International Journal of Pharmaceutical Research and Development (IJPRD)
Platform for Pharmaceutical Researches & Ideas
www.ijprd.com

DEVELOPMENT AND VALIDATION OF STABILITY-INDICATING RP-HPLC ASSAY METHOD FOR LEVODOPA AND CARBIDOPA IN LEVODOPA, CARBIDOPA AND ENTACAPONE ER TABLETS

Raj Kumar Dhawan[1]*,
Dr.R.Ravi[1], Dr.T.Subburaju[2], H.Revathi[1], C.Arul[3], K.Gopalakrishnan[4]

[1]Karpagam University, Pollachi main road, Coimbatore - 641021, Tamilnadu, India.
[2]Blue Mountain Botanicals, 262Q High Level Road, Fern Hill post,Ooty-643004.
[3]Hetero labs Ltd, 22-110, IDA, Jeedimetla, Hyderabad - 500055, Andhrapradesh, India.
[4]Actavis pharma Manufacturing Pvt Ltd, Sidco Pharmaceutical Complex 1st Floor, Alathur Near Sipcot Industrial Estate, Tiruporur, Chennai, Tamilnadu, India.

ABSTRACT

The objective of the current study was to develop and validate a simple, precise and accurate stability-indicating reverse-phase high-performance liquid chromatography (RP-HPLC) assay method for the determination of Levodopa and Carbidopa in Levodopa, Carbidopa and Entacapone ER tablets. RP-HPLC separation was achieved on an Inertsil ODS 3V, 250 × 4.6 mm, 5-µm column using a mobile phase containing a gradient mixture of mobile phase A and B. The flow rate of mobile phase was 1.5 ml/minute. The detection was carried out at 280 nm using a photodiode array detector. The drug was subjected to oxidation, hydrolysis, photolysis, humidity and heat to apply stress conditions. The method was validated for specificity, linearity, precision, accuracy, robustness and solution stability. The method was found to be linear in the drug concentration range of 80.200µg/ml to 240.600µg/ml and 20.169µg/ml to 60.506µg/ml with correlation coefficients of 0.99992 and 0.99993 for Levodopa and Carbidopa respectively. The precision (relative standard deviation, RSD) among six-sample preparation was 1.29% and 1.23% for Levodopa and Carbidopa respectively. Repeatability and intermediate precision (RSD) among six-sample preparation were 1.05% and 1.52% for Levodopa and Carbidopa respectively. Degradation products produced as a result of stress studies did not interfere with the detection of Levodopa and Carbidopa, therefore the method can be considered to be stability-indicating.

KEYWORDS : Levodopa, Carbidopa, Entacapone, RP-HPLC.

Available online on www.ijprd.com

Correspondence to Author

Raj Kumar Dhawan

Plot No.1054, behind ICICI ATM,
Pragathi Nagar, Hyderabad – 500072.

Email: dhawan_raj@yahoo.com

INTRODUCTION

Levodopa, Carbidopa and Entacapone ER tablets contain three active substances in one film coated tablet. Levodopa is used for the treatment of Parkinson's disease [1,2]. But with Carbidopa and Entacapone which improve the antiparkinson effects of Levodopa, the elimination half-life of Levodopa which is the active moiety of antiparkinson activity was 1.7h. Levodopa is extensively metabolized to various metabolites. Two major pathways are decarboxylation by dopa decarboxylase (DDC) and O-methylation by catechol-O-methyltransferase (COMT). Current evidence indicates that the symptoms of Parkinson's disease are related to depletion of dopamine in the corpus striatum. Administration of dopamine is ineffective in the treatment of Parkinson's disease apparently because it does cross the blood-brain barrier[3]. However, Levodopa, the metabolic precursor of dopamine, does cross the blood-brain barrier and presumably is converting to dopamine in the brain. This is thought to be the mechanism whereby Levodopa relieves the symptoms of Parkinson's disease. When Levodopa is administrated orally it is rapidly decarboxylated to dopamine in extra cerebral tissues so that only a small portion of a given dose is transported unchanged to the central nervous system. Carbidopa inhibits the decarboxylation of peripheral Levodopa, making more Levodopa available for transport to the brain. When co-administered with Levodopa, Carbidopa increases plasma levels of Levodopa and reduces the amount of Levodopa required to produce a given response by about 75% [4]. Carbidopa prolonged the plasma half-life of Levodopa from 50minutes to 1.5hours and decreased plasma and urinary dopamine and its major metabolite, homovanillic acid. Entacapone is a selective and reversible inhibitor of catechol-O-methyltransferase (COMT)[5]. When Entacapone is given in conjunction with Levodopa and Carbidopa, plasma levels of Levodopa are greater and more sustained than the administration of Levodopa and Carbidopa alone. It is believed that at a given frequency of Levodopa administration, these more sustained plasma levels of Levodopa results in more constant dopaminergic stimulation in the brain, leading to greater effects on the signs and symptoms of Parkinson's disease[6,7].

International Conference on Harmonization (ICH) has made the need of a stability-indicating assay method for every drug candidate mandatory. A stability-indicating assay method helps in establishing the inherent stability of the drug which in turn provides assurance on detection changes in identity, purity and potency of the product on exposure to various conditions. In this study, the drug candidate is exposed to a variety of stress conditions like acidic, alkaline, thermal, photolytic and oxidative stress. As per the ICH guidelines stress testing of the drug substance aids in identifying the likely degradation products, which in turn can help in establishing the degradation pathways and the intrinsic stability of the molecule and validate the stability indicating power of the analytical procedures used[8]. Several analytical techniques are available for estimation of Levodopa and Carbidopa in bulk dosage and product form by RP-HPTLC, HPLC and UV spectrophotometeric method. For this product HPLC method is available in united state of pharmacopoeia-35 (usp-35)[9,10,11,12]. But in this method flow rate is 2.0ml/minutes which results in high system back pressure and in mobile phase ion pair reagent is used which is a costly chemical. Keeping this objective in mind an attempt has been made to develop and validated stability-indicating RP-HPLC assay method for Levodopa and Carbidopa in Levodopa, Carbidopa and Entacapone ER tablets which would be highly sensitive, good resolution and reproducible. Various validation parameters of the analysis like specificity, precision, linearity and accuracy have been measured as per ICH guidelines[13].

MATERIALS AND METHOD

Chemicals, Materials and Equipments

The liquid chromatographic system was of Waters Alliance 2695 separation module with 2996 PDA Detector, which consisted of following components: a gradient pump, auto injector and

column oven. The chromatographic analysis was performed using Empower-2 software (Waters, Milford, USA) on an Inertsil ODS 3V, 250 mm x 4.6 mm 5 µm column. Qualified standards of Levodopa, Carbidopa, Entacapone and their impurities were gifted by Wockhardt research centre, Aurangabad, India. All other chemicals and reagents used were analytical grade and HPLC grade and purchased from Merck Chemicals, India. Tablets and placebo were obtained from Hetero labs, Hyderabad, India.

METHOD

Preparation of Buffer:

Dissolved 2.76gm of sodium dihydrogen orthophosphate monohydrate in 1000ml of water, pH was adjusted to 2.8 with 10% Orthophosphoric acid and filtered through 0.45µm membrane filter.

Mobile Phase A:

Prepared a mixture of buffer and acetonitrile in the ratio of 97:03% v/v and degassed.

Mobile Phase B:

Acetonitrile.

Diluent:

Diluted 1.0ml of Orthophosphoric acid in 1000ml of water and mixed.

Chromatographic conditions: [Table 1]

Column : Inertsil ODS 3V, 250mmx4.6mm,5µm
Flow rate : 1.5ml/minute
Mode : Gradient mode
Wavelength : 280nm
Injection volume : 20µL
Column oven temperature : 30°C
Sample temperature : 10°C
Run time : 23minutes

Table 1: Gradient program

Time (Minutes)	% of mobile phase A	% of mobile phase B
0	100	0
6	95	5
8	10	90
12	10	90
13	100	0
23	100	0

Preparation of Standard solution:

Standard solution of Levodopa and Carbidopa were prepared by dissolving 160mg of Levodopa and 40mg of Carbidopa in to a 50ml volumetric flask and dissolved with diluent. This solution was further diluted to obtain final concentrations of Levodopa 160 µg/ml and Carbidopa 40 µg/ml.

Preparation of sample solution:

Twenty tablets was weighed and powdered. The powder equivalent to 200mg of Levodopa was transferred to 250ml volumetric flask and treated with 150ml of diluent, sonicated using ultra sonicator. This solution was filtered through 0.45µm membrane filter. This filtered solution was diluted with diluent to get the final concentration of Levodopa 160 µg/ml and Carbidopa 40 µg/ml.

Procedure for forced degradation:

Degradation studies were performed in tablet solution containing Levodopa 160 µg/ml and Carbidopa 40 µg/ml.

Stress degradation by hydrolysis under acidic conditions:

For acid degradation, 2ml of 1N HCl was added and it was refluxed for 30minutes at 70°C. After 30minutes this solution was injected in stabilized chromatographic condition.

Stress degradation by hydrolysis under alkaline conditions:

For alkali degradation, 2ml of 1N NaOH was added and it was refluxed for 15minutes at 70°C. After 15minutes this solution was injected in stabilized chromatographic condition.

Oxidative degradation:

For oxidation, 2ml of 30% solution of hydrogen peroxide was added and it was refluxed for 15minutes at 70°C. After 15minutes this solution was injected in stabilized chromatographic condition.

Thermal degradation:
For dry heat, sample was exposed to heat at 70°C for about 24 hours. Treated sample solution was prepared and injected.

Photo-Degradation condition:
Sample was exposed to UV light up to 200watt hour/square meter and subsequently to cool fluorescent light to achieve an illumination of 1.2 million lux hours. Treated sample solution was prepared and injected.

Humidity Degradation condition:
Sample was exposed to humidity at 40°C/75%RH for about 96hours. Treated sample solutions was prepared and injected.

METHOD VALIDATION
The method was validated according to International Conference on Harmonization guidelines (ICH) for validation of analytical procedures.

Specificity
For the determination of specificity of the method blank, placebo and sample solution were spiked with known impurities at 1% level of sample concentration in triplicate, individual solutions of Levodopa, Carbidopa and Entacapone were prepared and injected.

Linearity
Linearity of response was performed using Levodopa standard solution in the range of 80.200µg/ml to 240.600µg/ml and Carbidopa standard solution in the range of 20.169µg/ml to 60.506µg/ml.

System suitability and system precision
Precision of the system was evaluated by analyzing six replicate injections standard preparations. System suitability parameters like tailing factor, theoretical plates and % RSD value was calculated.

Method precision
In method precision six sample solutions of Levodopa, Carbidopa and Entacapone ER tablets were prepared, injected and the percentage assay of Levodopa and Carbidopa for six samples was calculated.

Intermediate precision
Ruggedness of the method has been verified by analyzing six samples by different analyst, using different instrument and different column on different day. The percentage assay of analyte's and overall %RSD for both method precision and intermediate precision were calculated.

Accuracy:
To check the accuracy of method, known amount of Levodopa and Carbidopa working standard spiked with placebo at 50%, 100% and 150% of test concentration were prepared in triplicate at each level. Mean %recovery was calculated.

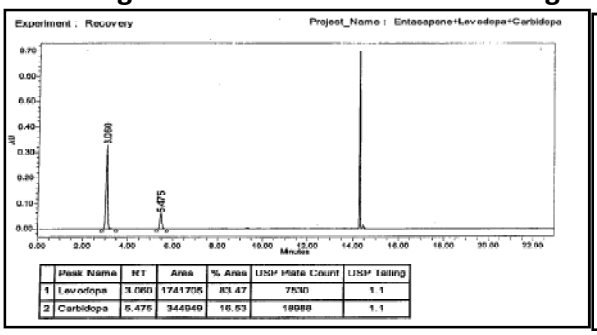
Robustness:
Robustness for the method was determined by analysis of samples under deliberately changed chromatographic conditions.

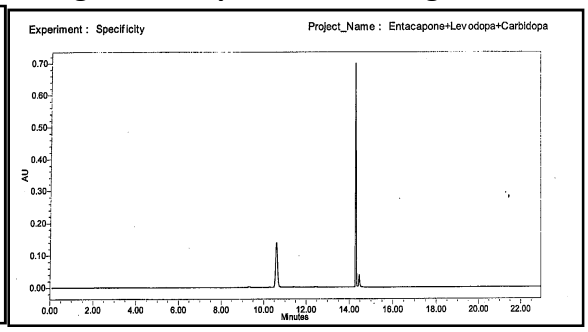
Stability in analytical solution
Stability of Levodopa and Carbidopa in analytical solution was verified by analyzing sample solution initially and also at different time intervals up to 24hours by storing the sample at 10°C.

RESULT AND DISCUSSION:

Development and optimization of Chromatographic conditions:
HPLC separations studies were carried out on the working standard solution of Levodopa (160 µg/ml) and Carbidopa (40 µg/ml). As per USP-35 the pH of mobile phase was 2.8 for the combination of Levodopa and Carbidopa tablets. However to achieve the better separation of levodopa and carbidopa in the present Levodopa, Carbidopa and Entacapone ER tablets, the mobile phase pH was kept at 3.0 and the column temperature was maintained 30°C. This resulted in good resolution and acceptable peak parameters. RT was found as 3.0 minutes for Levodopa and 5.5 minutes for Carbidopa. In this chromatographic condition, Entacapone also eluted at the RT 10.6, but along with some co-eluting peaks (placebo and impurities) [Fig.1and 2]. Therefore this method is not suitable for the estimation of Entacapone.

Fig 1: Standard solution chromatogram **Fig 2: Entacapone Chromatogram**





Stress Degradation Study [Table 2, 3]

ICH guidelines recommend 10-20% degradation for establishing stability indicating nature of the assay method. The degradation study (Liquid stage) indicated that Levodopa was susceptible to H_2O_2 and acidic hydrolysis more than alkali hydrolysis. For Carbidopa degradation was obtained in acidic, basic and H_2O_2 condition [Fig.3, 4, 5]. The degradation study (Solid stage) indicated that Levodopa and Carbidopa was susceptible to humidity stress more than photolytic and thermal stress condition [Fig.6, 7, 8]. Specificity of the method for the simultaneous estimation of levodopa and carbidopa in presence of their degradants was demonstrated by the absence of co-eulting peaks with main peaks.

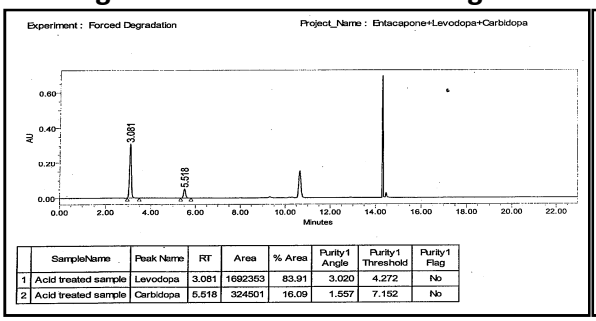
Table 2: Summary of Stress Degradation Study of Levodopa

Condition	%Assay	% Degradation	Peak purity of Levodopa		
			Purity Angle	Purity Threshold	Purity Flag
Untreated Sample	99.39	-	0.191	1.070	No
Acid Degradation	95.55	3.86	4.547	8.178	No
Base Degradation	97.61	1.79	2.936	4.117	No
Peroxide Degradation	96.99	2.41	3.020	4.272	No
Thermal Degradation	98.97	0.42	2.854	4.299	No
Photolytic Degradation	99.19	0.20	2.575	3.950	No
Humidity Degradation	96.06	3.35	2.838	3.980	No

Table 3: Summary of Stress Degradation Study of Carbidopa

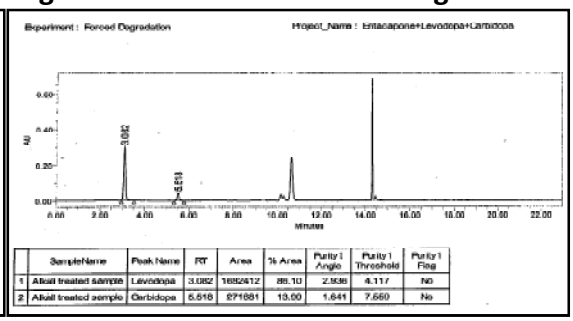
Condition	%Assay	% Degradation	Peak purity of Carbidopa		
			Purity Angle	Purity Threshold	Purity Flag
Untreated Sample	100.19	-	0.292	1.333	No
Acid Degradation	84.24	15.92	3.710	32.046	No
Base Degradation	78.71	21.44	1.641	7.559	No
Peroxide Degradation	92.76	7.42	1.557	7.152	No
Thermal Degradation	98.35	1.84	1.618	7.325	No
Photolytic Degradation	98.08	2.11	1.295	5.724	No
Humidity Degradation	91.56	8.61	1.589	6.684	No

Fig 3: Acid treated - chromatogram



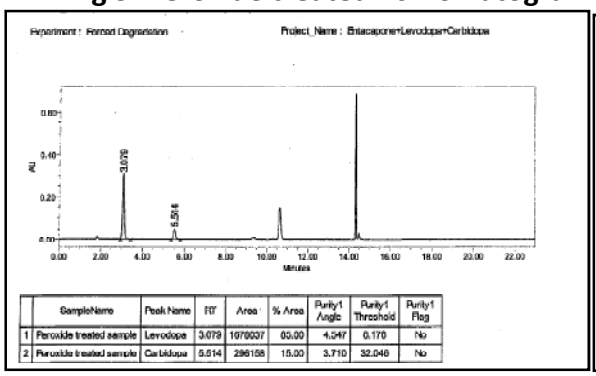
	SampleName	Peak Name	RT	Area	% Area	Purity1 Angle	Purity1 Threshold	Purity1 Flag
1	Acid treated sample	Levodopa	3.081	1692353	83.91	3.020	4.272	No
2	Acid treated sample	Carbidopa	5.518	324501	16.09	1.557	7.152	No

Fig 4: Alkali treated – chromatogram



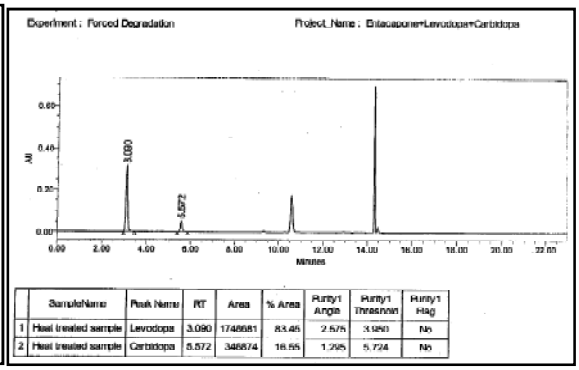
	SampleName	Peak Name	RT	Area	% Area	Purity1 Angle	Purity1 Threshold	Purity1 Flag
1	Alkali treated sample	Levodopa	3.082	1682412	86.10	2.936	4.117	NO
2	Alkali treated sample	Carbidopa	5.518	271681	13.90	1.641	7.660	No

Fig 5: Peroxide treated - chromatogram



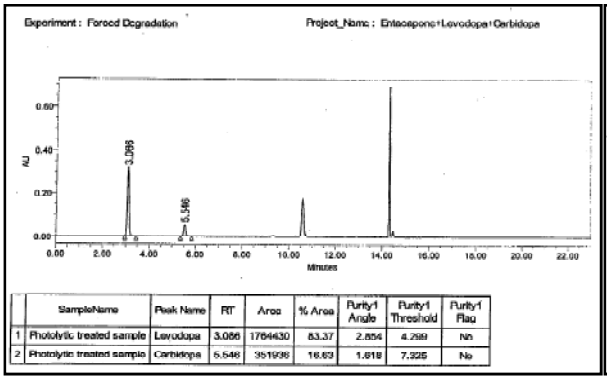
	SampleName	Peak Name	RT	Area	% Area	Purity1 Angle	Purity1 Threshold	Purity1 Flag
1	Peroxide treated sample	Levodopa	3.079	1979037	85.00	4.547	6.170	No
2	Peroxide treated sample	Carbidopa	5.514	296158	15.00	3.710	32.048	No

Fig 6: Heat treated - chromatogram



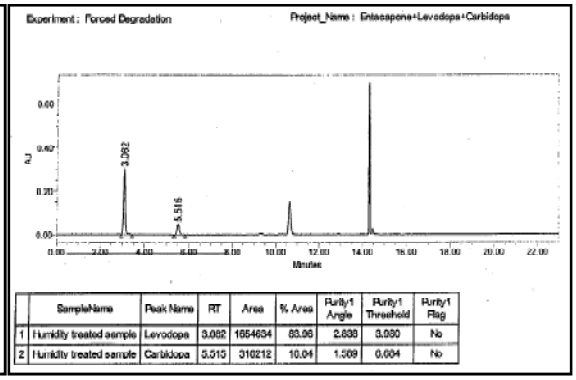
	SampleName	Peak Name	RT	Area	% Area	Purity1 Angle	Purity1 Threshold	Purity1 Flag
1	Heat treated sample	Levodopa	3.080	1748681	83.45	2.575	3.293	No
2	Heat treated sample	Carbidopa	5.572	346874	16.55	1.295	5.724	No

Fig 7: Photolytic treated-chromatogram



	SampleName	Peak Name	RT	Area	% Area	Purity1 Angle	Purity1 Threshold	Purity1 Flag
1	Photolytic treated sample	Levodopa	3.066	1764430	83.37	2.854	4.299	No
2	Photolytic treated sample	Carbidopa	5.548	351936	16.63	1.819	7.325	No

Fig 8: Humidity treated-chromatogram



	SampleName	Peak Name	RT	Area	% Area	Purity1 Angle	Purity1 Threshold	Purity1 Flag
1	Humidity treated sample	Levodopa	3.082	1654634	83.06	2.838	3.930	No
2	Humidity treated sample	Carbidopa	5.516	310212	16.04	1.509	6.664	No

Validation of the developed stability indicating method [Table 4]

Specificity

The Specificity of the method was determined by % assay difference between mean of spiked sample result and mean of method precision results. No interfering peak was eluted at the retention time of levodopa and carbidopa peak from blank and placebo.

Table 4: Validation Results

S.No.	Validation Parameters	Levodopa	Carbidopa
1.	Specificity	Specific	Specific
2.	System precision (%RSD)	0.20	0.22
	Theoretical plates	6979	22440
	Tailing factor	1.00	1.00
3.	Method precision (%RSD)	1.29	1.23
4.	Intermediate precision (%RSD)	1.05	1.52
5.	Accuracy-50% (%Recovery)	101.03%	99.45%

6.	Accuracy-100% (%Recovery)	99.64%	99.19%
7.	Accuracy-150% (%Recovery)	98.99%	99.14%
8.	Linearity- Correlation coefficient	0.99992	0.99993
9.	Stability in analytical solution (Cumulative %RSD)	0.22	0.68

Linearity

The data obtained in the linearity experiment was subjected to linear-regression analysis. A linear relationship between peak response and concentrations was obtained in the range of 80.200µg/ml to 240.600µg/ml for levodopa and 20.169µg/ml to 60.506µg/ml for carbidopa. The coefficient of regression for both the drugs was found to be nearer to 1.

System suitability and system precision

System suitability and system precision was daily performed during entire validation of this method. The results of system suitability and system precision were presented in [Table 4].

Method precision and Ruggedness

The developed method was found to be precise, as the overall % RSD for % assay of Levodopa and Carbidopa in both method precision and intermediate precision were less than 2.0.

Accuracy

Excellent recoveries were obtained at each level of spiked concentration. The result obtained (n = 3 for each level) indicated the mean recovery between 98% to 102% for both analytes.

Robustness [Table 5, 6]

The overall % RSD for % assay of method precision and Robustness variations for change in flow rate, change in wavelength, change in column oven temperature, change in pH of buffer and change in organic content in mobile phase was found to be less than 2.0.

Table 5: Robustness Results for Levodopa

Precision	-Flow	+Flow	-Temp	+Temp	-Org	+Org	-pH	+pH	-nm	+nm
99.69	99.64	99.89	99.30	99.42	97.20	97.51	98.05	98.19	99.81	99.90
97.38	97.43	97.22	99.41	99.50	98.66	98.85	98.80	98.91	97.34	97.37
99.88	99.69	99.59	101.03	101.13	98.45	98.16	98.10	98.22	99.93	99.87
98.59	-	-	-	-	-	-	-	-	-	-
99.59	-	-	-	-	-	-	-	-	-	-
101.18	-	-	-	-	-	-	-	-	-	-
Overall mean	99.23	99.22	99.56	99.60	98.96	98.98	99.03	99.07	99.27	99.27
Overall SD	1.226	1.275	1.156	1.168	1.264	1.229	1.167	1.139	1.264	1.260
Overall %RSD	1.24	1.29	1.16	1.17	1.28	1.24	1.18	1.15	1.27	1.27

Table 6: Robustness Results for Carbidopa

Precision	-Flow	+Flow	-Temp	+Temp	-Org	+Org	-pH	+pH	-nm	+nm
100.71	99.87	100.11	97.60	97.72	97.87	98.00	98.67	98.69	100.87	100.56
98.05	97.51	97.57	97.67	97.68	97.51	97.83	99.16	99.18	98.14	98.11
100.74	99.98	100.45	99.24	99.13	98.25	98.17	98.79	98.81	101.34	101.45
99.67	-	-	-	-	-	-	-	-	-	-
100.32	-	-	-	-	-	-	-	-	-	-
101.65	-	-	-	-	-	-	-	-	-	-
Overall mean	99.83	99.92	99.52	99.52	99.42	99.46	99.75	99.76	100.17	100.14
Overall SD	1.311	1.316	1.477	1.460	1.523	1.467	1.182	1.176	1.302	1.304
Overall %RSD	1.31	1.32	1.48	1.47	1.53	1.47	1.18	1.18	1.30	1.30

Stability in analytical solution

The sample solutions were not stable in room temperature (25°C). Hence the solution stability was performed at 10°C. The cumulative relative standard deviation was found below 2.0%. It

showed that the sample solution was stable up to 20 hours at 10°C.

CONCLUSION

This study indicates a simple, rapid and validated stability-indicating HPLC method for determination of Levodopa and Carbidopa in the presence of degradation products. All the degradation products formed during forced degradation studies were well separated from the analyte demonstrating that the developed method was specific and stability indicating. The developed and validated method was Specific and stability indicating, Linear, Precise, Accurate, Rugged and Robust for the determination of Levodopa and Carbidopa in Levodopa, Carbidopa and Entacapone ER tablets. The method can also be applied for marketed products.

ACKNOWELEDGEMENT

The authors are thankful to Wockhardt research centre, Aurangabad, India for providing working standards and their impurities. The authors also extend their thanks to hetero labs, Hyderabad, India for providing sample. The authors are also thankful to the vice chancellor, Karpagam University for providing necessary facilities and constant encouragement.

REFERENCES

1. Clane D.A, Definition of Parkinson's disease, Parkinsonism Related Disorders 2005, 11:S39-40.
2. David G.S, Anne B.Y., Treatment of Central Nervous System Degenerative Disorders. Joet G Hardman, Lee.E, Limbard, The Pharmacological Basis of Therapeutics, 10th edition, New York, Mc Graw Hill. 2001, P.552-560.
3. Rajput A, Rajput A.H, Parkinson's disease management strategies, Expert Rev Neurother. 2000,6(1):91-96.
4. John H., Parkinson's disease New Treatment Slow Onslaught of Symptoms, FDA Consumer. 1998; 32:34.
5. Castro A, Vallderoria F, Linazasoro G, Rodriguez-Oroz M.C, Stochi F, Marin C., Optimaization of use of LCE combination, Neurological. 2005, 20(4):180-188.
6. Lyons K.E, Pahwa R. Conversion from sustained release carbidopa and levodopa to carbidopa, levodopa and entacapone (Stalevo) in Parkinson disease patients. Clinical Neuropjarmacol. 2006,29(2):73-76.
7. Brooks D.J,Agid Y., Eggert K., Winder H., Ostergaard K., Holopainem A., TC-INIT Study Group, Treatment of end-of-dose wearing –off in parkinsons's disease, Europe Neurological. 2005;53(4):197-202.
8. ICH, Q1A (R2): Stability Testing of New Drug Substances and Products, International Conference on Harmonization, Geneva, February 2003.
9. United States of Pharmacopoeia Vol.2, Official monographs for USP 35, Carbidopa and Levodopa tablets 2012;2:2488.
10. Maitreyi Zaveri, Bhavita Dhru, Amit Khandhar., Simultaneous estimation of levodopa, carbidopa and entacapone in pharmaceutical dosage by validated reverse phase high performance liquid chromatography, International journal of institutional pharmacy and life sciences. 2012,2(1):10-19.
11. Deesha B.Gandhi and Priti J.Mehta. A Simultaneous RP-HPTLC method for determination of levodopa, carbidopa, and entacapone in combined tablet dosage form, Journal of Planar Chromatography. 2011;24:236-241.
12. P.C.Dmiani, A.C.Moschetti, A.J.Rovetto.F.Benavente and A.C.Olivieri., Design and optimization of a chemometrics assisted spectrophotmetric method for simultaneous determination of levodopa and carbidopa in pharmaceutical products, Analytica Chimica Acta. 2005,543:192-198.
13. ICH, Q1A (R2): Validation of Analytical procedure, International Conference on Harmonization, Geneva, February 2003.

*****