DEVELOPMENT AND VALIDATION OF A SIMPLE AND NOVEL RP-HPLC METHOD FOR THE SIMULTANEOUS DETERMINATION OF LEVODOPA AND CARBIDOPA IN BULK FORM AND PHARMACEUTICAL DOSAGE FORM ACCORDING TO ICH GUIDELINES

Surajit Barman[1], Priyadarshini Sahoo*[1], Sumanta Sen[2], Ramya Sr.S[3]

[1]Department of Pharmaceutical Analysis, Indira Gandhi Institute Of Pharmaceutical Sciences, Bhubanesswar,India.

[2]Department of Pharmaceutics, School of Pharmacy,Arka Jain University, Jamshedpur, Jharkand, India

[3]Department of Pharmaceutics, University College of Technology,Osmania University, Hyderabad, Telangana, India

Address of Correspondence:

Priyadarshini Sahoo**,**

Department of Pharmaceutical Analysis,

Indira Gandhi Institute Of Pharmaceutical Sciences

Bhubanesswar,India.

ABSTRACT

A new, simple, Accurate, precise, robust and rugged reverse phase-HPLC method was developed for the simultaneous estimation of the Levodopa and Carbidopa in pure and pharmaceutical dosage forms. Chromatogram was run through Hypersil C18 (250 mm×4.6 mm, 5µm) particle size. Mobile phase containing Potassium dihydrogen phosphate (0.03M) (pH-2.8): Methanol (75:25%) was pumped through column at a flow rate of 1.0ml/min. Temperature was maintained at Ambient. Optimized wavelength selected was 226 nm. Retention time of Levodopa and Carbidopawere found to be 1.693min and 3.235min ± 0.02 respectively. The precision %RSD of the Levodopa and Carbidopawere and found to be 0.435 and 0.039 respectively. %Recovery was obtained as 100.06% and 100.083% for Levodopa and Carbidoparespectively. Regression equation of Levodopa is y = 48138x +

5396.0., and y = 71.91x + 42.07 of Carbidopa. The LOD and LOQ values were found to be for the Levodopaand Carbidopaare 1.27µg/ml, 1.16µg/ml3.81µg/ml, 3.48µg/mland the proposed method was found to be simple, precise, accurate, rapid, economic and reproducible for the estimation of Levodopa and Carbidopa in pure form and pharmaceutical marketed formulation.

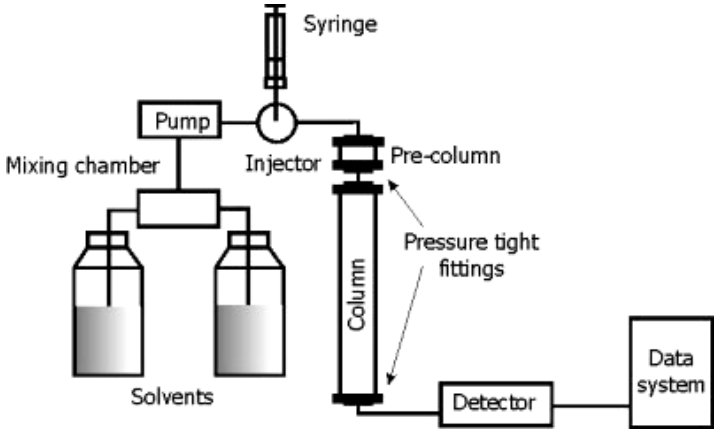
Keywords: Levodopa and Carbidopa, HPLC, Method Development, Validation.

INTRODUCTION

LIQUIDCHROMATOGRAPHY

Liquidchromatography(LC)isamethodofchromatographicseparationbasedon the difference in the distribution of species between two non-miscible phases, inwhich the mobile phase is a liquid which percolates through a stationary phasecontainedinacolumn.[1]

Liquid chromatography is mainly based on mechanisms of adsorption inliquid-solidchromatography,massdistributioninnormalphaseliquidchromatography (NPLC) and reversed-phase chromatography (RPLC), ionic in ion-exchangechromatography(IEC),sizeexclusionorstereochemicalinteractioninsize-exclusionchromatography(SEC)andaffinityinaffinitychromatography.[10]



Instrumentation

Themodulesof*HPLCunit*areillustratedinfigureno.2.Itconsistsofapumpunit,solventreservoirs,aninjector, acolumnanda detector.Theprincipleofoperationissimple.Thepumppushestheeluentthroughthecolumnata certainflowrate. When injecting the sample, the eluent passes through the injector and transfersthe sample in to the column. In the column, the sample components are separatedcomponentsaredetectedatthedetector.InmodernLCinstrumentstheoperationsarecontrolledbyacomputer.Inmostinstrumentsitispossibletocontrolthetemperatureof the eluent and column. In order to minimize peak broadening, the dead volume oftheunit,especiallyintheinjectionsystemandinthedetectormustbekeptsmall.

MATERIALS

Instruments:

WATERS HPLC, Model: Alliance 2695, Photo diode array detector (PDA), with an automated sample injector. The output signal was monitored and integrated using Empower 2 software. Agilent C8 (4.6 x 150 mm, 5 µm, Make: Waters) column was used for separations.

Chemicals :

Ortho-Phosphoric Acid, Potassium dihydrogen orthophosphate from Finar, Acetonitrile, Methanol,Water from Merck; Levodopa,Carbidopa provided by Sura Labs.

METHOD DEVELOPMENT:

Preparation Of The Levodopa And CarbidopaStandard And Sample Solution:

Preparation Of Standard Solution:

Accurately weigh and transfer 50 mg of Levodopa and 50 mg of Carbidopa into 50 ml of volumetric flask and add 10ml of water and sonicate 10min (or) shake 5min and make with water.

Further pipette required amount of Levodopa and Carbidopa from the above stock solutions into a 10ml volumetric flask and dilute up to the mark with Diluent.

Preparation Of Sample Stock Solution:

Commercially available six tablets ware weighed and powdered the powdered equivalent to the 585.58 mg of Levodopa and Carbidopaof active ingredients were transfer into a 50 ml of volumetric flask and add 10ml of methanol and sonicate for 20min (or) shake 10 min and make up with water.

Further pipette out required amountLevodopa and of Carbidopa from the above stock solutions into a 10ml volumetric flask and dilute up to the mark with Diluent.

Preparation Of Mobile Phase:

Transfer 1.36086gof Potassium dihydrogen phosphate into 1000ml of beaker and adjust pH 2.80 with orthophosphoric acid(OPA).

Transfer the above solution 750ml and 250ml of methanol is used as mobile phase. They are mixed and sonicated for 20minutes.

Optimized Chromatographic parameters:

Mobile phase : Potassium dihydrogen phosphate (0.03M) (pH-2.8): Methanol (75:25)

Auto sample temperature :Ambient

Injection volume : 20µL

Column : Hypersil C18 (250 mm×4.6mm,5µm) particle size

Detector wavelength : 226 nm

Flow rate : 1.0ml/min

Run time : 6 minutes

METHOD VALIDATION

1. System suitability: Tailing factor for the peaks due to Levodopa and Carbidopa in standard solution should not be more than 2.0. Theoretical plates for the Levodopa and Carbidopa peaks in standard solution should not be less than 2500.

2. Specificity: Solution of standard sample and placebo were prepared as per test procedure and injected into the HPLC system.

Acceptance criteria:

Chromatogram of standard and sample should be identical with near retention time.

Blank interference:

A study to establish the interference of blank was conducted. Solvent was injected into HPLC system as per the test procedure.

Acceptance criteria:

Chromatogram of blank should not show any peak at the retention time of analytepeak. There is no interference due to blank at the retention time of analyte.

3.Accuracy/Recovery: Recovery study can be performed in the concentration range of 50% to 150% of the target concentration of the test. Minimum 3 concentrations are recommended.

Acceptance criteria:

The average percentage recovery should be between 97-103% and relative standard deviation of these recovery concentrations was less than 2%.

4. Precision:

Preparation of sample:

Transfer the 802.04mg of sample into a 100ml of volume at flask and add 10ml of water and 10ml of methanol and sonicate 20min and makeup with water. Transfer the above solution into 5ml into 25ml volume metric flask dilute to the volume with water.

The method precision parameters were evaluated from sample chromatograms obtained, by calculating the % RSD of peek areas from 6 replicate injections.

Acceptance criteria: The injection reproducibility requirements are met if the %RSD for peak areas is not more than 2.0 and for retention time are not more than 2.0.

5. Linearity:

Prepare a series of standard solutions and inject into HPLC system. Plot the graph of standard versus the actual concentration in µg/ml and determine the coefficient of correlation and basis for 100% response.

Acceptance criteria:

Linearity regression coefficient of average peak area response of replicate injections plotted against respective concentration should not be less than 0.999. The % y-intercept as obtained from the linearity data (without extrapolation through origin 0, 0) should be within ±2.0.

Statistical Evaluation:

A graph between the concentration and the average area was plotted. Points for linearity were observed. Using the method of least squares, a line of best fit was taken and the correlation coefficient, slope and, y-intercept were calculated.

6. Robustness:

Effect of variation in flow rate:

Prepare the system suitability solution as per the test method and inject into the HPLC system with ±0.2 ml of the method flow. Evaluate the system suitability values as required by the test method for both flow rates. Actual flow rate was 1.0 ml/min and it was changed to 0.8ml/min and 1.2ml/min and inject into HPLC and system suitability was checked.

Effect of variation in Temperature:

Prepare the system suitability solution as per the test method and injected into the HPLC with ±5º Cof the method temperature. Evaluate the system suitability values as required by the test method for both temperatures.

7.LIMIT OF DETCTION:

The sensitivity of measurement of Levodopa and Carbidopa by use of proposed method was estimated in terms of the limit of detection (LOD). The LOD was calculated by the use of signal to noise ratio. In order to estimate the LOD value, the blank sample was injected six times and peak area of this blank was calculated as noise level. The LOD was calculated as three times the noise level.

$$LOD = 3.3\ \sigma / S$$

Where,σ = standard deviation of intercepts of calibration curves.

S = mean of slopes of the calibration curves.

The slope S may be estimated from the calibration curve of the analyte.

Minimum concentration of standard component in which the peak of the standard gets merged with noise called the LOD

$$LOD = 3.3* \sigma/S$$

Where;σ = standard deviation of response.

S = slope of calibration curve.

8. LIMIT OF QUANTIFICATION:

The sensitivity of measurement of Levodopa and Carbidopa by the use of proposed method was estimated in terms of limit of quantification (LOQ). The LOQ was calculated by the use of signal to noise ratio. In order to estimate the LOQ value, the blank sample was injected six times and thepeak area of this blank was calculated at noise level. TheLOQ was calculated as ten times the noise value gave the LOQ.

$$LOQ = 10\ \sigma / S$$

Where,σ = standard deviation of intercepts of calibration curves.

S = mean of slopes of the calibration curves.

The slope S may be estimated from the calibration curve of the analyte.

Minimum concentration of standard component in which the peak of the standard gets detected and quantification

$$LOQ = 10*\sigma/S$$

Where;σ = standard deviation of the response.

S = slope of the calibration curve.

RESULTS AND CONCLUSION

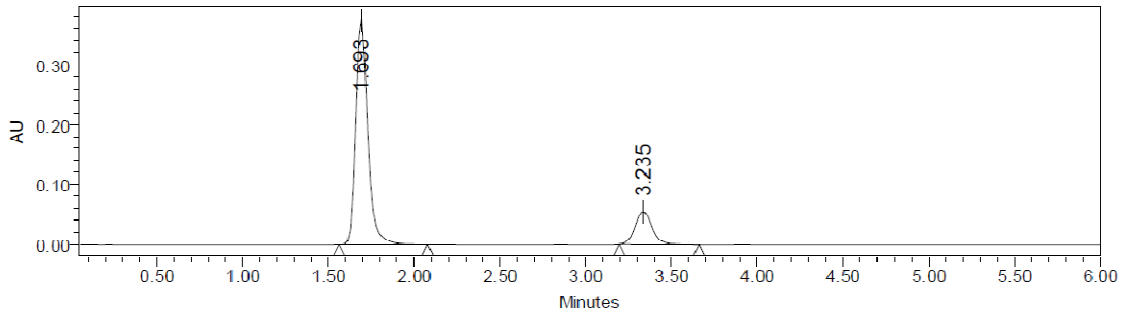


Fig no:Typical Chromatogram for optimized method

METHOD VALIDATION

SYSTEM SUITABILITY:

Table : System suitability data of Levodopa and Carbidopa

parameter	Levodopa	Carbidopa	Acceptance criteria
Retention time	1.691	3.299	_
Theoretical plates	5698	7529	>2500
Tailing factor	1.58	1.63	<2.00
% RSD	0.02	0.03	<2.00

SPECIFICITY:

Table :Specificity data for Levodopa and Carbidopa

S. no	Sample name	Levodopa		Carbidopa	
		Area	Rt	Area	Rt
1	Standard	3658985	1.691	6529	3.299
2	Sample	3785984	1694	6695	3.234
3	Blank	-	-	-	-

4	Placebo	-	-	-	-

RESULT

Chromatograms explain that retention time for standard, sample and commercial product of Levodopa and Carbidopa are same. This proves that, excipients have no effect on the analytical method. On the other hand, blank peak did not overlap drug peak. So the method is highly selective.

ACCURACY/RECOVERY:

Table : Accuracy data for Levodopa

S. no	Accuracy level	Injection	Sample area	Rt
1	50%	1	1928492	1.687
		2	1935674	1.691
		3	1927546	1.688
2	100%	1	3859865	1.688
		2	3865143	1.688
		3	3858748	1.688
3	150%	1	5785847	1.686
		2	5786423	1.685
		3	5789658	1.684

Table : Accuracy (%recovery) results of Levodopa

S. no	Accuracy Level	Sample name	µg/ml added	µg/ml found	% Recovery	% Mean
1	50%	1	40	39.949	99.872	99.979%
		2	40	40.098	100.245	
		3	40	39.929	99.822	
		1	80	80.071	100.088	

2	100%	2	80	80.180	100.225	100.124%
		3	80	80.048	100.060	
3	150%	1	120	120.080	100.066	100.091%
		2	120	120.092	100.076	
		3	120	120.159	100.132	

RESULT

Results of accuracy study are presented in the above table. The measured value was obtained by recovery test. Spiked amount of both the drug were compared against the recovery amount.

% Recovery was 100.00% for Levodopaand100.00% for Carbidopa. All the results indicate that the method is highly accurate**.**

PRECISION

Table : 17Precision studies for Levodopa and Carbidopa

S. no	Intraday precision for Levodopa			Intraday precision for Carbidopa		
	Peak area	Mean peak area	%RSD	Peak area	Mean peak area	%RSD
1	3658952	3665965	0.435	6598	6547	0.390
2	3659854			6529		
3	3659874			6537		
4	3658748			6538		
5	3698547			6546		
6	3659816			6534		

RESULT

Results of variability were summarized in the above table. The %RSD of peak areas was calculated for various run. Percentage relative standard deviation (%RSD) was found to be less than 2% which proves that method is precise**.**

LIMIT OF DETCTION

LOD= 3.3 σ / S

LOD for Levodopa = 1.27

LOD for Carbidopa = 1.16

LIMIT OF QUANTIFICATION

LOQ = 10 σ / S

LOQ for Levodopa = 3.81

LOQ for Carbidopa = 3.48

Where;

σ = standard deviation of the response.

S = slope of the calibration curve.

LINEARITY:

Table : Linearity data for Levodopa

S.no	Concentration (µg/ml)	Rt	Area
1.	40	1.689	1923835
2.	60	1.691	2899874
3.	80	1.692	3868985
4.	100	1.689	4835984
5.	120	1.688	5758747

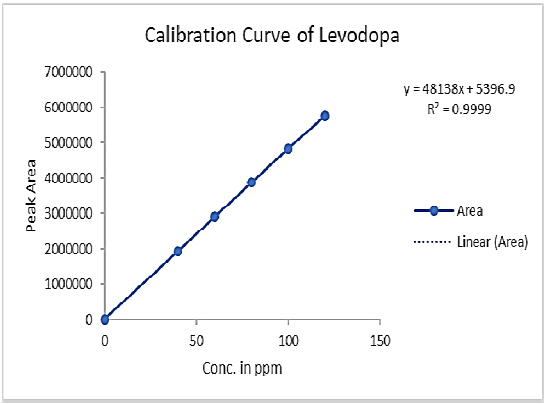
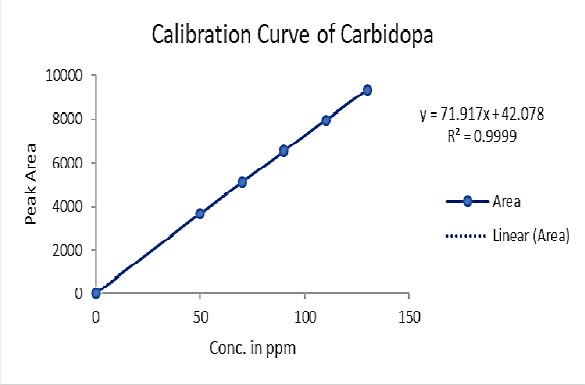


Table :Linearity data for Carbidopa

S.no	Concentration (µg/ml)	Rt	Area
1.	50	3.203	3675
2.	70	3.299	5108
3.	90	3.294	6529
4.	110	3.290	7954
5.	130	3.288	9349



ROBUSTNESS

Effect of variation in flow rate:

Prepare the system suitability solution as per the test method and inject into the HPLC system with ±0.2 ml of the method flow. Evaluate the system suitability values as required by the test method for both flow rates. Actual flow rate was 1.0 ml/min and it was changed to 0.8ml/min and 1.2ml/min and inject into HPLC and system suitability was checked.

Effect of variation in Temperature:

Prepare the system suitability solution as per the test method and injected into the HPLC with ±5º Cof the method temperature. Evaluate the system suitability values as required by the test method for both temperatures.

Table : Robustness data for Levodopa

Parameter	Rt	Theoretical plates	Tailing factor
Decreased flow rate (0.8ml/min)	1.868	5854	1.56
Increased flow rate (1.2ml/min)	1.544	5365	1.57
Decreased temperature (20^0c)	1.731	5418	1.53
Increased temperature (30^0c)	1.675	5496	1.54

Table : Robustness data for Carbidopa

Parameter	Rt	Theoretical plates	Tailing factor
Decreased flow rate (0.8ml/min)	3.621	7598	1.62
Increased flow rate(1.2ml/min)	2.998	7612	1.61
Decreased temperature (20^0c)	6.242	7251	1.64
Increased temperature (30^0c)	2.302	7195	1.61

CONCLUSION

In the present investigation, a simple, sensitive, precise and accurate RP-HPLC method was developed for the quantitative simultaneous estimation of Levodopa and Carbidopa in bulk drug and pharmaceutical dosage forms.

This method was simple, since diluted samples are directly used without any preliminary chemical derivatisation or purification steps. Levodopa was found to be readily sol in dil. hydrochloric and formic acids; practically insoluble in ethanol, benzene, chloroform, ethyl acetate, soluble in water.Carbidopa was found to be slightly soluble in water, Soluble in 100% ethanol and in methanol, freely soluble in 3N HCl, very slightly soluble in ethanol (96 per cent), practically insoluble in methylene chloride.
Potassium dihydrogen phosphate (0.03M) (pH-2.8): Methanol (75:25) was chosen as the mobile phase. The solvent system used in this method was economical. The %RSD values were within 2 and the method was found to be precise.

The results expressed in Tables for RP-HPLC method was promising. The RP-HPLC method is more sensitive, accurate and precise compared to the Spectrophotometric methods. This method can be used for the routine simultaneous determination of Levodopa and Carbidopa in bulk drug and in Pharmaceutical dosage forms.

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