



Chiral and non chiral determination of Dopa by capillary electrophoresis

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Abstract

The suitability of capillary electrophoresis for determining the enantiomeric purity of levodopa in a pharmaceutical formulation also containing benserazide was assessed. To this end, the pharmaceutical components were separated in a non-chiral medium that allowed the total amount of Dopa and that of benserazide to be quantified. The addition of a chiral crown ether to the background electrolyte allows to separate the enantiomers of this compounds. Optimizing the variables influencing the enantioresolution of Dopa affords a resolution high enough resolution to determine the amount of dextrodopa (the distomer) contained in levodopa (the eutomer) in a pharmaceutical. A relative limit of detection (RLD) is proposed as a measure of the lowest detectable enantiomeric impurity. The RLD for the determination of dextrodopa contained in levodopa was 0.1% and found to depend on the enantiomer migration order. The enantiomeric purity of levodopa in the pharmaceutical preparation and dextrodopa from Sigma was 99.5 and 99.95%, respectively.

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1. Introduction

Chiral capillary electrophoresis (Chiral CE) has been the subject of much attention and has been applied with success to the enantioseparation of different compounds [1]. These chiral separations are usually done by capillary zone electrophoresis with a background electrolyte containing a chiral selector to discriminate between the enantiomers.

The chiral selectors used for this purpose [2,3] include cyclodextrins, chiral crown ethers, dextrans, macrocyclic antibiotics and metal complexes.

Enantiomerism is a phenomenon of a high pharmaceutical relevance as drug enantiomers frequently exhibit differential pharmacological and toxicological properties [4]. The enantiomer possessing pharmacological activity is called the 'eutomer'; the other, called the 'distomer', is normally inactive or even toxic. The current general policy is to promote the commercialization of new drugs marketed as single enantiomers [5] wherever possible in order to reduce the required dose when the distomer is inactive (the so-called

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'isomeric ballast') or avoid secondary effects when it is toxic. The need has thus arisen for the pharmaceutical industry to develop fast, sensitive, selective analytical methods for controlling the enantiomeric purity of active principles.

CE has so far scarcely been used to determine enantiomeric purity owing to the high enantiomer resolution and sensitivity required [6]. Applications to real samples such as raw materials, pharmaceuticals or metabolites in biological fluids have been even scantier owing to the problems posed by matrix interferences from the excipients or other active principles in the pharmaceutical [1]. 3,4-Dihydroxyphenyl-L-alanine, which is widely known as 'L-Dopa' or 'levodopa', is a catecholamine used to treat Parkinson's disease (see Fig. 1). It is administered in an enantiomerically pure form as the racemic mixture has secondary effects such as dyskinesia and granulocytopenia [7]. In order to prevent its decarboxylation in extracerebral tissues and prolong its antiparkinsonian effect, it is formulated with an inhibitor of Dopa-decarboxylase. Benserazide (D,L-serine-2-[2,3,4-(trihydroxyphenyl)methyl]hydrazide) is one of such inhibitors. Although Dopa enantiomers have been successfully separated by CE using chiral crown ethers [8] and charged cyclodextrins [9] no quantitative method for the determination of its

enantiomeric purity in pharmaceuticals exists. The sole reported application of this type is based on reversed phase HPLC using chiral ligand-exchange principle [10] and the method is applied to several pharmaceutical preparations that contain Dopa and Carbidopa. The first enantiomer to elute is dextrodopa and separation factor of 1.6 is obtained.

The aim of this work is to show the ability of CE for determining high enantiomeric excesses. To serve as an example, it is studied the determination of Dopa enantiomeric purity in a pharmaceutical preparation marketed as single enantiomer that contains also Benserazide.

2. Experimental

2.1. Apparatus

Measurements were made on a Hewlett-Packard ^{3D}CE HPCE instrument (Waldbronn, Germany) equipped with a diode array detector. Hydrodynamic injection (50 mbar, 5 s) at the anode was used in all tests. Separations were conducted in fused silica capillaries (Sugelabor, Spain) that were 56 cm long × 50 μm ID or, for improved sensitivity, in extended-pathlength × 3 capillaries of the same length and ID obtained from Agilent. The experimental set-up also included a Selecta ultrasonic bath, a Crison micropH 2001 pH meter and an Alresa centrifuge.

2.2. Reagents

Benserazide, Dopa (in the levo, dextro and racemic forms), β-cyclodextrin, γ-cyclodextrin and heptakis(2,3,6)-tri-*o*-methyl-β-cyclodextrin were purchased from Sigma. Carboxymethyl-β-cyclodextrin, 2-hydroxypropyl-β-cyclodextrin, (+)-(18-crown-6)-2,3,11,12-tetracarboxylic acid (18C6H₄) and tris(hydroxymethyl)-aminomethane (Tris) were purchased from Fluka. Citric acid monohydrate was obtained from Merck. The background electrolyte (BGE) was prepared as follows: 0.121 g of Tris was weighed, adjusted with 0.1 M citric acid to pH 2.5, made to 100 ml with Milli-Q water and filtered. In this way, the BGE

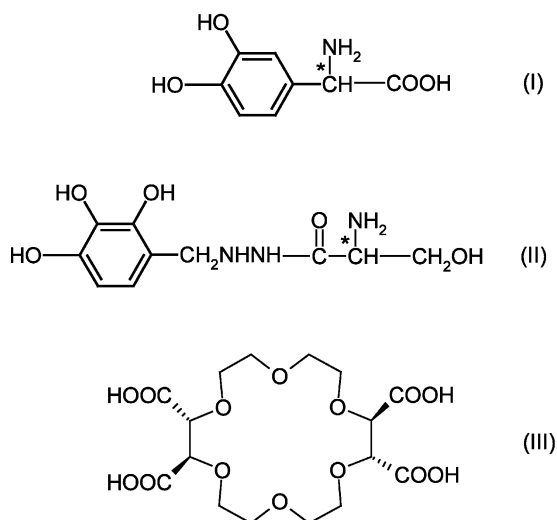


Fig. 1. Chemical structure of Dopa (I), benserazide (II) and 18C6H₄ (III). Asterisks denote asymmetric carbons.

was 10 mM in Tris and about in 75 mM citric acid. Dopa and benserazide solutions were prepared in 0.1 M HCl and protected from light in order to prevent its oxidation.

2.3. Procedure

Separations were conducted in the above-described Tris–citrate buffer, to which appropriate amounts of the chiral selector and organic modifier were added. Before each injection, the capillary was flushed with 3 min with the BGE and equilibrated at 30 kV for 3 min. After injection, a voltage of 30 kV was applied (direct polarity) and the electropherogram recorded. Finally, the capillary was flushed with 0.1 M NaOH and water for 3 min each. The analytes were detected at 198 and 230 nm, and the capillary temperature was set at 15 °C unless otherwise stated.

2.4. Samples

For the analysis of the pharmaceutical Madopar[®] 250 (Roche), ten tablets were ground and 0.2 g of the resulting powder was suspended in 30 ml of 0.1 M HCl. The suspension was sonicated for 10 min and centrifuged at 3000 rpm for 5 min. The solution was then filtered and diluted to 100 ml with 0.1 M HCl. The pharmaceutical preparation contains Benserazide and L-Dopa as active ingredients and microcrystalline cellulose, talc, povidone, magnesium stearate, gelatin and some pigments as excipients.

3. Results

The linear response interval for the detector usually precludes the joint determination of both enantiomers in products with a high enantiomeric excess [11]. The problem is usually solved by using two different injections [12]: one of a highly concentrated sample to determine the minor enantiomer and the other, more diluted, to quantify the major component. In this work, however, we used an alternative approach: the total analyte content was determined in a non-chiral medium and the distomer in a chiral medium. This method

has the advantage that the separation of the sample components under non-chiral conditions allows us to propose a new method for the analysis of benserazide and to save chiral selector. Subsequently, adding the chiral selector to the medium enables the determination of the distomer. Consequently, the determinations of total Dopa and dextrodopa are performed under similar conditions, which facilitates the implementation of the method.

3.1. Non-chiral determination of Dopa

An acid BGE was used to ensure that benserazide and Dopa would be present as cations. A Tris–citrate BGE at pH 2.5 was tested for this purpose. Under these conditions, both analytes were protonated and migrated before the electroosmotic flow. Resolution between analytes was excellent and exceeded that achieved in the sole CE separation of these components reported so far (Fig. 2). Higher pH values resulted in no improvement; rather, the analysis time was lengthened through deprotonation of the acid group in Dopa.

Calibration graphs were obtained from the electropherograms for synthetic mixtures of benserazide and levodopa at 230 nm, where both analytes exhibit absorption, using the peak area corrected for migration time versus concentration. Table 1 shows the figures of merit of the calibration curves. As can be seen, the results exhibit good correlation and expose the lack of systematic errors in the calibration—the intercepts are statistically insignificant. Aliquots of two ground samples from different batches were analyzed in duplicate, the results being consistent between both sets and with the nominal contents (Table 2).

3.2. Chiral separation of Dopa

The chiral separation of the Dopa enantiomers by use of a BGE containing various chiral selectors was assayed. Dopa possesses an aromatic group that facilitates inclusion in the hydrophobic cavity of cyclodextrins; also, the presence of positive charge in its structure allows it to interact with anionic cyclodextrins. However, none of the neutral cyclodextrins tested [viz. β -cyclodextrin, γ -

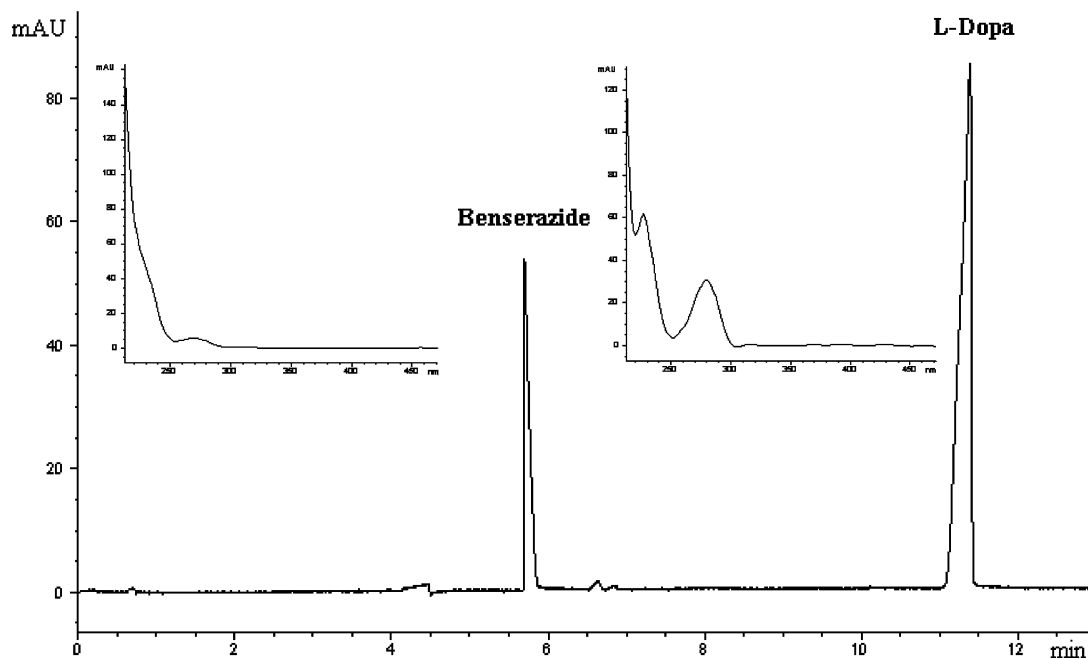


Fig. 2. Electropherogram for the pharmaceutical preparation Madopar® 250. 10 mM Tris–citric acid pH 2.5, 25 °C, 30 kV. UV–Vis spectra for benserazide (left) and levodopa (right).

cyclodextrin, heptakis(2,3,6)-tri-*o*-methyl- β -cyclodextrin and 2-hydroxypropyl- β -cyclodextrin] allowed the Dopa enantiomers to be efficiently separated, nor did an anionic cyclodextrin (viz. carboxymethyl- β -cyclodextrin).

We then tested (+)-(18-crown-6)-2,3,11,12-tetracarboxylic acid (18C6H₄), which possesses a

high enantiodiscriminating potential for primary amines. This selector has the disadvantage that its efficiency depends on the composition of the BGE; thus, sodium and potassium ions are to be avoided as they compete with the analyte for the ether cavity and inhibit enantiodiscrimination. The buffer used in the non-chiral separation was

Table 1

Figures of merit of the calibration curves used in quiral and nonchiral determinations, as well as to determine LOD and quantification (LOQ)

Curve ^a	Concentration range (mM)	Number of data points	Intercept ^c	Slope ^c	Correlation coefficient	LOD ^b (mM)	LOQ ^b (mM)	
<i>Non chiral determinations^c</i>								
Benserazide	0.24–2.4	6	0.48 ± 0.19	13483 ± 187	0.9994	0.079	0.19	
L-Dopa	1.2–12	6	0.17 ± 0.45	8439 ± 85	0.9999	0.15	0.42	
<i>Chiral determinations^d</i>								
L-Dopa	0.025–0.080	4	0.23 ± 0.22	0.2219	86467 ± 4210	0.997	0.013	0.025
D-Dopa	0.025–0.080	4	0.49 ± 0.20	0.2019	98425 ± 3384	0.9984	0.011	0.025

^a Expressed as corrected area by migration time vs. concentration.

^b Calculated as the signal exceeding three times (LOD) or ten times (LOQ) the standard deviation of the intercept.

^c Analytes detected in a conventional capillary at 230 nm.

^d Analytes detected in a light path extended X3 capillary at 198 nm.

^e Confidence intervals given as standard deviations.

Table 2
Quantification of Madopar[®] 250 tablets

Madopar [®] 250 mg	Benserazide ^a (mg/g)	L-Dopa ^b (mg/g)
Batch J3	51.62 ± 0.41	200.05 ± 2.13
Batch N25	51.93 ± 1.07	203.14 ± 0.50

The obtained value is the average of two aliquots injected in duplicate and the confidence intervals are given as standard deviations.

^a Nominal value = 50 mg/g.

^b Nominal value = 200 mg/g.

compatible with this chiral selector, even though Tris is a primary amine, because it possesses a very small inclusion constant [13]. A 10 mM concentration of 18C6H₄ afforded resolution of the Dopa enantiomers. The migration order of the analytes was checked by analyzing racemic mixtures enriched with the L-isomer. The D-enantiomer was found to migrate more slowly than the L-enantiomer, which indicates that D-Dopa forms a stronger complex with the chiral crown ether.

Determining enantiomeric purity requires a high chiral resolution in order to avoid overlap resulting from the high concentration of the major enantiomer. Chiral resolution was found to increase with increasing 18C6H₄ concentration up to 12 mM, beyond which it leveled off or even decreased. Such a concentration of the chiral selector was thus adopted for further testing.

The pH of the BGE can influence the enantiomer resolution as 18C6H₄ is a polycarboxylic acid with pK_a values of 2.1–4.9. Tests in acidic medium were performed in order to ensure full protonation of the amino group in Dopa (pK_{a2} = 8.72), and the pH range 2.5–4.5 was used to examine the effect of the degree of ionization of 18C6H₄. The resolution values obtained were close to 2 and decreased slightly with increasing pH. All other tests were conducted at pH 2.5, which provided slightly better enantiomeric separations.

The addition of an organic modifier such as methanol to the solvent altered the polarity of the medium, which strongly influences host–guest equilibria. This effect was examined by supplying the BGE with increasing proportions of this solvent from 0 to 25%. The presence of methanol increased chiral resolution and migration times.

The increased resolution can be ascribed to increased inclusion of the analyte in the ether cavity. In fact, inclusion is facilitated by a decreased polarity of the medium, which favors host–guest interactions and hence complexation. As a result, migration times increased from 10 min with 0% MeOH to 20 min with 25% MeOH. We chose 15% MeOH to be the optimum concentration of organic modifier as a compromise between resolution and analysis time.

One other factor influencing enantioresolution is the capillary temperature, the effect of which was examined over the range 15–35 °C. As a rule, the inclusion constant and chiral resolution increases with decreasing temperature [14]. However, decreasing the temperature also increases analysis times as it reduces the electrophoretic mobility of the analytes. A temperature of 15 °C was chosen in order to ensure optimal resolution in subsequent tests.

Under the selected conditions (viz. pH 2.5, 12 mM 18C6H₄, 15% MeOH, 15 °C), the resolution for Dopa enantiomers was close to 4. Also, benserazide enantiomers were separated with a resolution of 9 and posed no interference with Dopa enantiomers. However, the corrected peak areas for benserazide enantiomers were found to be 60:40, which indicates that the benserazide studied was not a racemate. This assertion could not be confirmed by CE as the enantiomers are not commercially available. The optical rotatory dispersion (ORD) spectrum exhibited a negative signal that confirmed the presence of an enantiomeric excess of the levorotatory form in the benserazide used.

3.3. Enantiomeric purity of Dopa

The good resolution achieved for the dilute racemate of Dopa allowed the enantiomeric purity of levodopa in the studied pharmaceutical to be determined. Quantifying large enantiomeric excesses requires not only a high sensitivity but also a high resolution and both parameters must be optimized jointly.

In resolving synthetic mixtures of dextrodopa at a concentration near its limit of detection (LOD) and excess levodopa, a loss of resolution was

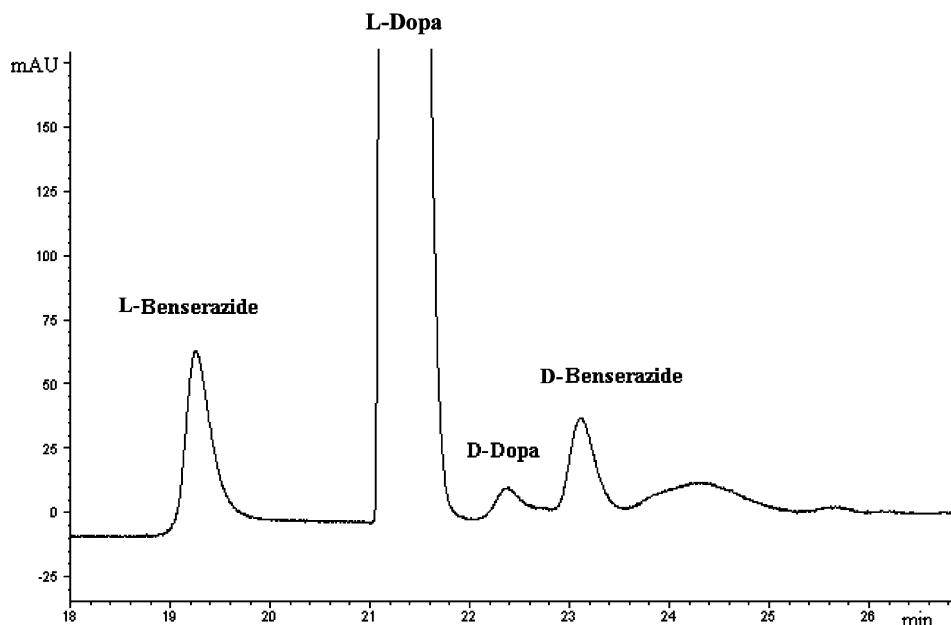


Fig. 3. Electropherogram obtained for the pharmaceutical preparation in chiral conditions. Tris–citric acid (10 mM) pH 2.5, 12 mM 18C6H₄, 15% MeOH, 15 °C.

found to occur because of the peak broadening of the major enantiomer. Moreover a high levodopa concentration produces tailing and the appearance of additional small peaks that preclude the determination of dextrodopa. These shortcomings were previously reported [15] and arise from overload of the capillary and adsorption effects.

Consequently, the highest enantiomeric purity that can be detected is restricted by the LOD of one enantiomer in the presence of the other rather than by the LOD of the technique concerned [1]. The following relative limit of detection (RLD), which takes account of this effect, is proposed:

$$\text{RLD} = \frac{\text{LD}_{\min}}{C_{\text{maj}}^{\max}} \quad (1)$$

where LD_{\min} is the LOD of the technique for the minor enantiomer and C_{maj}^{\max} the maximum concentration of the major enantiomer that can be injected without resolution, solubility or peak shape problems. The RLD thus defined for mixtures with a high enantiomeric excess is a measure of the smallest enantiomeric impurity that can be detected.

In order to reduce the LOD for the method, the analytes were measured in an extended-pathlength capillary, using a wavelength of 198 nm (where they exhibit a high absorbance). The LOD for dextrodopa ($\text{LD}_{\text{D-Dopa}}$) was estimated from the calibration graph run at low concentrations of this enantiomer. The limit thus obtained, 10^{-5} M, was consistent with that determined by visual inspection. The maximum eutomer concentration in each injection, C_{maj}^{\max} , was determined by injecting various levodopa solutions in the concentration range 10–50 mM. Based on the results, levodopa concentrations above 10 mM resulted in tailing in peak and in the appearance of small additional peaks that precluded the determination of dextrodopa, so this concentration was taken to be C_{maj}^{\max} . From Eq. (1), an RLD value of 0.1% was calculated.

The enantiomeric purity of the pharmaceutical was established by injecting a solution containing a levodopa concentration about 10 mM. The dextrodopa impurity was clearly resolved from the major peak and was determined to be 0.5% (Fig. 3). This is consistent with the results obtained by Husain et al. [10] for the enantiomeric purity of

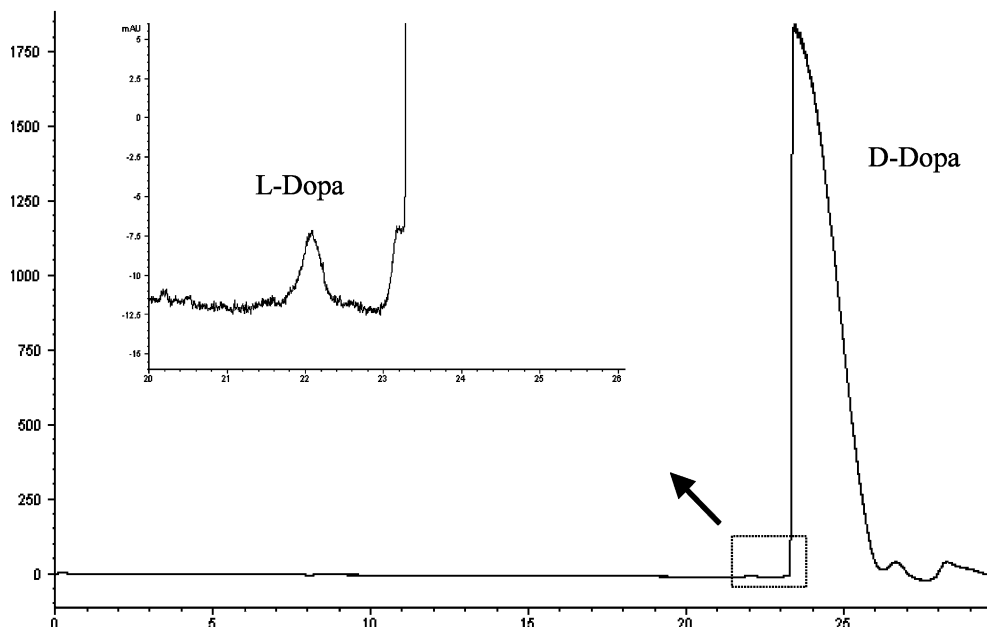


Fig. 4. Detection of levodopa impurity contained in dextrodopa (conditions as in Fig. 3). Residues of dextrodopa after the main peak can also be observed due to the high concentration of this analyte injected.

levodopa in pharmaceuticals using chiral HPLC. Our analyses also showed the corrected area ratio between the two benserazide enantiomers to be 70:30, which indicates that the studied product possesses an enantiomeric ratio different from that in the benserazide from Sigma.

The RLD, and hence the maximum determinable enantiomeric purity, was influenced by the enantiomer migration order. This was confirmed in the reverse situation, i.e. in determining the levodopa impurity accompanying the dextrodopa used in this work: the enantiomer migration sequence was more favorable and the $C_{\text{maj}}^{\text{max}}$ value for dextrodopa increased to 80 mM—higher levels were precluded by poor solubility—with no resolution or interference problems. Based on the LOD for levodopa (Table 1), RLD was calculated to be 0.02%.

In order to determine the amount of levodopa impurity contained in the dextrodopa used, a solution of the latter at a 80 mM concentration was injected. The results show a 0.05% of the levo isomer (Fig. 4). The enantiomeric purity was thus 99.95%, which is consistent with the minimum

enantiomeric purity for dextrodopa stated by the manufacturer.

4. Conclusions

The highly efficient resolution of the Dopa enantiomers achieved by using (+)-(18-crown-6)-2,3,11,12-tetracarboxylic acid affords application of the proposed method to the determination of enantiomeric purity. A RLD is proposed as a measure of the minimum chiral impurity that can be detected. As shown above, the RLD for the determination of dextrodopa contained in levodopa was 0.1% and found to depend on the enantiomer migration order. Thus, the RLD for levodopa contained in dextrodopa was only 0.02%. The proposed method was used to determine the enantiomeric purity of levodopa in a pharmaceutical preparation and also in pure dextrodopa. The results revealed that levodopa enantiomeric purity and dextrodopa enantiomeric purity was 99.5 and 99.95%, respectively. The CE method is a good alternative to the reported

HPLC method because a similar resolution factor for Dopa enantiomers is obtained and the RLD is low enough to determinate dextrodopa impurity in commercial formulations of levodopa.

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