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Food Chemistry 92 (2005) 381–386
Food Chemistry
www.elsevier.com/locate/foodchem

Analytical, Nutritional and Clinical Methods

Determination of levodopa by capillary zone electrophoresis using an acidic phosphate buffer and its application in the analysis of beans

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Received 8 December 2003; received in revised form 16 October 2004; accepted 18 October 2004

Abstract

A simple capillary zone electrophoresis method has been developed for the quantification of levodopa in broad bean and lentil. The dependence of effective mobility of levodopa on pH was investigated in the range of 1.64–11.20; and the simulated apparent dissociation constant values of levodopa were 2.30, 8.11 and 9.92, respectively, which were consistent with literature values. Meanwhile, we obtained the isoelectric point for levodopa was 5.20. Levodopa could be well separated from sample matrix and determined with conditions of 35 mM NaH_2PO_4, pH 4.55, 17.5 kV and 30 °C. A plot of the peak area on levodopa concentration was linear over the range of 5.0–300 μg/mL with a correlation coefficient of 0.9994. The method was validated for the quantification of levodopa in beans. The recoveries of added levodopa in different samples were 99.8% and 105.0%, respectively.

Keywords: Levodopa; Capillary zone electrophoresis; Apparent dissociation constant; Broad bean; Lentil

1. Introduction

The unusual amino acid levodopa (3,4-dihydroxyphenylalanine, L-DOPA) is the precursor required by the brain to produce dopamine, a neurotransmitter (chemical messenger in the nervous system). People with Parkinson's disease have depleted levels of dopamine and levodopa is used to increase dopamine in the brain, which reduces the symptoms of Parkinson's disease. Nevertheless, auto-oxidation of levodopa generates toxic metabolities, such as free radicals, semi-quinones and quinones. In vitro, levodopa is a powerful toxin that is lethal to the culture of neurons, and a few animal studies have shown that chronic levodopa may be toxic in vivo, too (Melamed, Offen, Shirvan, & Ziv, 2000).

As one of the antinutritional compounds, levodopa was first isolated 90 years ago from the fruit of broad bean (Brain, 1976). Recent literature also reported that minute quantity of levodopa exists in some beans (Viswanathan, Thangadurai, Vendan, & Ramesh, 1999; Vadivel & Janardhanan, 2002). A variety of analytical methods have been developed in order to measure levodopa levels in different sample matrices, such as HPLC (Cedarbaum, Williamson, & Kutt, 1987; Sagar & Smyth, 2000), spectrofluorimetry (Ma, 1999), circular dichroism (Shen, 1992), flow injection analysis (Marcolino-Junior, Teixeira, Pereira, & Fatibello-Filho, 2001), spectrophotometric (Coello, Maspoch, & Villegas, 2000; Hassib & El-Khateeb, 1990), photokinetic (Martinez-Lozano, Perez-Ruiz, Tomas, & Val, 1991), and catalytic anodic stripping voltammetric (Jin, Qian, & Fang, 1990) methods, etc. Botanists quantitatively estimated the concentration of levodopa in vegetal samples even by measuring the UV absorption at 283 nm after correction for background absorption yet (Brain, 1976;

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doi:10.1016/j.foodchem.2004.10.009

Vadivel & Janardhanan, 2002; Viswanathan et al., 1999). However, these methods suffer from limitations such as material waste and time consumption, because a number of preliminary steps are often required to obtain the species from the sample matrix.

Capillary electrophoresis (CE) has a theoretical resolution higher than that of other instrumental analysis techniques such as HPLC and this high efficiency is due to its characteristic flat flow profile. Presently, owing to its high resolving power, short analysis time, low solvent consumption and simple pretreatment requirements, CE has been used as an attractive method for separating and measuring different food components such as proteins, amino acids and nucleotides, etc. (Cortacero-Ramírez, Segura-Carretero, Cruces-Blanco, Hernáinz-Bermúdez de Castro, & Fernández-Gutiérrez, 2004; Mohamed, Rayas-Duarte, Gordon, & Xu, 2004). So far, analysis for levodopa accomplished in natural samples using CE has not been reported. The relative researches were only seen determination of levodopa in composite tablet sample with amperometric detection (Zhang, Chen, Hu, & Fang, 2001) and assessed the enantiomeric purity of levodopa in the pharmaceutical preparation (Blanco & Valverde, 2003).

The apparent dissociation constant (pK_a) is a biologically important parameter that is important for a thorough understanding of absorption, transport, and receptor binding of drugs at the molecular level (Takács-Novák et al., 1990). Although determination of a pK_a value using CE has been reported in literature, but obtain three pK_a values in an experiment was rare. The objective of this work was to apply the capillary zone electrophoresis (CZE) technique to the determination of pK_a values and quantification of levodopa in beans using acidic extracts, which rely on the solubility and stability of levodopa in an acidic environment. This work demonstrated that CE is a simple and powerful separating and determining approach in the food analysis realm.

2. Experimental

2.1. Apparatus

A Beckman P/ACE system 5510 (Beckman Coulter Instrument, Fullerton, CA, USA) was used in all the electrophoretic experiments. The system was controlled by the P/ACE station software and was equipped with a capillary cartridge, containing a 47 cm (40 cm from inlet to the detector) ×75 μm i.d. fused-silica capillary (Yongnian Photoconductive Fiber Factory, Hebei, China). The separation was carried out in one with a 100×200 μm detector window. Detection was performed by a photodiode array (PDA) detector that monitoring at 210 nm.

The capillary was treated prior to its first use by flushing with 1.0 M HCl for 10 min, 0.5 M NaOH for 15 min, and distilled water for 15 min. Between two runs, a rinse-cycle was used with 1.0 M HCl for 0.5 min, 0.5 M NaOH for 0.5 min, distilled water for 0.5 min and running buffer for 1 min. Samples were injected by applying a pressure of 0.5 psi for 3 s.

2.2. Reagents and materials

Levodopa was purchased from the National Institute for Control of Pharmaceutical and Bio-products, Beijing, China. Broad bean (*Vicia faba* L.) and lentil (*Lens culinaris* Medik.) were provided by Economic Crops Research Institute, Gansu Academy of Agricultural Science, China. All other chemicals were of analytical reagent grade.

Stock solution 0.50 mg/mL for levodopa was prepared in 10 mM HCl solution, from which analytical solutions were prepared by appropriate dilution with distilled water. The running buffers were prepared from 100 mM phosphate solution that was diluted with distilled water. The desired pH was adjusted by addition of 0.2 M HCl or 0.2 M NaOH.

2.3. Sample extraction

All the samples were crushed before extraction. The shatter of broad bean skin or the powder of lentil seed were dipped in 5 mL 10 mM HCl solutions, and extracted with an ultrasonic bath for 60 min. Then, the extracted mixtures were centrifuged. After being centrifuged, the extracts were poured off and the residue extracted once again using the same procedure. Combined twi-extracts and filtered through a filter paper as the sample extracts. The sample extracts and electrolyte solutions were filtered through a 0.45 μm membrane filter prior to use.

Sample extraction procedure was performed three times for each sample, and the three sample extracts were used to determination, respectively.

3. Results and discussion

3.1. Determination of pK_a

In this work, no neutral substance was used for electroosmotic flow (EOF) marker and the EOF mobility (μ_{EOF}) was calculated with the method of the migration time of EOF corresponding to the abrupt change in electric current (Li et al., 2001)

$$\mu_{EOF} = \frac{L^2}{t_i V}, \quad (1)$$

where L is the total length of the capillary; V is the applied voltage; $t_i = (t_1 + t_2)/2$, t_1 and t_2 are the times corresponding to the start and end point of abrupt change profile in electric current. So, the effective mobility (μ_{eff}) of the analyte can be calculated as

$$\mu_{eff} = \mu_{app} - \mu_{EOF} = \left(\frac{l}{t_m} - \frac{L}{t_i}\right)\frac{L}{V}, \quad (2)$$

where μ_{app} is the apparent mobility; t_m is the migration time of the analyte; l is the length of capillary from inlet to the detector.

The dependence of μ_{eff} on pH in range of 1.64–11.20 was tested and shown in Fig. 1, and taking into account the pK_a values of levodopa. This relationship is based upon the principle that a compound shows its maximum μ_{eff} when it is completely ionized; when it has no μ_{eff} in its neutral form and when it has intermediate μ_{eff} at pH around its pK_a. When the dependence of μ_{eff} on pH was considered in three sub-ranges, they can be simulated individually with following equation (Li et al., 1999):

$$\mu_{eff} = \frac{\mu_1 - \mu_2}{1 + e^{\frac{pH - pK_a}{c}}} + \mu_2, \quad (3)$$

where μ_1 and μ_2 are the effective mobilities at low and high pH values, respectively; c is a constant; and pK_a is the apparent dissociation constant of levodopa when ion strength is ignored. The simulated parameters were shown in Table 1. Then the determined pK_a values were obtained and they were well consistent with literature values, which were sought out from Internet as they were not embodied in common handbooks on chemistry.

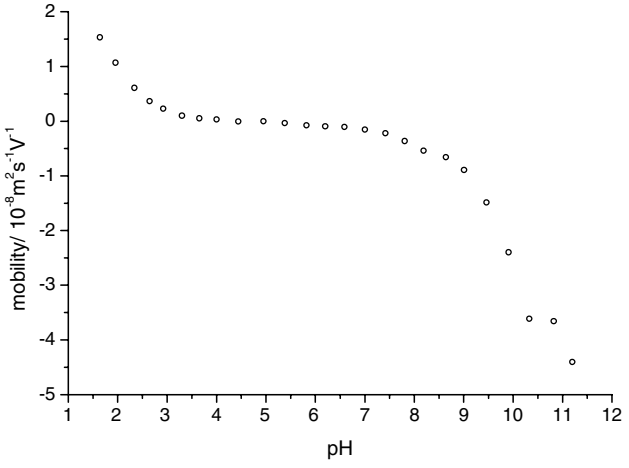

Fig. 1. Influence of the effective mobility on pH. Analytical conditions: 20 mM NaH_2PO_4 buffer; applied voltage 20.0 kV; temperature 25 °C; sample injection time 3 s with a 0.5 psi pressure, UV 210 nm.

To explain the pK_a value attached for which hydroxyl group, levodopa molecule was drawn with Hyperchem (Hyperchem 4.0, Hypercube, Inc., 1994) and pre-optimized using MM+ molecular mechanics force field. A more precise optimization was done with semi-empirical PM3 method in Hyperchem. The calculation was carried out at the restricted Hartree–Fock level with no configuration interaction. The molecular structure was optimized using the Polak–Ribiere algorithm until the root mean square gradient was 0.001. The bond lengths of N–H and O–H, the net charges on nitrogen atom and oxygen atoms of carboxyl and hydroxyl were calculated and shown in Fig. 2. By comparison the data, it was readily understood that the *p*-position more dissociable than *m*-position in the molecule.

It was demonstrated that the levodopa molecule becomes protonated at low pH values (pH < 2.30) to give the cationic form, and a marked positive μ_{eff} could be observed. When pH was equal to 2.30, the carboxyl group achieved dissociation equilibrium and when pH values were between 2.30 and 8.11, the levodopa molecule bearing both a positive and a negative charge, existed as a zwitterion (Skoog, West, & Holler, 1995). The zwitterion of levodopa, containing as it does a positive and a negative charge, has no tendency to migrate to an electric field, whereas the singly charged anionic and cationic species are attracted to electrodes of opposite charge. No μ_{eff} of levodopa occurs in an electric field when the pH of the buffer is such that the concentrations of the anionic and cationic forms are identical. The pH at which no μ_{eff} occurs is the isoelectric point (p*I*), and that is an important physical constant for characterizing

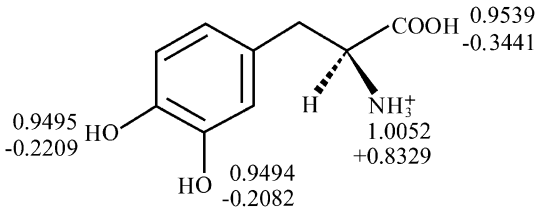

Fig. 2. Bond lengths of N–H, O–H and the net charges of N, O atoms.

Table 1
Nonlinear simulated results of effective mobility on pH

Part	pH range	μ_1 (10^{-8} m^2 s^{-1}V^{-1})	μ_2 (10^{-8} m^2 s^{-1}V^{-1})	c	pK_a	Literature value[a] of pK_a
1	1.64–5.38	1.5333	−0.0370	0.3243	2.30	2.30 (WEB, 2004a)
2	6.20–9.01	−0.0959	−0.8928	0.4082	8.11	8.2 (WEB, 2004b)
3	8.19–11.20	−0.5422	−4.4049	0.4024	9.92	9.9 (WEB, 2004c)

[a] The literature values were sought out from Internet.

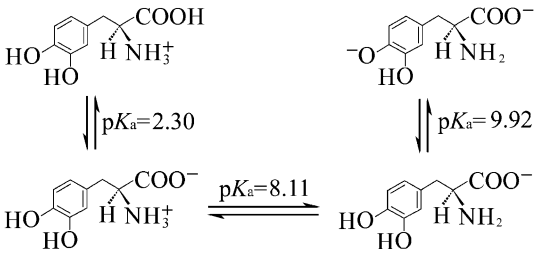

Fig. 3. Scheme of levodopa molecule dissociations.

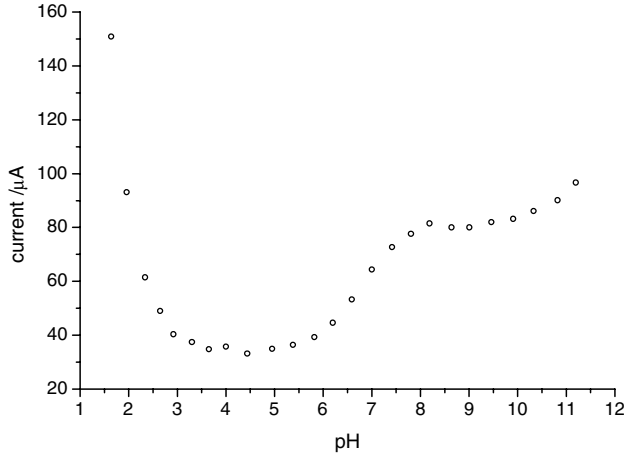

Fig. 4. Influence of the electric current on pH. Analytical conditions were the same as in Fig. 1.

amino acids. The pI was readily related to the ionization constants for the species. Thus, for levodopa

$$pI = (pK_{a1} + pK_{a2})/2 = 5.20.$$

When pH was equal to 8.11, the NH_3^+-group achieved dissociation equilibrium, and when pH values were higher than 8.11, the levodopa molecule gave the anion form and presented a negative μ_{eff}. When pH was equal to 9.92, the hydroxyl group in *para* position achieved dissociation equilibrium. The pK_a values and their corresponding equilibria were illustrated in Fig. 3. The change of electric current (Fig. 4) is also a demonstration for above conclusion.

3.2. Separation conditions

The effect of dissociations as discussed above and appearance of pseudo-peaks in electropherograms showed that levodopa was unstable in neutral and basic environment, so it could be analyzed only under acidic conditions. In addition, Fig. 4 showed that the electric current in pH range of 3–6 was lower and changed little. Considering the separation efficiency (evaluated with the theoretical plate number) and the symmetry of the peak shape, the pH of buffer was kept at 4.55 for subsequent experiments.

The concentration of phosphate was tested from 10 to 60 mM, and 35 mM phosphate was selected as the optimum concentration used in the analysis of the samples. It has been reported that addition of organic modifier to the buffer is an important parameter to improve the separation selectivity, efficiency and resolution as it modified the partition coefficient, mobile phase polarity and EOF (Liu, Wang, Zhang, Chen, & Hu, 2000). However, in the experiment, we tested mixing the phosphate buffer with different concentrations of ethanol, methanol and acetonitrile, respectively. The results indicated that organic modifiers had not improved the separation efficiency and instead, made the peak shapes deteriorated. So, the running buffer was comprised of only phosphate.

The effects of applied voltage and temperature were also investigated. The results indicated that higher voltage or temperature corresponded to shorter analysis time, but it implied increasing Joule heat generation. In this work, 17.5 kV and 30 °C were selected as the separation voltage and temperature.

3.3. Determination of samples

The linear relationship between the peak areas and the corresponding concentrations of levodopa were tested in the range of 5.0–500 μg/mL, but the peaks unusable for the quantification when concentration above 300 μg/mL due to the data deviation from the straight-line. The calibration curve was found only in the range of 5.0–300 μg/mL with a correlation coefficient of 0.9994. The linear regression equation is as follows:

$$A = 7792.8C + 124424.5,$$

where A and C are the peak area and the concentration (μg/mL) of levodopa, respectively. The limit of detection (LOD) calculated as the analyte concentration produced a peak three times higher than the baseline noise was 0.7 μg/mL.

The reproducibility of migration time and peak area were investigated, and its relative standard deviation (RSD) were 0.41% ($n = 4$) and 3.85% ($n = 4$), respectively.

When broad bean skin and lentil seed samples extraction, we tested water, ethanol and methanol as extraction solvents, and performed same procedure. The extraction efficiencies of organic solvents did not higher than water, might be because levodopa is a water-soluble substance. So, we selected water as extraction solvent. Each of broad bean skin and lentil seed samples weighted and extracted three times for determination.

Fig. 5 demonstrates the electropherograms of extracts of broad bean skin and lentil seed. The peak of levodopa was identified by increasing the peak area after adding the standard substance of levodopa in the sample solution and by referenced their UV absorption spectra.

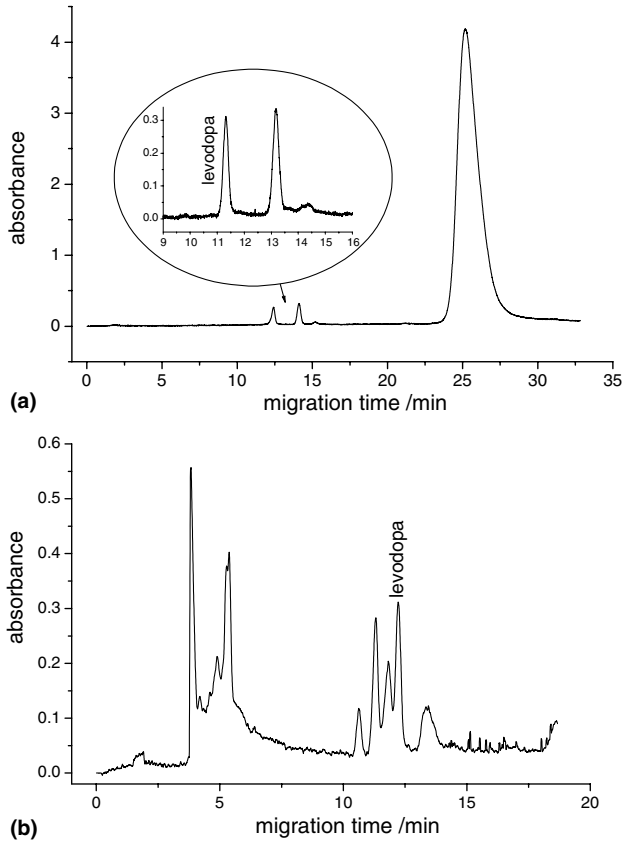

Fig. 5. Electropherograms of the sample solutions: (a) broad bean skin; (b) lentil seed. Analytical conditions: 35 mM NaH_2PO_4 buffer, pH 4.55, applied voltage 17.5 kV, temperature 30 °C; sample injection time 3 s with a 0.5 psi pressure, UV 210 nm.

The determination results of levodopa in broad bean skin and lentil seed samples shown in Table 2. The three times extraction repeatability of broad bean skin and lentil seed sample within 4.3% and 5.9%, respectively. The recoveries of the method were determined by standard addition method, and the results were also listed in Table 2.

3.4. Samples stability

Levodopa was present in small quantity compared with commonly consumed legumes that could only be eliminated after repeated boiling and decanting of seeds in water for at least 4 times (Viswanathan et al., 1999), another study reported that dry-heat treatment eliminated more than 45% of it (Siddhuraju, Vijayakiunari, & Janardhanan, 1996).

Table 2
Analysis result of levodopa in samples

Sample	Concentration of extract (μg mL^{-1})	Levodopa content (%)	Recovery (%)
Broad bean skin	11.7 ($n = 3$)	0.012	99.8
Lentil seed	22.0 ($n = 3$)	0.015	105.0

The stability of levodopa in acidic aqueous solution was demonstrated by heating the broad bean skin sample in boiling water bath for 5, 10, and 20 min, respectively. The migration time and peak area of analyte did not changed.

Another test was conducted by leaving extracts of the broad bean skin sample for 1–4 days at room temperature. The levodopa peak also has not obviously changed within 3 days. The extracts that had been placed for 4 days showed only one overlapped large peak in electropherogram, and levodopa peak could not be observed any more.

The results of this study showed that CE is an efficient tool to determine pK_a values. The sufficient separation capability was crucial to the interference with the co-existent component in UV absorption, and it was appropriate that adopt CE as an approach for the determination of levodopa in beans. In addition, the method is simple, making sample preparation very easy; and no organic solvents are employed, the cost of reagents and capillaries is reduced compared with that of HPLC solvents and columns; furthermore, the method is rapid. Thus, it is expected that analytical conditions similar to those developed in this work can be applied not only to beans, but also to other food samples.

Acknowledgement

This project was financial supported by National Natural Science Foundation of China (No. 20275014).

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