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DEVELOPMENT OF AN HPLC METHOD FOR THE SIMULTANEOUS DETERMINATION OF LEVODOPA AND CARBIDOPA IN BULK AND TABLET FORMS

O. A. O. MOHAMMED[1,2], S. W. SHANTIER[1], I. O. ABU REID[3], E. A. GADKARIEM[1]

1. Department of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Khartoum, Sudan, P .O. Box 1996
2. National Drug Quality Control Laboratory, Khartoum, Sudan
3. Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Ribat National University, Khartoum, Sudan

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Abstract: A selective, accurate and precise HPLC method has been developed for the simultaneous determination of levodopa and carbidopa in bulk and tablet form. Best resolution was achieved on a C_{18} column (5 µm particle size, 250 mm×4.6 mm I.D.) using a mixture of phosphate buffer pH 2.8 and acetonitrile (95: 5 v/v) as the mobile phase pumped at a flow rate of 1 ml/min. The constructed calibration curves were linear in the concentration range (25-250 µg/ml and 2.5 – 25 µg/ml for levodopa and carbidopa, respectively) with correlation coefficients close to 1.0. The accuracy and the precision of the developed method were very good (RSD < 2%). The validity of the proposed method was confirmed through the statistical comparison of the obtained data with those of the official USP method.

Keywords: Chromatography; HPLC; Levodopa; Carbidopa; Official method.

Corresponding Author: S. W. SHANTIER

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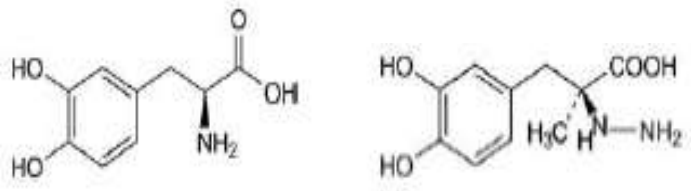
INTRODUCTION

Levodopa (Figures 1a), a naturally occurring amino acid, is the immediate precursor of the neurotransmitter dopamine. The actions of levodopa are mainly those of dopamine.

Unlike dopamine, levodopa readily enters the CNS and is used in the treatment of conditions, such as Parkinson's disease, that are associated with depletion of dopamine in the brain.

Carbidopa (Figures 1b), is a peripheral dopa-decarboxylase inhibitor with little or no pharmacological activity when given alone in usual doses. It inhibits the peripheral decarboxylation of levodopa to dopamine, unlike levodopa, it does not cross the blood-brain barrier, and effective brain concentrations of dopamine are produced with lower doses of levodopa. The combination of levodopa and carbidopa is used in the treatment of Parkinson's disease [1]. Levodopa and carbidopa tablets are available in dose ratio of 1:4 or 1:10 carbidopa to levodopa.



(a) **(b)**

Figure 1: Chemical structure of (a) levodopa and (b) carbidopa

Both the British Pharmacoepia (BP) and United States Pharmacoepia (USP) (2, 3) use reversed phase high performance liquid chromatographic method for the determination of the two drugs combination. Literature search revealed that various spectrophotmetric and chromatographic methods also have been used for the analysis of the two drugs in combination (4-13).

In this study, we are reporting a simple and accurate High performance liquid chromatographic method using a relatively cheap mobile phase constituents for the simultaneous determination of levodopa and carbidopa with optimum resolution between the two analytes within a reasonable analysis time.

MATERIALS AND METHODS

Instruments and apparatus

Experiments were performed on a Shimadzu Prominence HPLC system consisted of: degasser (Model DGU-20A5), pump (Model LC-20AD), Rheodyne manual injector fitted with 20 µl loop,

variable wavelength UV–VIS detector (ModelSPD-20A). Chromatographic separations were carried out on an Intersustain® (15 µm particle size, 250 mm×4.6 mm I.D.) from GL Sciences Inc., Japan. A Shimadzu UV-1800 240V spectrophotometer.

Standard and sample:

Levodopa (99.3%) and Carbidopa (100.2%) were kindly provided by National Medicines Quality Control Laboratory (NMQCL) - Sudan. Levocar® tablets (25/250 mg) and Credanil® tablets (25/250 mg) were purchased from the local market.

Chemicals and reagents

Chemicals

Sodium dihydrogen phosphate dehydrate, phosphoric acid (analytical grade), acetonitrile and sodium 1-decanesulfonate (HPLC grade) were purchased from Scharlau, Spain.

Diluent

Phosphoric acid (0.1M) was used as the diluent throughout the experimental work.

Phosphate buffer pH 2.8

Eleven grams of sodium dihydrogen phosphate were dissolved in 900 ml of distilled water, the pH was adjusted to 2.8 using phosphoric acid and the volume was made to one litre with distilled water.

Preparation of standards and solutions

Levodopa Standard stock solution

An accurately weighed about 125 mg of the working standard were transferred into a 100 ml volumetric flask, 70 ml of the diluent were added and the solution was sonicated for 5 minutes. The volume was then completed to the mark with the same diluent (Solution A; 1.25 mg/ml).

Carbidopa standard stock solution

An accurately weighed about 125 mg carbidopa of the working standard were transferred into a 100 ml volumetric flask, 70 ml of the diluent was added and the solution was sonicated for 5 minutes. The volume was then completed to the mark with the same diluent (Solution B; 0.125 mg/ml).

Linearity standard solution

A five points calibration solution was prepared by transferring aliquot volumes (2-20 ml) from each stock standard solution (Solution A and B) into five separate 100 ml volumetric flasks; the volumes of the flasks were then made to mark with the diluent.

Assay standard solution

This solution was prepared by transferring 10 ml each of solution A and B into a 100 ml volumetric flask and making the volume to mark with the diluent.

Sample preparation

An amount of powdered tablets equivalent to 25 mg carbidopa and 250 mg levodopa was accurately weighed and transferred into 100 ml volumetric flask, 70 ml of the diluent were added and solution was sonicated for 15 minutes with occasional swirling. The volume was then completed to the mark with diluent and filtered through 0.45µ nylon filter (Solution C). 5 ml of solution C were diluted to 100 ml using the diluent.

Procedure

Determination of detection wavelength

Two milliliters of solution A and B were transferred to two separate 100 ml volumetric flasks and diluted to mark with the diluent. The solutions were then scanned in a UV spectrophotometer in the range 190-400 nm to determine the suitable detection wavelength of each analyte.

Method development and optimization

Mobile phases containing different proportions of acetonitrile: phosphate buffer pH 2.8 pumped at 1 ml/minute flow rate were tried. Standard mixture containing 0.25 mg/ml levodopa and 0.025 mg/ml carbidopa was injected every time.

Method validation

Linearity, precision, accuracy and robustness were investigated as method validation parameters. The compound peaks were identified by their retention times and compared with the standards. Quantification was performed with five point external calibration curves. Precision was determined as repeatability (three samples) and intermediate precision (three samples on a different day). Accuracy was determined by sample spiking and robustness was determined by intentionally varying the method parameters within small range.

Linearity

Triplicate 20 µl injections were made from each linearity standard solution. The calibration curve of each analyte was obtained by plotting its average peak area against its corresponding concentration. The regression analysis data (slope, intercept and correlation coefficient) were calculated, further the limit of detection (LOD) and limit of quantitation (LOQ) were calculated from the regression analysis data according to the following formulae (11):

LOD = 3.3 σ/S and LOQ = 10 σ/S

Where σ = the standard deviation of the response, S = the slope of the calibration curve

Accuracy

To assess the accuracy of the method, recovery studies were carried out by spiking the sample with standard to 20%, 100%, and 200% of the nominal sample concentration (n = 3). The recovery% was then calculated using the following equation (13):

$\%\ recovery = (C_s - C_u)/C_A \times 100$

Where

C_s =concentration of spiked samples.

C_u = concentration of unspiked samples.

C_A = concentration of analyte added to the test sample

Precision

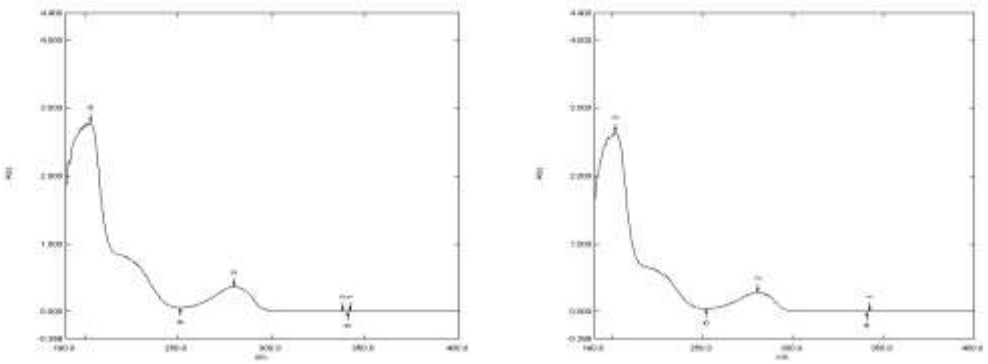
The method's within the day precision was determined by analyzing three sample solutions containing different concentrations of the analytes in the range of (20 -200%) of the nominal sample concentration, prepared by proper dilution from Solution C. Each sample was injected three times; the mean, standard deviation (SD) and the relative standard deviation (RSD) were calculated. To determine the between the days precision the procedure was repeated by a different analyst on a different day using a different instrument.

Robustness

Robustness was investigated by varying the following method critical parameters: pH (± 0.1), organic solvent ratio (± 1%), flow rate (± 0.1 ml/min), column supplier (different brand) and detection wavelength (± 5 nm).

RESULTS AND DISCUSSION

Due to their structural similarity, the two analytes showed very similar absorption features in the ultraviolet region (Figures 2a and 2b). The wavelength away from the stray light region and giving reasonable response for both analytes (280 nm) was selected as the chromatographic detection wavelength.



(a) **(b)**

Figure 2: UV spectrum of (a) levodopa and (b) Carbidopa

Method development and optimization

The official USP method for the determination of levodopa and carbidopa combination in tablets uses a mobile phase consisting of pH 2.8 buffer and a small amount of sodium-1- decane sulfonate; (polarity index = 10.20), although the mobile phase flow rate was 2 ml/min still the elution of carbidopa was very much delayed (11.7 minutes against 3.2 minutes for levodopa) leading to unnecessarily long analysis time.

In this study the main objectives were to overcome this problem and to avoid using the ion-pairing reagent for its cost and deleterious effect on the column. Since the two molecules are zwitterionic and possess very similar chemical structure it is possible to affect their separation by suppressing the ionization of one functional group, mainly the carboxylic function by using mobile phase of acidic pH namely 2.8 and exploiting the ionization of the amino functions to derive the separation of the two analytes. Different volume ratios of acetonitrile and the phosphate buffer pH 2.8 were tried at a flow rate of 1 ml/min.

Table (1) summarizes the results of the preliminary trials carried out to optimize the chromatographic conditions which can give satisfactory resolution for the two drugs.

The use of 30% acetonitrile in the mobile phase (polarity index = 8.88) resulted in completely overlapping peaks, with the reduction of acetontrile to 10% (polarity index = 9.76) the two

peaks were almost separated; however carbidopa peak was broad and not symmetrical. The best separation with a reasonable analysis time was obtained when the proportion of acetonitrile in the mobile phase was 5% (polarity index = 9.98) as shown in Fig. 3.

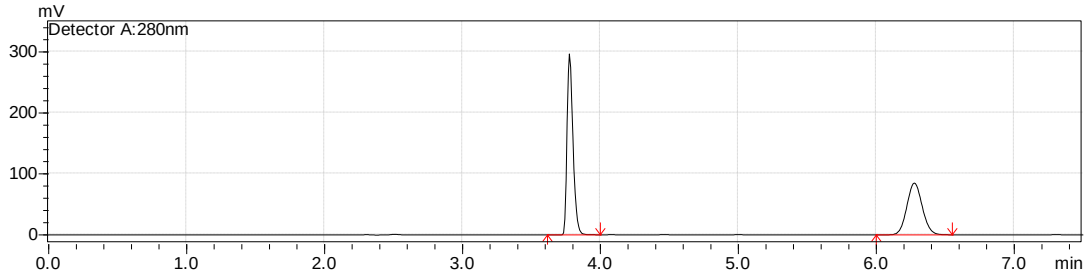


Figure 3: Typical chromatogram for drugs mixture using 5: 95 v/v acetonitrile: buffer solution (25:2.5µg/ml levodopa: carbidopa)

Method validation

Linearity

The constructed calibration curves were linear over the concentration range 25-250 µg/ml and 2.5-25 µg/ml for levodopa and carbidopa, respectively with correlation coefficients close to 1.0 for both analytes, indicating the proportionality of their responses with the concentration. Table 2 summarizes the regression analysis data and the analytes LOD and LOQ.

Accuracy

The obtained high percentage recoveries (minimum 97.53% and maximum 100.40%) indicate the accuracy of the developed method and freedom from interference. The recovery data at three levels (20, 100 & 200 %) is presented in Table 3.

Precision

The method repeatability precision (within the day) was proved by the low RSD% values; 0.12 - 1.14% and 0.12- 0.44% for levodopa and carbidopa, respectively. Further the intermediate precision (between the days) performed with a different analyst on a different day using a different instrument resulted in RSD% values of 0.14 and 0.71 % for levodopa and carbidopa, respectively.

Robustness

Robustness was assessed by intentionally varying the studied parameters within small range. The obtained results showed that the developed method can withstand small deliberate changes without affecting the method's system suitability parameters.

Assay

The developed method was applied for the determination of content uniformity. Good assay results were obtained for levodopa and carbidopa (99.70 ± 0.16 and 99.88 ± 0.20; n=3, respectively).

The validity of the method was also assessed by comparing the statistical results obtained with those of the official USP liquid chromatography method. As the calculated t- values were less than tabulated ones (n =4, P=0.05), the result of the developed method can be considered as accurate and precise as the official liquid chromatographic method (Table 4).

CONCLUSION

The developed method proved to be selective, accurate and precise for the analysis of levodopa and carbidopa in bulk and tablets form. The wide linearity range of the developed method allows the analysis of the marketed products of the two drugs having different dose ratios using one standard preparation. The method can also be used for the routine analysis of the drugs either in single or combination forms.

Conflict of Interests

The authors declare that there is no conflict of interest and the current work was self sponsored.

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Table 1. Results of optimization trials of the system

System	T_0	Levodopa				Carbidopa				Resolution
		RT	T. plate	T. F.	K_1	RT	T. plate	T. F.	K_2	
ACN: Buffer (70:30%)	Overlapped									
ACN: Buffer (30:70)	Overlapped									
ACN: Buffer (5:95%)	2.4	3.8	2966.80	1.01	0.58	6.27	10645.62	1.15	1.6	4.40
0.24g/l SDS in ACN: Buffer (5:95%)		3.8	3021.36	1.03		6.20	10622.41	1.15		4.34

Table 2. Regression analysis data for levodopa and carbidopa

Parameter	Levodopa	Carbidopa
Retention time (minutes)	3.99	6.20
Concentration range (µg/ml)	25-250	2.5-25
Slope± SE (*10^6)	15.32 ± 0.12	11.50±0.16
Intercept± SE (*10^3)	51.43±15.34	-3.24 ±1.98
Correlation coefficient (r^2)	0.9998	0.9994
Limit of detection (µg/ml)	3.77	0.98
Limit of quantitation (µg/ml)	11.44	2.96

Table 3. Percentage recovery at three levels

Analyte	Percentage recovery mean ± SD; n = 3		
	20 %	**100 %**	**200 %**
Levodopa	100.40 ± 0.36	99.07 ± 0.12	98.57 ± 0.38
Carbidopa	97.53 ± 0.15	97.97 ± 0.06	99.80 ± 0.90

Table 4: Results of the proposed method compared to the official method

		% content ± SD	**t - calculated (t - tabulated)**	**F-calculated (F- tabulated)**
Developed method	Carbidopa	99.88 ± 0.20	0.55 (2.78)	14.4 (19
	Levodopa	99.70 ± 0.16		
USP method	Carbidopa	98.00 ± 0.17	0.85 (2.78)	12.2 (19)
	Levodopa	99.12 ± 0.17		

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