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HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF DRUG RELEASE OF LEVODOPA AND CARBIDOPA IN ENTACAPONE, LEVODOPA AND CARBIDOPA TABLETS

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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 method of analysis of pharmaceutical formulation - Entacapone, Levodopa and Carbidopa tablets for the treatment of Parkinson's disease. The paper presents a simple and efficient HPLC method that has been developed for a multi component drug formulation for estimation of % drug release of levodopa and carbidopa. This HPLC method uses 'Cosmosil 5PE-MS 150 x 4.6 mm, 5 µ' HPLC column, combination of 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 0.020 to 0.40 mg/mL and 0.005 to 0.100 mg/mL of Levodopa and Carbidopa respectively), and specific for blank and placebo solution ensuring suitability of the method for quantitative determination of % drug release of Levodopa and Carbidopa in presence of Entacapone in multi component pharmaceutical formulation.

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.

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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 [1].

There is no pharmacopoeial or literature reference of a suitable HPLC method to estimate % drug release of Levodopa and Carbidopa in presence of each other and Entacapone in the proposed triple combination formulation. Proposed method was developed to cater to this need of pharmaceutical industry.

Literature survey revealed few methods for individual or combination product analysis such as;

spectroscopic methods for simultaneous estimation of levodopa and carbidopa [4]. Method for *in-vitro* release of drugs is also found but with longer run time [3, 5, 7]. Estimation methods by liquid chromatography for levodopa and carbidopa have been reported using electrochemical detector [6] and fluorescence detector [18]. Spectrophotometric determination of entacapone was reported in single drug product [19, 22], HPLC method in combination product was also reported [20, 21], USP monographs for determination of Levodopa and carbidopa for single and dual drug combination have also been reported [30, 31, 32, 33, 34, 35].

In the present study, we propose a rapid and robust HPLC method for simultaneous estimation of Levodopa {(2S)-2-amino-3-(3,4-dihydroxyphenyl) propanoic acid}[LD] and Carbidopa {(2S)-3-(3,4-dihydroxyphenyl)-2-hydrazino-2-methyl propanoic acid} [CD] in presence of Entacapone {(2E)-2-cyano-3-(3,4-dihydroxy - 5 -nitrophenyl) - N, N-diethyl-2-propenamide}[EN].

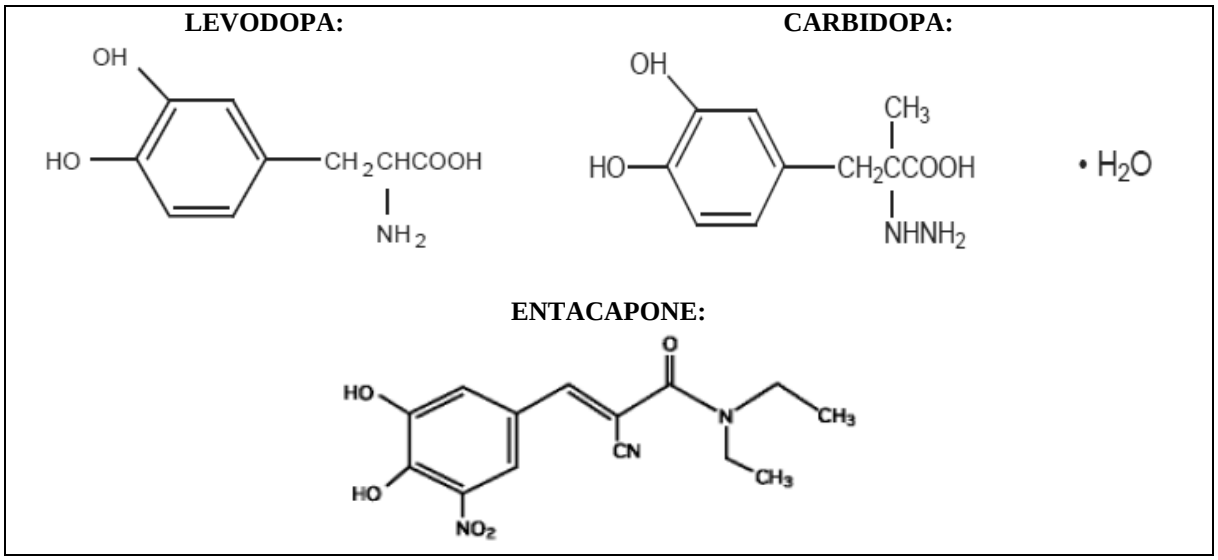


FIG.1 CHEMICAL STRUCTURES OF LEVODOPA, CARBIDOPA AND ENTACAPONE

2. MATERIAL AND METHODS:

2.1. 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 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. Working standards of entacapone, levodopa and carbidopa were obtained from Macleods Pharmaceuticals Limited, Mumbai, India.

2.2 Spectrophotometric analysis for λ_{max}: Solutions of levodopa, carbidopa and entacapone were prepared in diluent containing orthophosphoric acid and tetrahydrofuran and the absorption spectrum was obtained in the UV range using UV-Vis Spectrophotometer (Make). λ_{max} was determined from the respective absorption spectrum of the drugs. Both the drugs show λ_{max} at about 280nm.

2.3. 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 100µl capacity. Cosmosil 5PE-MS 150 x 4.6 mm, 5 µ (Nacalai Tesque, USA) HPLC column was used for chromatographic separation. Phosphate buffer and methanol were used as mobile phase in gradient mode. Buffer was composed of 10 mM potassium dihydrogen orthophosphate solution with pH adjusted to 2.5 using orthophosphoric acid. Flow rate was 1.5 mL/min and detection was carried out at 280 nm based on their wavelength maxima as per UV spectrum. Labsolutions software was used for data collection.

2.4. Solution Preparation:

2.4.1 Standard Preparation:

Levodopa standard solution: About 42 mg of Levodopa was accurately weighed and transferred to a 25 mL volumetric flask. About 15 mL of diluent was added and sonicated to dissolve Levodopa completely. This solution was allowed to equilibrate to room temperature, diluted to volume with diluent and mixed.

Carbidopa standard solution: About 45 mg of Carbidopa was accurately weighed and transferred to a 100 mL volumetric flask. About 70 mL of diluent was added and sonicated to dissolve Carbidopa completely. This solution was allowed to equilibrate to room temperature, diluted to volume with diluent and mixed.

Standard solution: 5 mL of each of Levodopa standard solution and Carbidopa standard solution was transferred to a 50 mL volumetric flask, diluted to volume with dissolution medium and mixed.

2.4.2 Sample Preparation: 750 mL of dissolution medium was poured in each vessel. Sufficient time was allowed for the dissolution medium to equilibrate at 37°C $\pm$ 0.5°C. Stirring element speed was adjusted to 50 rpm. One tablet was placed in each basket. The apparatus was lowered in the dissolution medium and started.

At the end of specified time, 10 mL aliquot was withdrawn from a zone midway between the surface of the dissolution medium and the top of the rotating basket using a sampling cannula with pre - filter attached to the end of it and filtered immediately through Whatman GF/C (25 mm) filter, discarding first 5 ml of the filtrate.

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

2.5.1 Specificity: Specificity for blank, placebo and Entacapone was established by injecting blank solution, placebo solution, Levodopa standard solution, Carbidopa standard solution and Entacapone standard solution. (**Fig. 3**)

2.5.2 Solution Stability: Solution stability was evaluated by storing sample solution at 10°C till 24 hrs.

2.5.3 Filter Compatibility: Sample solution was prepared by spiking Levodopa and Carbidopa into placebo powder containing Entacapone equivalent to one dosage unit. At filtration stage, solution was filtered through Whatman GF/C filter (25 mm). First 5.0 mL of filtrate was discarded. The filtrate was collected for further analysis. The unfiltered sample solution was centrifuged.

Each of the above solutions thus obtained (filtered sample solution and centrifuged sample solution) were analysed as described in the methodology. The results were calculated.

2.5.4 Filter Saturation: Sample solution was prepared by spiking Levodopa and Carbidopa into placebo powder containing Entacapone equivalent to one dosage unit. At filtration stage, three filtrates were obtained using three separate Whatman GF/C filters (25 mm) by discarding 1 mL, 3 mL and 5 mL respectively. The filtrates were collected for further analysis.

2.5.5 Accuracy: Accuracy study was performed from 12.5% to 120% of the target concentration of individual active ingredient. Recovery solutions were prepared by spiking levodopa and Carbidopa to placebo powder containing Entacapone in dissolution medium.
(**Table 6**).

2.5.6 Linearity: A series of solutions were prepared by quantitative dilutions of the stock solution of standard solutions to obtain solutions as mentioned in the following table from 8% to 150% of the target concentration of individual active ingredient. Each solution was injected and the peak area was recorded. Slope, Y-intercept and Correlation coefficient of the regression line were calculated. (**Table 7**)

2.5.7 Repeatability:

2.5.7.1 Precision: Precision test was carried out by spiking levodopa and Carbidopa to placebo powder containing Entacapone equivalent to one dosage unit to obtain solutions at 100 % level of target concentration.

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

2.5.7.2 Intermediate precision: For intermediate precision, analysis was carried out by different analyst, on a different day, using a different HPLC and different dissolution apparatus. The absolute difference between the mean % release results obtained in precision and intermediate precision was calculated. (**Table10**)

2.5.8 Robustness: The Dissolution method was carried out as described in the methodology and by making the following alterations in the dissolution conditions.

- Changing the volume of dissolution medium (742.5 mL, 757.5 mL
- Changing the strength of dissolution medium (0.08N, 0.12N)

3. RESULTS AND DISCUSSION:

3.1. Preliminary studies:

3.1.1 Selection of dissolution medium: Dissolution medium was chosen based on USFDA recommendation of 0.1 N Hydrochloric acid for Carbidopa and Levodopa in Carbidopa, Levodopa and Entacapone tablets.

3.1.2 Selection of wavelength: Wavelength was selected based on absorbance maxima of both the drugs as per UV spectrum. 280 nm was optimum for both the active ingredients. (**Fig. 2**)

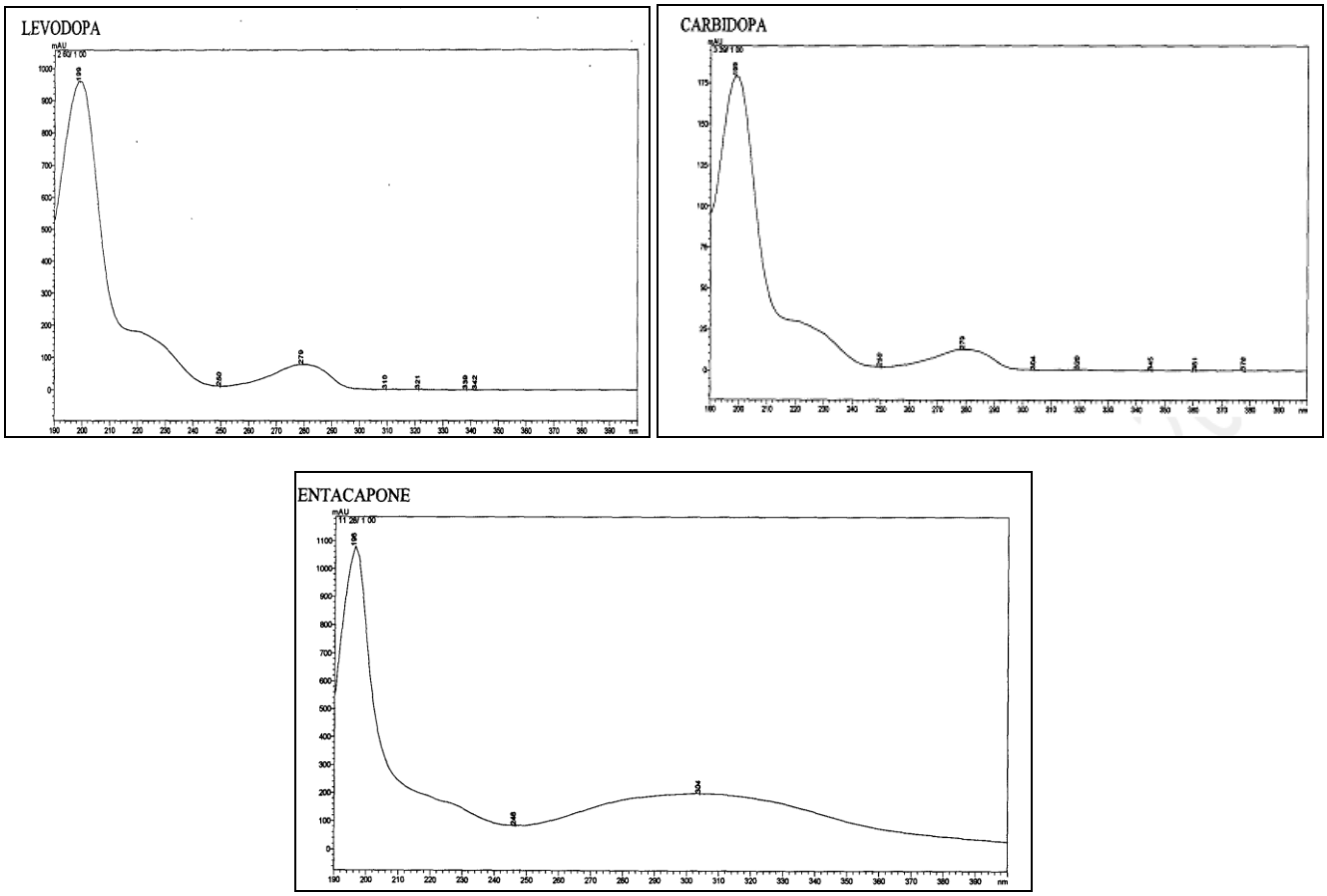


FIG. 2: UV ABSORPTION SPECTRA OF LEVODOPA, CARBIDOPA AND ENTACAPONE.

3.1.3 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. With reference to the specified pH range of HPLC column, pH 2.5 was evaluated and found to be optimum.

3.1.4 Selection of HPLC column: Levodopa and Carbidopa elute early on an octadecyl phase. In order to retain them, a more polar phase was evaluated and selected for method development. Cosmosil 5PE-MS 150 x 4.6 mm, 5 µ was the column of choice.

Conventional 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. 150 mm column was chosen to achieve a shorter run time.

3.1.5 Selection of HPLC pump mode: Entacapone do not elute early with a low solvent mobile phase. Hence, gradient mode was chosen and optimized to elute Entacapone in the same run and for separation of active ingredients with a flow rate of 1.5ml/min and run time of 9 minutes. (**Table 1**)

TABLE 1: GRADIENT TIME PROGRAM

Time (min)	Buffer (% v/v)	Methanol (% v/v)
0 → 3	100	0
3 → 3.1	100 → 10	0 → 90
3.1 → 6	10	90
6 → 6.1	10 → 100	90 → 0
6.1→ 9	100	0

3.1.6 Selection of diluent: For better solubility and stability of Levodopa and Carbidopa, combination of Orthophosphoric acid and Tetrahydrofuran in ratio 70:30 was chosen as diluent.

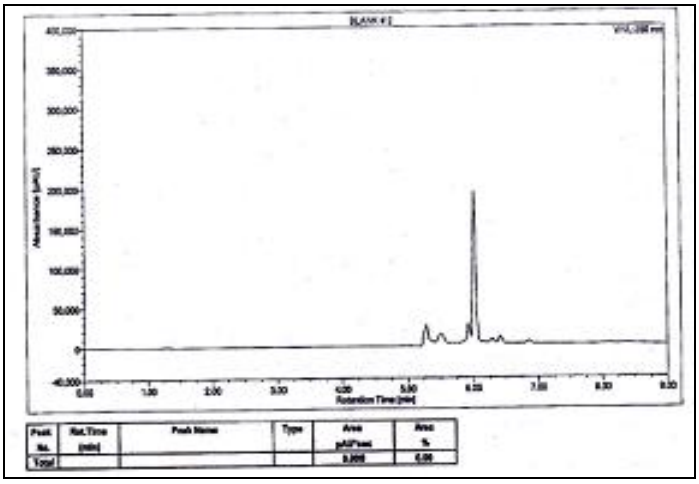
3.2. Method Validation:

3.2.1 Specificity:

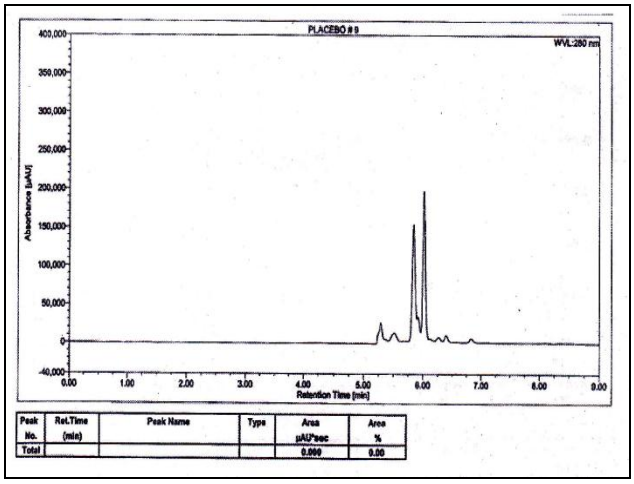
TABLE 2: VALUES OF RETENTION TIME OBTAINED

Sr. No.	Sample Details	Retention Time (min)
1	Blank	No interference observed
2	Placebo Solution	No interference observed
3	Levodopa	2.25
4	Carbidopa	4.24
5	Entacapone	5.83

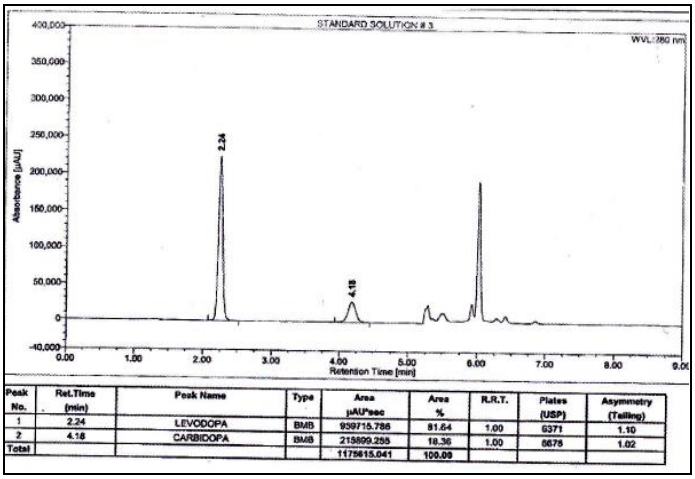
As shown in **Table 2**, No interference from blank, placebo and Entacapone was observed at retention times of Levodopa peak and Carbidopa peak.



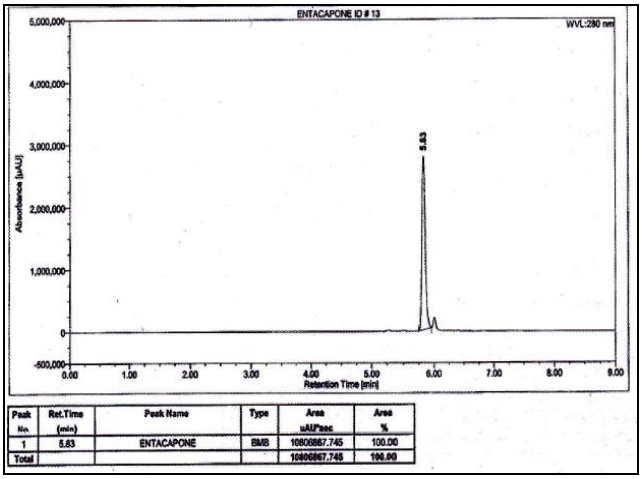
CHROMATOGRAM OF BLANK



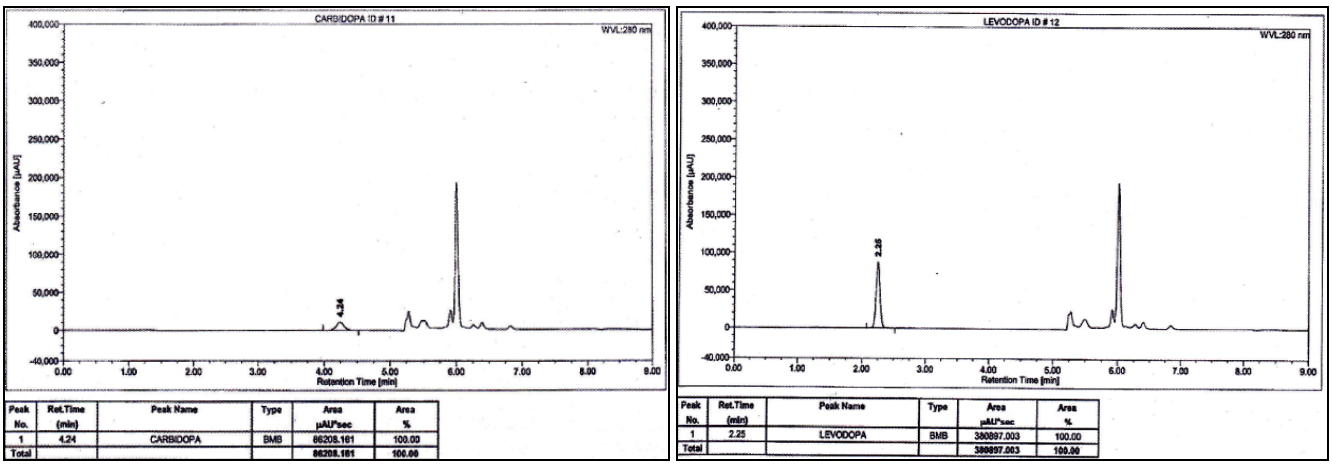
CHROMATOGRAM OF PLACEBO WITH ENTACAPONE



CHROMATOGRAM OF STANDARD



CHROMATOGRAM OF ENTACAPONE STANDARD



CHROMATOGRAM OF CARBIDOPA STANDARD **CHROMATOGRAM OF LEVODOPA STANDARD**

FIG. 3: CHROMATOGRAMS FOR SPECIFICITY TO CONFIRM NO INTERFERENCE AT RETENTION TIME OF PEAKS OF INTEREST

3.3.2 Solution Stability: The absolute difference between the % release of sample solution when stored for 24 hours at 10°C and % release of initial was within the acceptance criteria of not more than 2. (**Table 3)**

TABLE 3: OBSERVATION OF SOLUTION STABILITY

Time (hours)	Levodopa			Carbidopa		
	Area	% Release	Absolute difference	Area	% Release	Absolute difference
0	383262	101.9	-	80233	96.1	-
12	382619	101.8	0.1	79647	95.4	0.7
24	383091	100.2	1.7	78956	94.6	1.5

The sample solution was found to be stable till 24 hours, when stored at 10°C.

3.3.3 Filter Compatibility: The absolute difference between the results obtained for filtered solution and centrifuged solution was calculated. (**Table 4**)

TABLE 4: FILTER COMPATIBILITY RESULTS OF THE PROPOSED METHOD

Filter Type	Levodopa			Carbidopa		
	Area	% Release	Absolute Difference	Area	% Release	Absolute Difference
Centrifuge	364625	99.7	-	80808	101.3	-
Whatman GF/C filter	366618	100.3	0.6	81157	101.7	0.4

Since the absolute difference between the results obtained for filtered sample solution and centrifuged sample solution was within acceptance criteria of NMT 2, Whatman GF/C (25 mm) filter is considered as suitable for sample filtration.

Each of the filtered solutions thus obtained were analysed as described in the methodology. The absolute difference between the results obtained for consecutive filtered solutions was calculated. (**Table 5**).

3.3.4 Filter Saturation:

TABLE 5: FILTER SATURATION RESULTS OF THE PROPOSED METHOD

Volume Discarded	Levodopa			Carbidopa		
	Area	% Release	Absolute Difference	Area	% Release	Absolute Difference
1 mL	365740	100.0	-	81084	101.7	-
3 mL	366187	100.2	0.2	81108	101.7	0.0
5 mL	366624	100.3	0.1	80921	101.5	0.2

Since the absolute difference between the results obtained for two consecutive filtered solutions was within acceptance criteria of NMT 2, volume of 5 mL was considered as sufficient to saturate the filter.

3.3.5 Accuracy:

TABLE 6: ACCURACY RESULTS OF THE PROPOSED METHOD

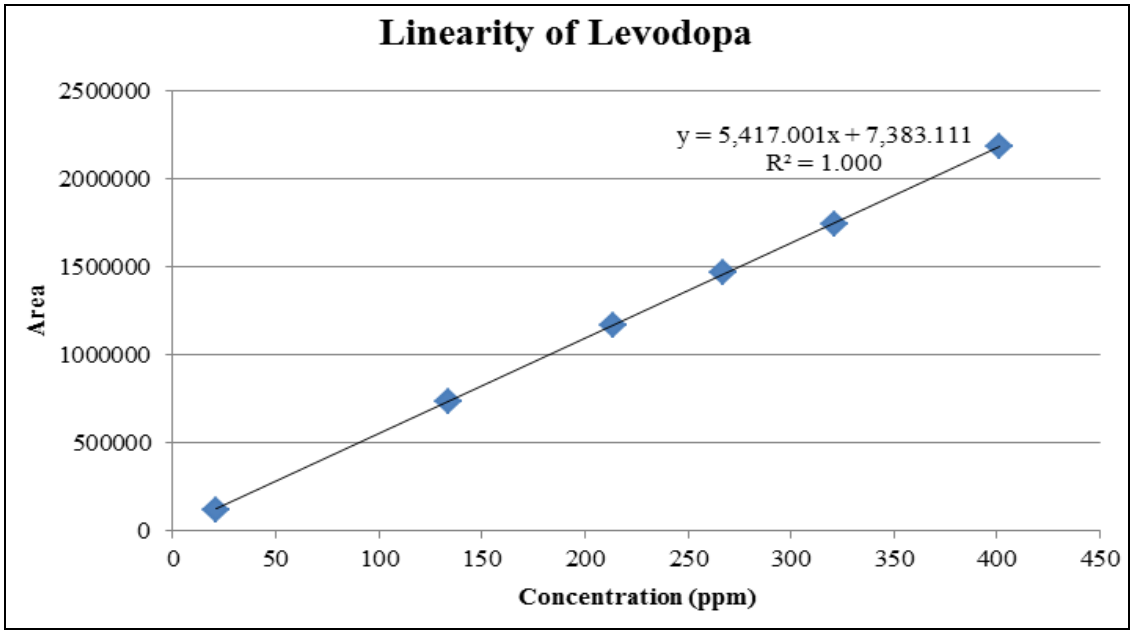
	Levodopa			Carbidopa		
Level	**API Spiked (mg)**	**Area**	**% Recovery**	**API Spiked (mg)**	**Area**	**% Recovery**
	25.2116	193570	100.1	6.251	44175	102.4
12.5 %	25.4509	193592	99.2	6.251	44055	103.1
	25.6702	192973	98.0	6.251	44000	103.0
	201.8224	1526569	98.6	50.008	347567	101.7
100%	201.5233	1526541	98.8	50.008	347031	101.5
	201.4934	1525994	98.8	50.008	346880	101.5
	242.2168	1802524	97.1	60.010	413266	100.8
120%	241.8978	1802568	97.2	60.010	413652	100.9
	242.0074	1800264	97.0	60.010	412909	100.7
		Mean % Recovery	98.3		**Mean % Recovery**	101.8

The % recovery was within 95-105% (**Table 6**). Hence method is considered to be accurate.

3.3.6 Linearity:

TABLE 7: LINEARITY RESULTS OF THE PROPOSED METHOD

% Level	Levodopa		Carbidopa	
	Concentration (ppm)	**Area**	**Concentration (ppm)**	**Area**
8	20.80	115019	5.06	25219
50	133.70	735280	33.41	162512
80	213.92	1168438	53.66	257718
100	267.40	1465216	66.82	321435
120	320.88	1736532	80.99	393997
150	401.11	2179072	100.23	478962
Slope		5417.001		4790.148
Y-Intercept		7383.111		1729.732
Correlation coefficient		1.000		1.000



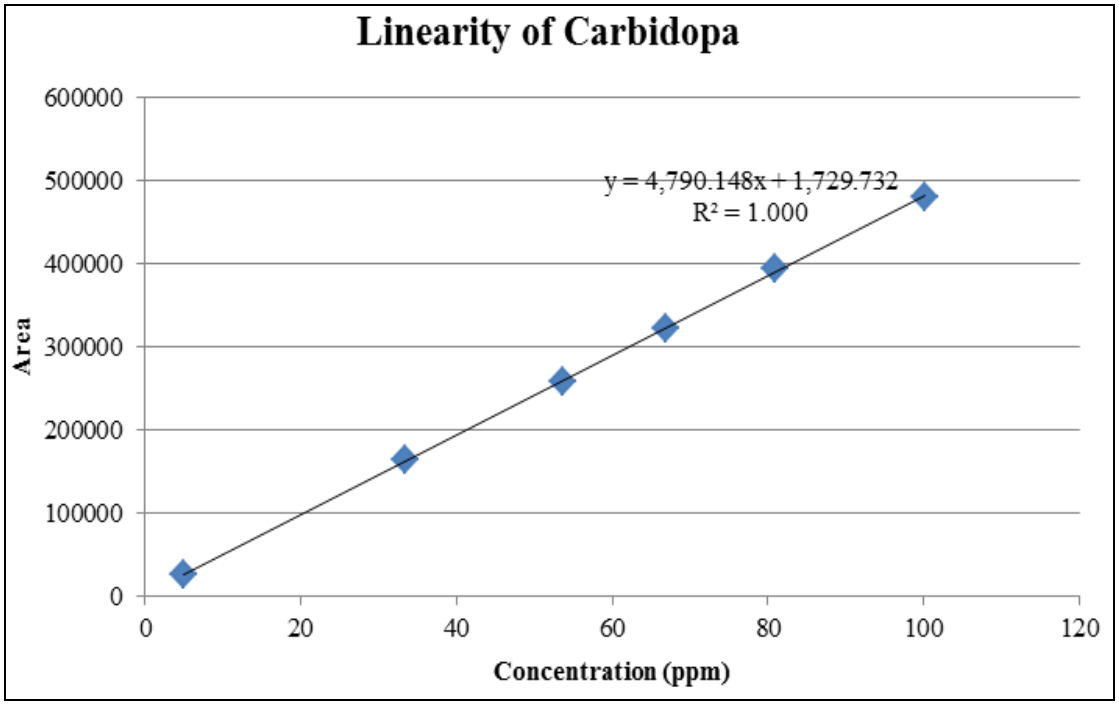


FIG. 4: LINEARITY PLOT

Linearity plot of Levodopa and Carbidopa (**Fig. 4**) and results (**Table 7)** shows that the correlation coefficient is within acceptance criteria of not less than 0.99. Hence the method is linear.

3.3.7 Repeatability:

3.3.7.1 Precision:

TABLE 8: PRECISION RESULTS OF THE PROPOSED METHOD

	Levodopa		Carbidopa	
	Area	**% Release**	**Area**	**% Release**
Sample-1	1443722	98.1	298726	95.1
Sample-2	1469334	99.9	297066	94.6
Sample-3	1461117	99.3	298870	95.1
Sample-4	1444689	98.2	298671	95.1
Sample-5	1471629	100.0	296375	94.3
Sample-6	1461307	99.3	298047	94.9
Mean		99.1	**Mean**	94.9
% RSD		0.82	**% RSD**	0.35

3.3.7.2 Intermediate precision:

TABLE 9: INTERMEDIATE PRECISION RESULTS OF THE PROPOSED METHOD

	Levodopa		Carbidopa	
	Area	**% Release**	**Area**	**% Release**
Sample-1	1510380	100.4	325657	97.5
Sample-2	1533018	101.9	325950	97.6
Sample-3	1510459	100.4	325677	97.5
Sample-4	1532532	101.9	325973	97.6
Sample-5	1512934	100.6	326026	97.6
Sample-6	1532413	101.9	325942	97.6
Mean		101.2	**Mean**	97.6
% RSD		0.78	**% RSD**	0.05

TABLE 10: COMPARISON OF PRECISION AND INTERMEDIATE PRECISION RESULTS OF THE PROPOSED METHOD

Content	Mean % Release in Precision	Mean % Release in Intermediate Precision	Absolute difference
Levodopa	99.1	101.2	2.1
Carbidopa	94.9	97.6	2.7

The absolute difference between the mean % release results obtained in precision (**Table 8**) and intermediate precision (**Table 9**) was within the acceptance criteria of not more than 5.0 Also difference between precision and intermediate precision was within 5% (**Table 10**). Hence, the method for estimation of % release is precise.

3.3.8 Robustness:

TABLE 11: ROBUSTNESS RESULTS OF THE PROPOSED METHOD

Unit	% Release of Levodopa				
	Unaltered	Dissolution Volume 742.5 mL	Dissolution Volume 757.5 mL	Strength of Dissolution medium 0.08N	Strength of Dissolution medium 0.12N
1	103.8	97.3	100.6	96.8	89.9
2	98.1	102.1	99.1	102.1	97.7
3	98.0	98.0	97.0	92.1	91.3
4	91.0	104.1	100.3	91.6	101.6
5	103.1	100.6	97.4	92.0	96.6
6	100.8	99.4	101.1	101.0	98.4
Mean	99.1	100.3	99.3	95.9	95.9
% RSD	4.7	2.5	1.7	5.0	4.6
Unit	**% Release of Carbidopa**				
	Unaltered	**Dissolution Volume 742.5 mL**	**Dissolution Volume 757.5 mL**	**Strength of Dissolution medium 0.08N**	**Strength of Dissolution medium 0.12N**
1	104.5	97.2	99.3	94.4	88.7
2	97.8	102.0	99.0	99.3	95.5
3	97.6	99.4	96.2	91.7	88.5
4	91.0	103.7	100.6	91.6	98.3
5	102.4	100.6	96.7	89.2	97.3
6	101.4	99.7	101.6	98.0	95.8
Mean	99.1	100.4	98.9	94.0	94.0
% RSD	4.8	2.2	2.2	4.2	4.6

TABLE 12: COMPARATIVE % RELEASE RESULTS FOR ROBUSTNESS

Sr. No.	Changed Parameter	Levodopa		Carbidopa	
		Mean % Release	Absolute Difference	Mean % Release	Absolute Difference
1	Unaltered	99.1		99.1	-
2	Volume 742.5 mL	100.3	1.2	100.4	1.3
3	Volume 757.5mL	99.3	0.2	98.9	0.2
4	0.08N	95.9	3.2	94	5.1
5	0.12N	95.9	3.2	94	5.1

TABLE 13: SYSTEM SUITABILITY RESULTS FOR ROBUSTNESS

Sr. No.	Changed Parameter	Levodopa			Carbidopa		
		Tailing Factor (NMT2.0)	Theoretical Plates (NLT 2000)	% RSD of peak area (NMT 2.0)	Tailing Factor (NMT2.0)	Theoretical Plates (NLT 2000)	% RSD of peak area (NMT 2.0)
1	Unaltered	1.10	6371	0.04	1.02	6678	0.11
2	Volume 742.5 mL	1.07	5576	0.21	1.01	6628	0.26
3	Volume 757.5mL	1.07	5678	0.18	1.02	6659	0.19
4	0.08N	1.07	5422	0.10	1.04	6296	0.43
5	0.12N	1.13	5390	0.12	1.03	6286	0.29

The system suitability parameters (**Table 13**) were not significantly changed with altered conditions.
The absolute difference in the results obtained under normal condition and robustness study of change in dissolution medium volume and dissolution medium strength (**Table 11, 12**) were within the acceptance criteria of NMT 10.

CONCLUSION: A simple and efficient method for estimation of % Release of Levodopa and Carbidopa in triple drug combination product was developed and 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 estimation of % Release of Levodopa and % Release of Carbidopa in a pharmaceutical formulation.

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CONFLICT OF INTEREST: The authors declare that they have no conflict of interests.

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