quantification for the minor enantiomer, precision and accuracy were investigated in CE. Reproducibility of the migration times and peak areas of the enantiomers of dopa and carbidopa were studied for racemic compounds and are summarized in Table 3. These characteristics are acceptable for the usage of this method for the quantification of small enantiomeric impurities of D-dopa or D-carbidopa in L-dopa or L-carbidopa, respectively.

L-Dopa and L-carbidopa were injected alone and no trace of the other enantiomer was detectable. For both compounds the first migrating peak was identified as the L-enantiomer. The linearity and recovery were investigated by adding the racemic compound to the standard L-enantiomer of dopa or carbidopa in the range of 0.1–1.5%. Linear regression analysis, plotting the analyte peak area (y) versus the percentage of impurity (x), gave the following equations:

Dopa: $y = 0.009x + 0.0001 \quad (R^2 = 0.9994)$

Carbidopa: $y = 0.009x + 0.0001 \quad (R^2 = 0.9998)$

The adequate linearity of the calibration graphs is demonstrated by the determination of correlation coefficients ($R^2 > 0.999$) obtained for the regression lines.

Most CE separations for standard mixture of enantiomers report a determination of the minor enantiomer at the level 0.5–1.0% [24–35]. Only in a few studies are enantiomeric impurities determined at the level of 0.1%, which is what is required by the regulatory acts for the impurities in drugs [31,32]. The requirement applies also to enantiomeric impurities. The method proposed in this study allows the determination ($S/N > 3$) of the minor enantiomer at the 0.1% level. Typical electropherograms of dopa and carbidopa enantiomers containing low levels of their stereoisomers are presented in Figs. 4 and 5. The data about the precision and accuracy of this method in the concentration range of minor enantiomer 0.1–1.5% are summarized in Table 4. Limits of detection (LODs) and quantification (LOQs) for dopa and carbidopa, corresponding to signal-to-noise ratios of 3 and 10, respectively, were calculated from linear regression analysis made by plotting the analyte peak height versus the percentage of impurity. The LOD, the lowest concentration of D-dopa that could be detected, was 0.05%, which corresponds to a concentration of 2.59 μg/ml. A LOD of 0.03% (1.5 μg/ml) was obtained for the D-enantiomer of carbidopa. The LOQs, the lowest concentration of D-dopa and D-carbidopa that could be determined in a purity determination were 0.17% (8.62 μg/ml) and 0.10% (5.0 μg/ml), respectively.

4. Conclusions

The low pH CE methods have been developed for the enantiomeric purity determination of dopa and carbidopa at the level 0.1–1.5%. The anionic cyclodextrin derivatives, single isomer HDAS-β-CD and CM-β-CD were successfully used to resolve the stereoisomers of dopa and carbidopa, respectively. Acceptable measures of linearity, accuracy and precision of both migration time and peak area were obtained. Both chiral CE methods are capable of generating precise data.

Table 3
Reproducibility of the migration times and the peak area percentage of racemic dopa and carbidopa in CE ($n = 5$)[a]

		First enantiomer (RSD %)	Second enantiomer (RSD %)
Dopa	average migration time (min)	14.31 (1.17)	20.24 (2.42)
	average peak area (%)	49.23 (0.77)	50.77 (0.74)
Carbidopa	average migration time (min)	27.58 (2.91)	29.42 (2.95)
	average peak area (%)	50.01 (0.52)	49.99 (0.52)

[a] Separation conditions as in Section 2.3.

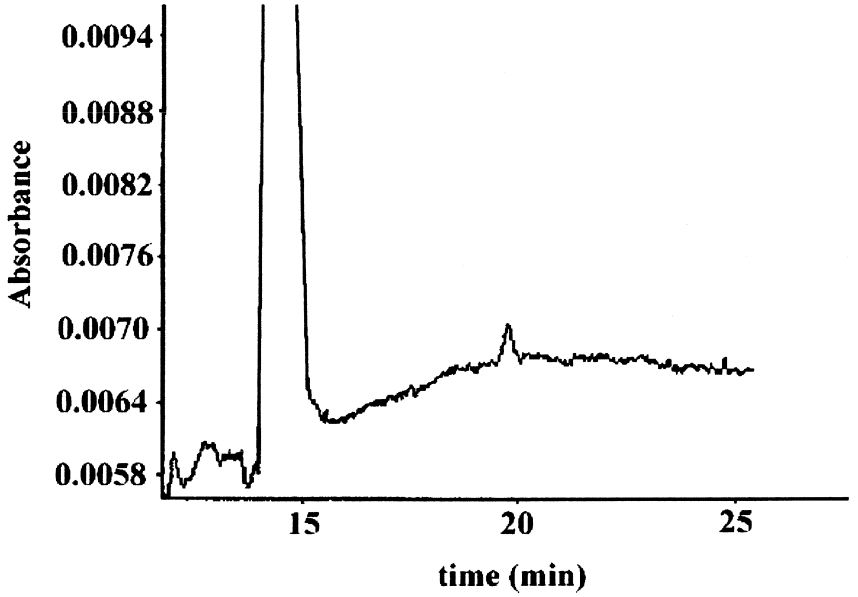

Fig. 4. Typical electropherogram of L-dopa containing 0.2% of D-dopa. Buffer: 20 m*M* HDAS-β-CD in 20 m*M* sodium dihydrogen phosphate adjusted to pH 2.5 with phosphoric acid; applied voltage: +20 kV; electrokinetic injection: +10 kV, 10 s. Other conditions as in Section 2.3.

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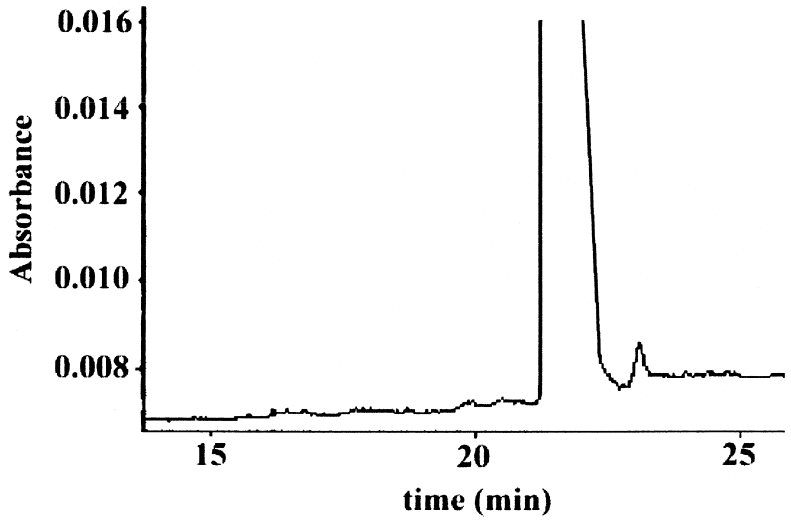

Fig. 5. Typical electropherogram of L-carbidopa containing 0.2% of D-carbidopa. Buffer: 20 m*M* CM-β-CD in 20 m*M* sodium dihydrogen phosphate adjusted to pH 2.5 with phosphoric acid; applied voltage: +30 kV; electrokinetic injection: +10 kV, 7 s. Other conditions as in Section 2.3.

Table 4
Precision and accuracy results for dopa and carbidopa[a]

Compounds	Nominal concentrations (%)	Mean calculated concentrations (%)	Precision (RSD %)	Accuracy (%) (deviation of means from calibration curve)
Dopa	1.5 ($n=3$)	1.51	1.67	100.74
	1.0 ($n=3$)	0.98	2.83	97.78
	0.5 ($n=3$)	0.51	1.74	102.22
	0.2 ($n=3$)	0.207	0.05	103.89
	0.1 ($n=3$)	0.095	2.50	94.44
Carbidopa	1.5 ($n=4$)	1.49	0.37	99.31
	1.0 ($n=4$)	1.01	1.80	101.44
	0.5 ($n=4$)	0.50	0.32	100.34
	0.2 ($n=4$)	0.204	3.72	101.81
	0.1 ($n=4$)	0.096	3.30	95.82

[a] Separation conditions as in Section 2.3.

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