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Stability Indicating HPLC Method for Simultaneous Estimation of Entacapone, Levodopa and Carbidopa in Pharmaceutical Formulation

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Abstract

In pharmaceutical industry, researchers aim at catering to the need of robust analytical methods for analysis of generic drug products. The paper deals with the pharmaceutical formulation - Entacapone, Levodopa and Carbidopa tablets for the treatment of Parkinson's disease. The paper presents a simple and efficient stability indicating HPLC method that has been developed in a multi component drug formulation for simultaneous estimation of Entacapone, Levodopa and Carbidopa in presence of their related impurities. This HPLC method uses 'Cosmosil PE 150 × 4.6 mm, 5 µ' HPLC column, phosphate buffer pH 2.5 and Methanol as mobile phase in gradient mode with UV detection at 280 nm. The method was validated and found to be precise, robust, accurate, linear (in range 0.05 to 0.15 mg/ml, 0.012 to 0.15 mg/ml and 0.003 to 0.038 mg/ml of Entacapone, Levodopa and Carbidopa respectively), and specific for 15 known impurities ensuring suitability of the method for quantitative determination of Entacapone, Levodopa and Carbidopa.

Introduction

Parkinson's disease is a progressive, neurodegenerative disorder of the extrapyramidal nervous system affecting the mobility and control of the skeletal muscular system. Symptoms of Parkinson's disease are related to depletion of dopamine. But administration of dopamine is ineffective in the treatment of Parkinson's disease. This is because it does not cross the blood-brain barrier. However, levodopa, the metabolic precursor of dopamine, does cross the blood-brain barrier, and presumably is converted to dopamine in the brain. Carbidopa inhibits the decarboxylation of peripheral levodopa, making more levodopa available for transport to the brain. Entacapone is a selective and reversible inhibitor of catechol-O-methyltransferase (COMT). When entacapone is given in conjunction with levodopa and carbidopa, plasma levels of levodopa are greater and more sustained than after administration of levodopa and carbidopa alone.

It is very difficult to develop a stability indicating method for such triple combination drug products that is capable of analyzing each active ingredient in presence of their related impurities.

Literature survey revealed few methods for individual or combination product analysis, Spectroscopic methods for simultaneous estimation of Levodopa and Carbidopa [1], and LC estimation of Entacapone in tablets [2]. A method for in-vitro release of drugs is also found [3-5]. Publications were found on LC method for estimation using electrochemical detector [6] and LC method for estimation of levodopa and carbidopa using fluorescence detector [7-18]. Few official pharmacopial monographs for single and dual drug combinations [19-24] were also found. In the present study, we propose a rapid and stability indicating HPLC method for simultaneous estimation of Levodopa [(2S)-2-amino-3-(3,4-dihydroxyphenyl) propanoic acid], Carbidopa [(2S)-3-(3,4-dihydroxyphenyl)-2-hydrazino-2-methylpropanoic acid] and Entacapone [(2E)-2-cyano-3-(3,4-dihydroxy-5-nitrophenyl)-N,N-diethyl-2-propenamide] in presence of their 15 process related or degradation impurities [25-29].

Materials and Methods

Reagents and materials

All analytical reagent grade (AR Grade) reagents were used for method development purpose. Acetonitrile (Merck) and Tetrahydrofuran (Merck) were used for standard and sample solution preparation. Orthophosphoric acid (Rankem) and Potassium dihydrogen orthophosphate (Merck) were used for mobile preparation. Milli-Q water (HPLC grade) was used for all solution preparations. Impurities and working standards of Entacapone, Levodopa and Carbidopa were obtained from Macleods Pharmaceuticals Limited, Mumbai, India.

Chromatographic system and conditions

Development study was performed on Shimadzu HPLC, consisting of UV-Visible, photodiode array detector and a quaternary gradient pump. Sample loop in the system was of 100 µl capacity. Cosmosil 5PE 150 × 4.6 mm, 5 µ (Nacalai Tesque, USA) HPLC column was used for chromatographic separation. Mobile phase consisted of phosphate buffer and methanol in gradient mode. Buffer consisted of 10mM potassium dihydrogen orthophosphate solution with pH adjusted to 2.5 using orthophosphoric acid. Flow rate was 1.0 mL/min and detection was carried out at 280 nm based on there wavelength maxima as per UV spectrum. Labsolutions software was used for data collection. For intermediate precision study, Agilent HPLC system
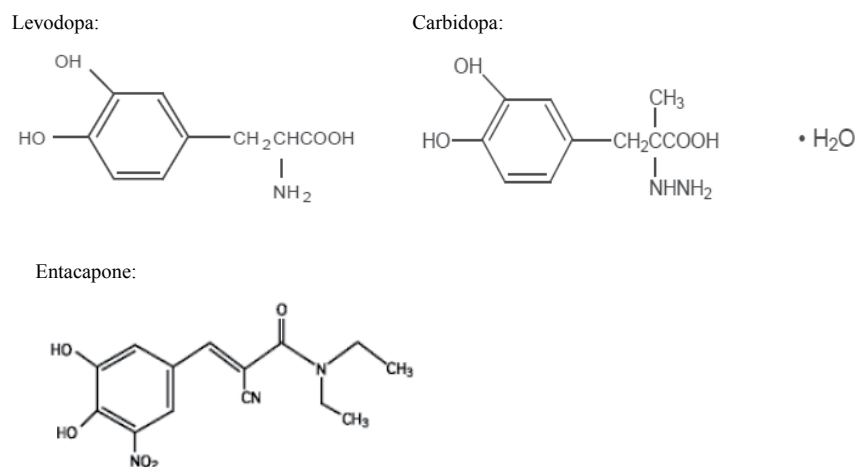
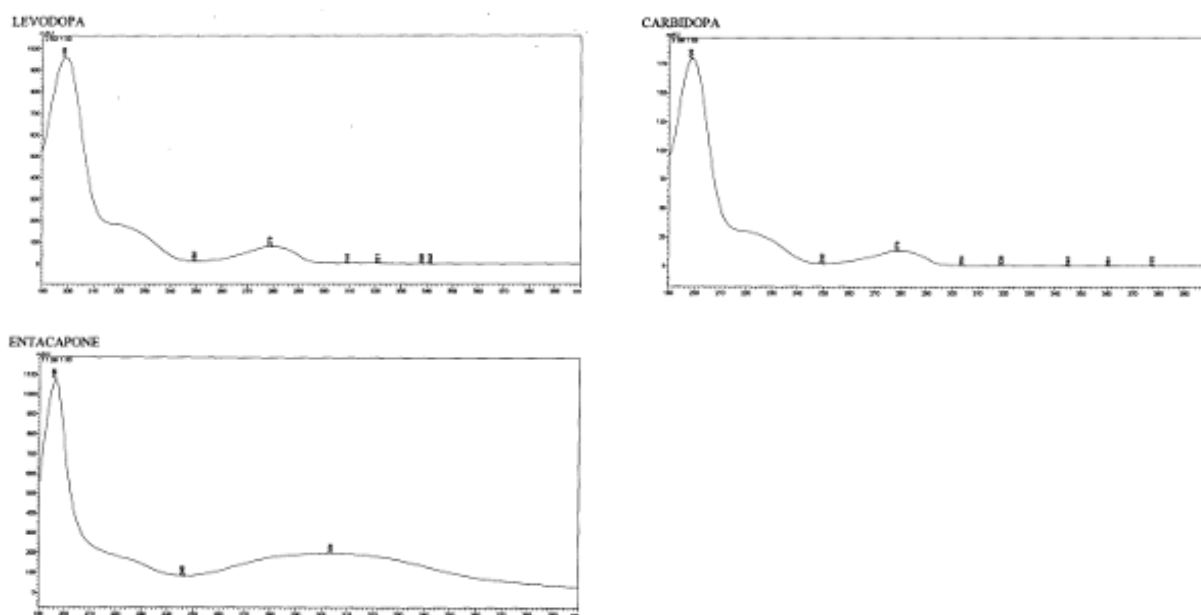**Figure 1:** Chemical structures of analyzed substances.

Figure 2: UV Spectrum.

with gradient pump, UV-visible detector and Chemstation software was used (Figure 1).

Results and Discussion

Preliminary studies

There is no pharmacopoeial or literature reference of a suitable stability indicating assay test for the proposed triple combination formulation. The development of Assay method was initiated from USP method for Levodopa tablets which use a simple HPLC method including octadecyl stationary phase and phosphate buffer pH 2.0/ acetonitrile for mobile phase.

Selection of wavelength: Wavelength was selected based on absorbance maxima of three drugs as per UV spectrum. 280 nm was optimum for all the active ingredients (Figure 2).

Selection of mobile phase: Due to difference in acidity of levodopa/ carbidopa and entacapone, low pH was selected to achieve optimum separation of all the peaks. Looking at the pH range of HPLC column, pH 2.5 was evaluated and found to be optimum.

Selection of HPLC column: Entacapone elute late on a C_{18} column even with 60% solvent in mobile phase (Figure 3). Levodopa and Carbidopa are polar in nature which makes them elute early on a non polar octadecyl (Inertsil ODS 250 mm × 4.6 mm, 5 μ) phase. In order to elute Entacapone early, a more polar phase was evaluated and selected for method development. Cosmosil PE, 150 × 4.6 mm, 5 μ was the column of choice. Phenyl phase is polar in nature but do not last long at low pH due to its weak bonding. Cosmosil PE column has an ethyl group attached to phenyl group which makes this column a rugged stationary phase with better column life than phenyl column. A 150 mm column was chosen to achieve a shorter run time.
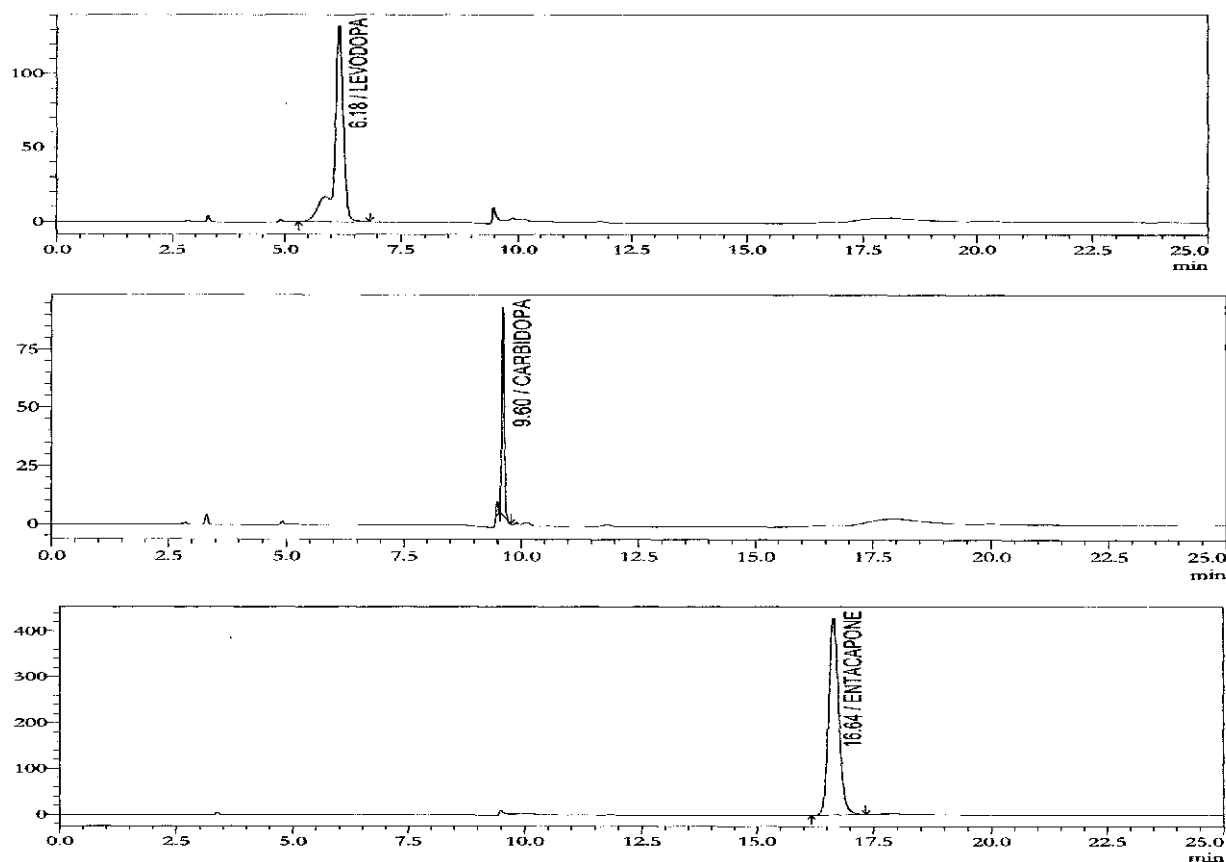
Figure 3: Preliminary chromatogram with C18 phase.

Time (min)	Phosphate Buffer (% v/v)	Methanol (% v/v)
0 → 5	98	2
5 → 6	98→40	2→60
6 → 13	40	60
13 → 14	40→98	60→2
14 → 18	98	2

Table 1: Gradient time program.

Selection of HPLC pump mode: Entacapone do not elute early with a low solvent mobile phase. Hence, gradient mode was chosen and optimized for separation of active ingredients with a flow rate of 1 ml/min and run time of 17 minutes (Table 1).

Selection of diluent: The difference in solubility of the active ingredients makes it difficult to finalize an optimum diluent. Levodopa and Carbidopa dissolves in acidic and aqueous condition whereas entacapone dissolves in less polar solvent like acetonitrile. Looking at the difference in solubility, a combination of 1% orthophosphoric acid and acetonitrile in the ratio 60:40 (diluent 1) was suggested for stock solution preparation. But entacapone has a tendency to precipitate on standing with acetonitrile and methanol in diluent. For better solubility of entacapone and stability of solutions with improved peak shapes, second dilution was performed in 1% orthophosphoric acid and tetrahydrofuran in the ratio 80:20 (diluent 2). Higher percent of tetrahydrofuran is not recommended due to its corrosive nature.

Solution preparation

Standard preparation: About 50 mg of Entacapone and about 31.25 mg of Levodopa was accurately weighed and dissolved in 50 ml of Diluent 1 (Solution A). About 31.25 mg of Carbidopa was dissolved in 100 ml of diluent 2 (Solution B). Further, 10 ml of (Solution A) and 5 ml of (Solution B) was diluted to 100 ml with diluent 2.

Sample Preparation: To prepare the sample, 5 intact tablets were transferred to a volumetric flask of 500 mL; 250 ml of diluent (1) was added to it and was sonicated for 30 minutes to dissolve. It was then cooled to room temperature and made up to mark with the same diluent. Filtered through 0.45 µ nylon filter, and further diluted 5 ml of the above solution to 100 mL with diluent (2), and mixed.

Method validation

Once optimum separation conditions are achieved, method is validated to ensure its suitability and reliability for routine use in estimation of % content of active ingredients in a pharmaceutical formulation. Validation parameters adopted are as follows:

Specificity: Specificity for blank, placebo, and known impurities was established by injecting known concentration of impurity solutions
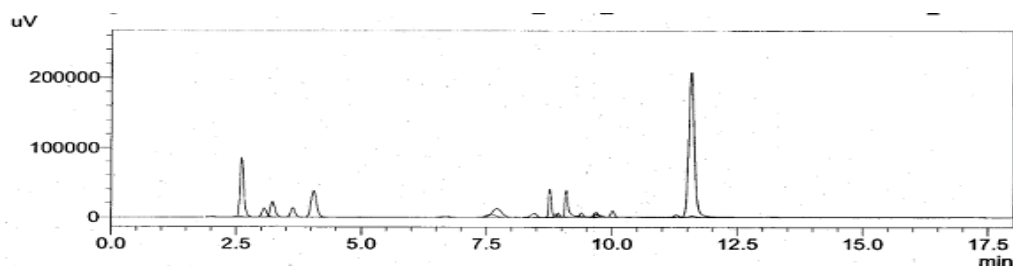
Figure 4: Overlaid Chromatogram of impurity solution for specificity.

Sr. No.	Sample Details	Retention Time (min)
1	Blank	No peak detected
2	Placebo Solution	No peak detected
3	Levodopa Related compound A	2.4
4	L-Tyrosine	3.1
5	Methyldopa	3.3
6	3-methoxy tyrosine	4.1
7	Methyldopa Methyl ester	7.6
8	Carbidopa Related compound A	7.9
9	1-veratryglycine	8.9
10	3-Acetyl-L-Tyrosine	9.0
11	3,4-Dihydoxyphenylacetone	9.2
12	Entacapone Impurity C	9.7
13	Entacapone Impurity F	9.7
14	Cyclohexylidine Carbidopa -Methyl Ester	10.0
15	Entacapone De-nitro	10.4
16	Entacapone Impurity A	11.3
17	Entacapone Methoxy Impurity	13.2
18	Levodopa	2.7
19	Carbidopa	3.9
20	Entacapone	11.8

Table 2: Values of retention time obtained.

(Figure 4). Specificity for unknown impurities was established by performing forced degradation study on tablet formulation as shown in Table 2. Peaks of interest were subjected to peak purity assessment using photodiode detector. All the peaks were found to be spectrally pure and no co-elution of any impurity was observed.

As shown in Table 3, No interference from blank, placebo and known impurities was observed at retention times of Entacapone, Levodopa and Carbidopa peaks.

Solution stability: Solution stability was evaluated by storing solutions at 25°C and 10°C. Carbidopa degraded by 2% at 25°C, whereas, solutions were found to be stable till 24 h when stored at 10°C (Tables 4 and 5).

Accuracy: Since Entacapone, Levodopa, and Carbidopa tablets have 7 strengths [(200+200+50), (200+175+43.75), (200+150+37.5), (200+125+31.25), (200+100+25), (200+75+18.75), and (200+50+12.5)], accuracy study was performed at 50% of the lowest concentration and 150% of the highest concentration of individual active ingredient. Recovery solutions were prepared by spiking Entacapone; Levodopa and Carbidopa API to placebo powder to obtain solutions of desired concentration (Table 6).

Linearity: A series of solutions were prepared by quantitative dilutions of the stock solution of standard to obtain solutions as mentioned in the following table. Each solution was injected and the peak area was recorded. Slope, Y-intercept and Correlation coefficient of the regression line were calculated (Table 7). In above, 200+200+50 mg strength was taken into consideration. By establishing linearity in entire working range, samples of all the 7 strengths can be analyzed against a single standard corresponding to any strength.

Precision

Repeatability: Six sample preparations were prepared and injected. The mean and relative standard deviation of the results was calculated. The results obtained for assay are tabulated in Table 8.

Intermediate precision: For intermediate precision analysis was carried out different day, using a different HPLC and different column. The absolute difference between the mean assay results obtained in repeatability and intermediate precision was calculated (Figure 5).

The obtained results for % assay and overall comparative data presented in the following Table 9.

The absolute difference between the mean assay results obtained in repeatability and intermediate precision is within the acceptance criteria of not more than 2.0. Hence, the method is precise.

Robustness: The Assay method was carried out as described

Forced Degradation Condition	% Degradation	% Impurity
Acid Hydrolysis: Exposure to acidic condition with 5M hydrochloric acid	About 5.7% Degradation of carbidopa observed	Methyldopa: About 1.16% DHP: About 1.16% Total Impurity: 5.34%
Base Hydrolysis: Exposure to basic condition with 5M sodium hydroxide	About 5.5% Degradation of carbidopa observed	Methyldopa: About 1.06% DHP: About 3.18% Total Impurity: 4.24%
Oxidative Degradation: Exposure to Oxidative condition with 3% hydrogen peroxide	About 9.5% Degradation of carbidopa observed	Methyldopa: About 1.20% DHP: About 2.24% Unknown Impurity about 6.5% Total Impurity: 9.94%
Thermal Degradation: Exposure to 80°C for 24 hrs	About 2.02% Degradation of carbidopa observed	Methyldopa: About 2.83% Total Impurity: 2.83%
Photostability: Exposure to UV Radiation NLT 1.2 million lux hours	About 1.6% Degradation of carbidopa observed	Methyldopa: About 2.13% Total Impurity: 2.13%
Humidity Degradation: Exposure to 40°C temperature and 75% Relative humidity	About 5.6% Degradation of carbidopa observed	Methyldopa: About 2.7% DHP: About 3.8% Total Impurity: 6.5%

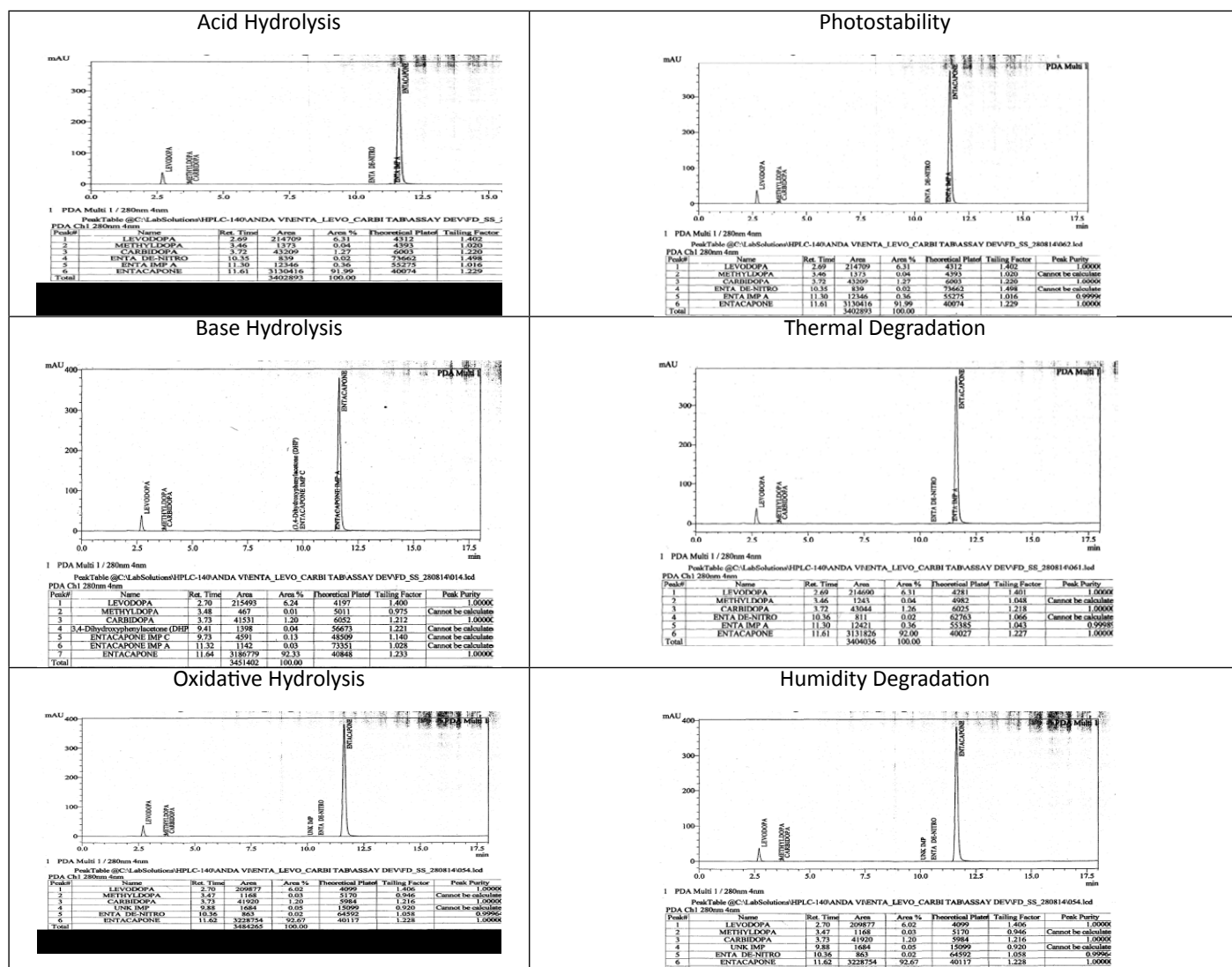**Table 3:** Observations of forced degradation study.

Table 4: Chromatograms of Forced Degradation.

Time (hours)	Entacapone			
	AT 25°C		AT 10°C	
			Area	% Difference w.r.t. Initial
Initial	3163365	-	3208318	-
24 hours	3161080	0.07	3199728	0.26
Time (hours)	Levodopa			
	AT 25°C		AT 10°C	
			Area	% Difference w.r.t. Initial
Initial	213017	-	218864	-
24 hours	214085	-	218927	-
Time (hours)	Carbidopa			
	AT 25°C		AT 10°C	
			Area	% Difference w.r.t. Initial
Initial	44007	-	43818	-
24 hours	42816	2.71	43589	0.52

Table 5: Observation of solution stability.

	% Recovery		
Level	**Entacapone**	**Levodopa**	**Carbidopa**
50%	99.2	100.0	98.7
100%	99.4	100.2	99.0
150%	99.6	98.5	99.2

Table 6: Accuracy results of the proposed method.

Entacapone			Levodopa			Carbidopa		
% Level	**Concen-tration (ppm)**	**Area**	**% Level**	**Concen-tration (ppm)**	**Area**	**% Level**	**Concen-tration (ppm)**	**Area**
50	50.33	1644591	12	12.08	105951	12	3.05	19826
60	60.39	1974936	25	24.17	211972	24	6.09	40184
66	66.43	2161375	50	50.35	436302	50	12.69	83963
75	74.48	2445512	75	74.52	631283	75	18.78	124561
100	100.65	3276792	100	100.70	860553	100	25.38	167461
120	120.78	3943031	120	120.84	1020742	120	30.46	201900
150	150.98	4926510	150	151.05	1295253	150	38.07	253235
Slope	2590.1783		**Slope**	8494.4494		**Slope**	6650.2203	
Y-Intercept	5024.5917		**Y-Intercept**	4069.0608		**Y-Intercept**	493.9482	
Correlation coefficient	1.000		**Correlation coefficient**	0.9998		**Correlation coefficient**	1.0000	

Table 7: Linearity results of the proposed method.

	% Assay		
	Entacapone	**Levodopa**	**Carbidopa**
Sample-1	98.8	100.9	95.6
Sample-2	99.4	101.0	96.3
Sample-3	99.3	101.2	95.5
Sample-4	98.8	101.1	95.7
Sample-5	99.0	101.4	96.4
Sample-6	98.5	101.3	96.1
Mean	98.96	101.15	95.9
% RSD	0.06	0.03	0.03

Table 8: Precision results of the proposed method.

in the methodology and by making the following alterations in the chromatographic conditions.

➢ Changing the flow rate of mobile phase (0.8 mL/min, 1.2 mL/min)

➢ Changing the pH of buffer of mobile phase (pH=2.3, pH=2.7)

The results for system suitability are presented in Tables 10-12.

The system suitability parameters like % relative standard deviation for five replicate injections of standard solution, tailing factor and theoretical plates were not significantly changed with altered conditions. Hence, it is concluded that, the method is robust to deliberate changes made in analytical method.

Conclusion

A simple and efficient stability indicating HPLC method for simultaneous estimation of Entacapone, Levodopa and Carbidopa in
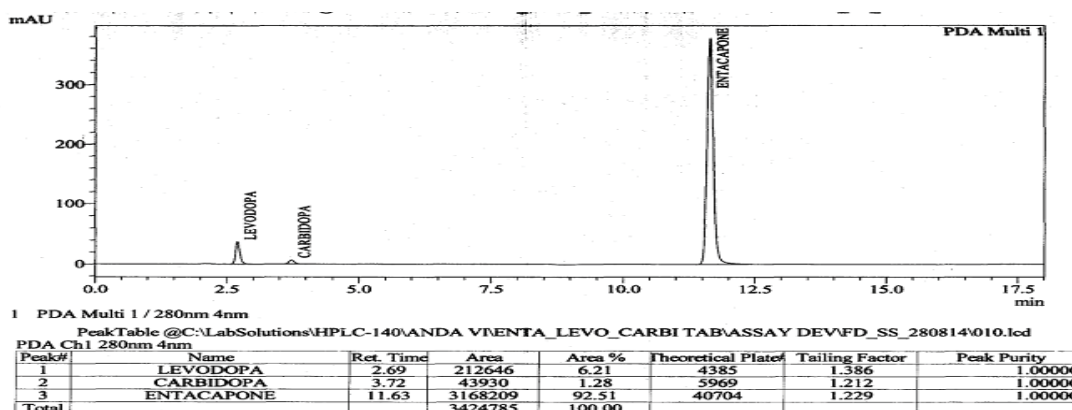
Figure 5: Representative chromatogram of sample.

	% Assay		
	Mean Assay in Repeatability (Precision)	Mean Assay in Intermediate Precision	Absolute difference
Entacapone	98.96	100.15	1.19
Levodopa	101.15	101.18	0.03
Carbidopa	95.9	96.6	0.6

Table 9: Comparison of precision and intermediate precision results of the proposed method.

Altered condition	Retention time (min)	Tailing Factor	Theoretical plates	% RSD	%Assay
Normal (unaltered) (Repeatability)	11.6	1.19	44742	0.08	98.9
Flow rate of mobile phase (0.8 mL/min)	12.94	1.20	43095	0.08	100.5
Flow rate of mobile phase (1.2 mL/min)	10.61	1.18	47443	0.09	99.4
pH of buffer of mobile phase (pH=2.3)	10.30	0.98	71000	0.07	100.0
pH of buffer of mobile phase (pH=2.7)	10.30	1.01	69703	0.03	100.8

Table 10: Robustness results of the proposed method (For Entacapone).

Altered condition	Retention time (min)	Tailing Factor	Theoretical plates	% RSD	%Assay
Normal (unaltered) (Repeatability)	2.60	1.37	5402	0.15	101.15
Flow rate of mobile phase (0.8 mL/min)	3.22	1.36	5928	0.22	101.9
Flow rate of mobile phase (1.2 mL/min)	2.18	1.32	4493	0.12	102.3
pH of buffer of mobile phase (pH=2.3)	2.37	1.00	7508	0.11	100.4
pH of buffer of mobile phase (pH=2.7)	2.33	1.04	7097	0.08	99.4

Table 11: Robustness results of the proposed method (For Levodopa).

Altered condition	Retention time (min)	Tailing Factor	Theoretical plates	% RSD	%Assay
Normal (unaltered) (Repeatability)	3.62	1.19	6330	0.10	95.9
Flow rate of mobile phase (0.8 mL/min)	4.51	1.18	6907	0.12	96.9
Flow rate of mobile phase (1.2 mL/min)	3.04	1.17	5204	0.12	96.6
pH of buffer of mobile phase (pH=2.3)	3.27	0.94	6436	0.94	96.0
pH of buffer of mobile phase (pH=2.7)	3.05	1.01	6201	0.09	96.5

Table 12: Robustness results of the proposed method (For Carbidopa).

presence of 15 impurities has been developed. Method was validated for specificity, accuracy, linearity, precision and robustness ensuring suitability of the method for quantitative analysis. The results indicated that this method is suitable for simultaneous estimation of Entacapone, Levodopa and Carbidopa in a pharmaceutical formulation.

Acknowledgements

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