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Simultaneous Estimation of Levodopa, Carbidopa and Entacapone in Bulk and Pharmaceutical Formulations by RP-HPLC Method

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ABSTRACT

A new, simple, precise, accurate and reproducible RP-HPLC method was developed and validated for the simultaneous estimation of levodopa, carbidopa and entacapone in bulk and pharmaceutical formulations. Separation of levodopa, carbidopa and entacapone was successfully achieved on a phenomenex C18 (250mm x 4.6mm x 5µm). The mobile phase consisted of 0.1M Ammonium acetate buffer: methanol (60:40 *v/v*) at a flow rate of 1.0 ml/min. The response was found to be linear in the concentration range of 50µg/ml to150 µg/ml for levodopa, 12.5 µg/ml to 37.5 µg/ml for carbidopa and 200 µg/ml to 600 µg/ml for entacapone. The value of the correlation coefficient was found to be 0.999, 1.000 and 0.999 for levodopa, carbidopa and entacapone respectively. The LOD and LOQ for levodopa were found to be 0.926 µg/ml and 3.088 µg/ml, respectively. The LOD and LOQ for carbidopa were found to be 0.1917 µg/ml and 0.6389 µg/ml, respectively and LOD and LOQ for entacapone were found to be 2.068 µg/ml and 6.893µg/ml respectively. This method was found to be good percentage recovery for levodopa, carbidopa and entacapone were found to be 99.00, 99.00 and 100.00 respectively indicates that the proposed method is sufficiently accurate. The specificity of the method shows good correlation between retention times of standard with the sample so, the method specifically determines the analyte in the sample without interference from excipients that are commonly present in tablet dosage forms. The method was extensively validated according to ICH guidelines for linearity, range, accuracy, precision, specificity and robustness.

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

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INTRODUCTION

Levodopa is the naturally occurring from dihydroxyphenylalanine and the immediate precursor of dopamine[1]. Unlike dopamine itself, it can be taken orally and crosses the blood-brain barrier. It is rapidly taken up by dopaminergic neurons and converted to dopamine. It is used for the treatment of parkinsonian disorders and is usually given with agents that inhibit its conversion to dopamine outside of the central nervous system[2]. The chemical structure of the levodopa is given in Figure 1.

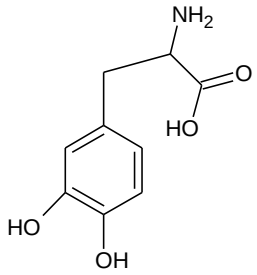

Figure 1: Chemical structure of Levodopa

Carbidopa is an inhibitor of DOPA decarboxylase, preventing conversion of levodopa to dopamine[3]. It is used in parkinson disease to reduce peripheral adverse effects of levodopa. It has no antiparkinson actions by itself[4]. The chemical structure of the carbidopa is given in Figure 2.

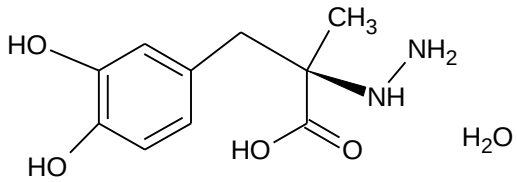

Figure 2: Chemical structure of Carbidopa

Entacapone is a selective, reversible catechol-O-methyl transferase inhibitor for the treatment of Parkinson's disease[5]. It belongs to the class of nitrocatechols. When administered concomitantly with levodopa and a decarboxylase inhibitor, increased and more sustained plasma levodopa concentrations are reached as compared to the administration of levodopa and a decarboxylase inhibitor. The chemical structure of the entacapone is given in Figure 3.

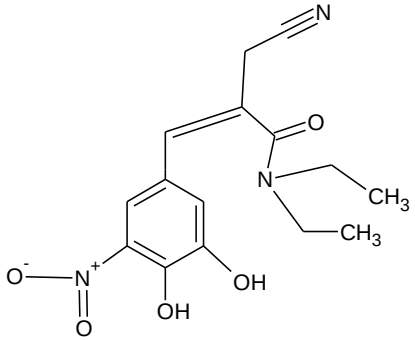

Figure 3: Chemical structure of entacapone

Only a few HPLC methods have been reported in the literature for the simultaneous determination of levodopa, carbidopa and entacapone in bulk and pharmaceutical formulations[6-8]. The aim of the present investigation was to develop and validate a more sensitive, precise and accurate RP-HPLC method with UV detection for the simultaneous quantification of the above said three drugs in bulk and in its pharmaceutical formulations.

MATERIALS AND METHOD

Pure standards and Chemicals

Levodopa, Carbidopa and Entacapone was a gift sample by Lara drugs Pvt Ltd., Hyderabad. Ammonium acetate, methanol of HPLC grade was purchased from Merck (India) Ltd., Mumbai and HPLC grade water from milli Q water.

Instrument and Chromatographic Conditions

The separation was carried out on HPLC system with Waters 2695 alliance with binary HPLC pump, Waters 2998 PDA detector, Waters Empower2 software with phenomenex C18, (250 mm × 4.6mm; 5 µm) column. The mobile phase consisting of 0.1M ammonium acetate buffer: Methanol was degassed and was pumped from the solvent reservoir in the ratio of 60:40 *v/v* was pumped into the column at a flow rate of 1.0 ml/min. The column temperature was 30°C. The detection was monitored at 230 nm and the run time was 14 min. The volume of injection loop was 10µl prior to injection of the drug solution the column was equilibrated for at least 15 min with the mobile phase flowing through the system.

Preparation of standard solutions

Accurately weigh and transfer 50 mg of levodopa, 12.5 mg of carbidopa and 100 mg of entacapone into 100 ml of volumetric flask and add 10 ml of mobile phase and sonicate for 10 min and makeup the volume upto the mark with mobile phase. This solution is used as stock standard solution. Pipette out 5.0 ml of stock standard stock into 25 ml volumetric flask and dilute to volume with mobile phase.

Preparation of Sample Solutions

Accurately weigh 704 mg of sample. Transfer the sample powder into 100 ml of volumetric flask add 10 ml mobile phase and sonicate for 20 min to completely dissolve the drugs. The volume was made up to the mark with mobile phase and filter through the 0.45 µm filter paper. Transfer 5 ml of above prepared solution into 25 ml volumetric flask and make up the volume to mark with mobile phase.

System suitability studies

System suitability for chromatographic separation was checked on each day of validation to evaluate the components of the analytical system in order to show that the performance of the system meet the standards required by the method. System suitability parameters established for the developed method include number of theoretical plates (efficiency), resolution and tailing factor.

Linearity and Range

The linearity of the method was determined at five concentration levels (50µg/ml to150 µg/ml for levodopa, 12.5 µg/ml to 37.5 µg/ml for carbidopa and 200 µg/ml to 600 µg/ml for entacapone). The calibration curve was constructed by plotting response factor against concentration of drugs. The slope and intercept value for calibration curve was Y = 43363 (R^2=0.999) for levodopa, Y = 25862 (R^2=1) for carbidopa and Y = 43363 (R^2=0.999) for entacapone. The results shows an excellent correlation exists between peak area and concentration of selected drugs within concentration range indicated above.

Accuracy and Precision

The accuracy of the method was determined by recovery experiments. The recovery studies were carried out 3 times. The percentage recovery and standard deviation of the percentage recovery were calculated. From the data obtained, added recoveries of standard drugs were found to be accurate. The precision of the method was demonstrated by inter-day and intra-day variation studies. In the intraday studies, six repeated injections of standard and sample solutions were made and the response factor of drug peaks and percentage RSD were calculated. In the inter-day variation studies, six repeated injections of standard and sample solutions were made for three consecutive days and response factor of drugs peaks and percentage RSD were calculated.

Robustness

Robustness of the method was determined by making slight changes in the chromatographic conditions. It was observed that there were no marked changes in the chromatograms which demonstrated that the RP-HPLC method developed is robust.

Limit of quantification (LOQ) and detection (LOD)

Limit of quantification and detection were predicted by plotting linearity curve for different nominal concentrations of levodopa, carbidopa and entacapone. RSD (σ) method predicted using following formulas (a) and (b). Precision was established at this predicted level was applied; the LOQ and LOD values were

(a) $LOQ = 10\ \sigma / S$

(b) $LOD = 3.3\ \sigma / S$

Where σ = residual standard deviation of response

S = slope of the calibration curve.

RESULTS AND DISCUSSION

System Suitability Studies

The column efficiency, resolution and tailing factor were calculated for the standard solutions (Table 1).The values obtained demonstrated the suitability of the system for the analysis of this drug combinations, system suitability parameters may fall within ± 2 %Relative standard deviation range during routine performance of the method.

Table 1: System suitability parameters

Parameter	Levodopa	Carbidopa	Entacapone
Retention time	5.029	7.189	10.943
Theoretical plates	11035	7740	10961
Tailing factor	1.08	1.12	1.04
% RSD	1.0	0.3	0.9

Linearity and range

The range of linearity of the method was 50-150 µg/ml for levodopa, 12.50-37.50 µg/ml for carbidopa and 200-60 µg/ml for entacapone. The calibration curve was constructed by plotting response factor against concentration of drugs. The slope and intercept value for calibration curve was Y = 43363 (R^2=0.999) for levodopa, Y = 25862 (R^2=1) for carbidopa and Y = 43363 (R^2=0.999) for entacapone. The results shows an excellent correlation exists between peak area and concentration of levodopa, carbidopa, entacapone within concentration range indicated above. The results for calibration data are shown in Table 2 and calibration curves are given in Figure 4, 5 & 6.

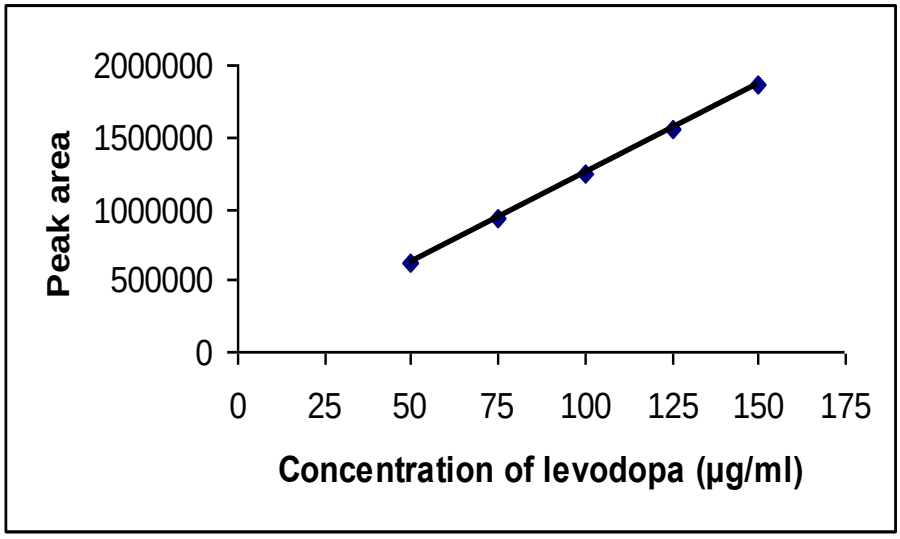

Figure 4: Linearity curve for levodopa

Table 2: Linearity studies for levodopa, carbidopa, entacapone by proposed method.

Linearity of levodopa		Linearity of carbidopa		Linearity of entacapone	
Conc. µg/ml	Peak area	Conc. µg/ml	Peak area	Conc. µg/ml	Peak area
50	622869	12.5	1293127	200	2401780
75	934315	18.75	1939647	300	3603348
100	1245103	25	2586255	400	4807833
125	1558889	31.25	3232814	500	6007595
150	1868780	37.50	3879380	600	7205867

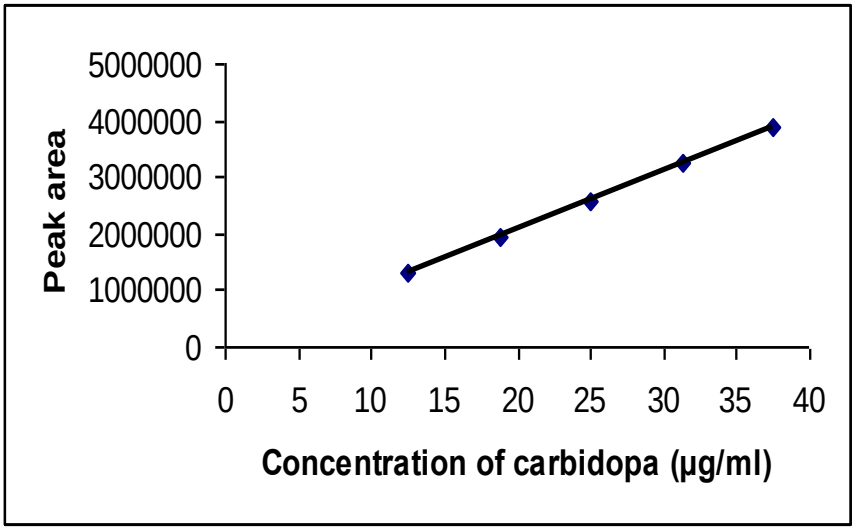

Figure 5: Linearity curve for carbidopa

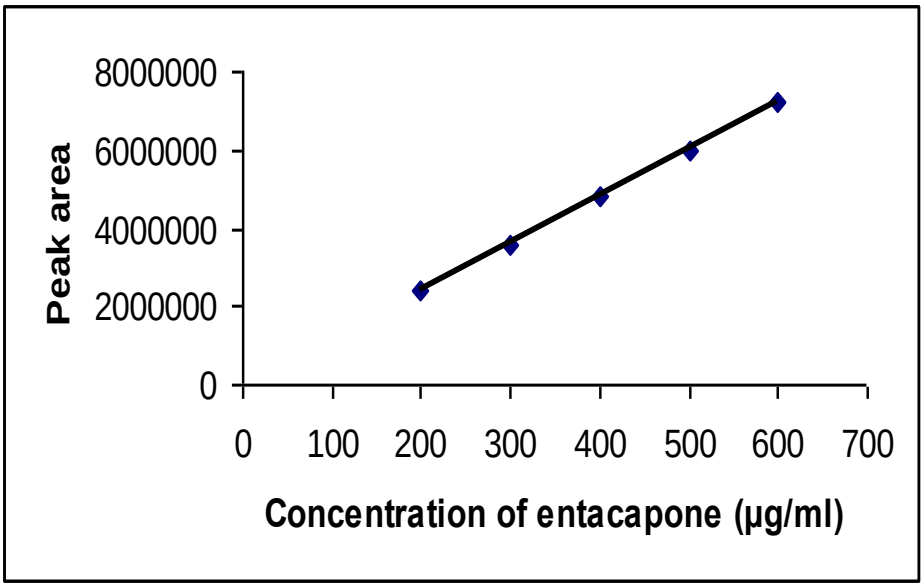

Figure 6: Linearity curve for entacapone

Accuracy and Precision

The results of accuracy of the method were determined by recovery experiments. The percentage recovery and standard deviation of the percentage recovery were calculated. From the data

obtained, added recoveries of standard drugs were found to be accurate (Tables 3, 4 & 5). The precision of the method was demonstrated by inter-day and intra-day variation studies. In the intraday studies, six repeated injections of standard and sample solutions were made and the response factor of drug and percentage RSD were calculated. In the inter-day variation studies, six repeated injections of standard and sample solutions were made for three consecutive days and response factor of drug and percentage RSD were calculated. The chromatograms of three different levels shown in Figure 7, 8 & 9. From the results, the developed RP-HPLC method was considered to be precise (Table 6).

Table 3: Accuracy for levodopa

Accuracy level	Sample weight	µg/ml added	µg/ml found	% Recovery	% Mean
50%	352.00	49.50	49.56	100	100
	352.00	49.50	49.55	100	
	352.00	49.50	49.53	100	
100%	704.00	99.00	99.06	100	100
	704.00	99.00	99.03	100	
	704.00	99.00	99.01	100	
150%	1056.00	148.50	148.66	100	100
	1056.00	148.50	148.65	100	
	1056.00	148.50	148.68	100	

Table 4: Accuracy for carbidopa

Accuracy level	Sample weight	µg/ml added	µg/ml found	% Recovery	% Mean
50%	352.00	12.375	12.44	101	101
	352.00	12.375	12.42	100	
	352.00	12.375	12.46	101	
100%	704.00	24.750	24.96	101	101
	704.00	24.750	25.00	101	
	704.00	24.750	24.91	101	
150%	1056.00	37.125	37.37	101	101
	1056.00	37.125	37.36	101	
	1056.00	37.125	37.33	101	

Table 5: Accuracy for entacapone

Accuracy level	Sample weight	µg/ml added	µg/ml found	% Recovery	% Mean
50%	352.00	200.00	198.17	99	99
	352.00	200.00	198.76	99	
	352.00	200.00	198.10	99	
100%	704.00	400.00	396.59	99	99
	704.00	400.00	396.41	99	
	704.00	400.00	396.41	99	
150%	1056.00	600.00	594.57	99	99
	1056.00	600.00	594.26	99	
	1056.00	600.00	594.52	99	

Table 6: Precision studies

Sample Wt(mg)	Levodopa		Carbodopa		Entacapone	
	Peak area	% Assay	Peak area	% Assay	Peak area	% Assay
704.00	1243093	99	2581148	100	4805022	99
704.00	124602	99	2574741	99	4802014	99
704.00	1247200	99	2605955	100	4865191	100
704.00	1244328	99	2596319	100	4806546	99
704.00	1239171	99	2595253	100	4826360	100
704.00	1241294	99	2512860	97	4832164	100

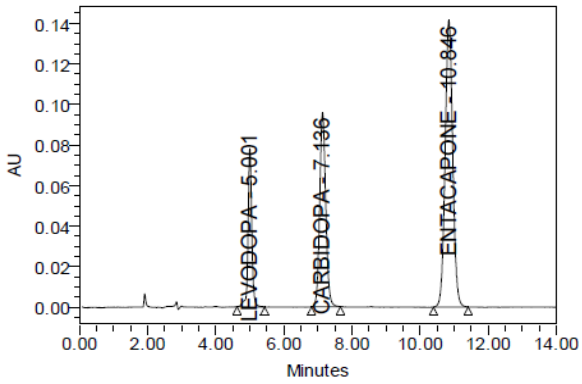

Figure 7: Chromatogram of levodopa, carbidopa and entacapone at 50 % accuracy level

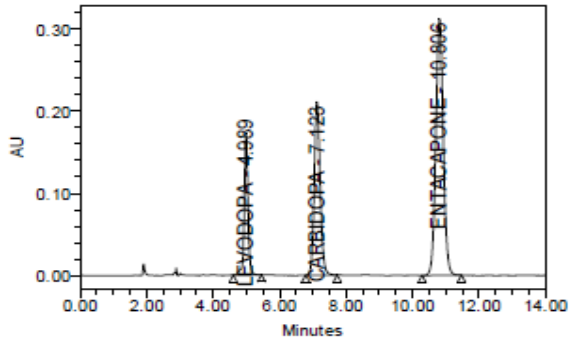

Figure 8: Chromatogram of levodopa, carbidopa and entacapone at 100 % accuracy level

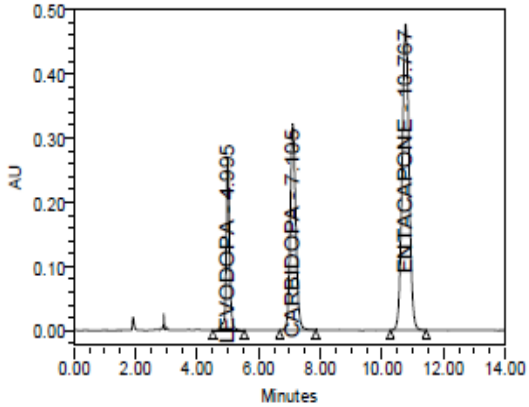

Figure 9: Chromatogram of levodopa, carbidopa and entacapone at 150 % accuracy level

Robustness

Robustness of the method was determined by making slight changes in the chromatographic conditions. It was observed that there were no marked changes in the chromatograms which demonstrated that the RP-HPLC method developed is are robust (Table 7, 8, 9).

Table 7: Robustness for levodopa

Sample. No	Sample Name	RT	Area	Theoretical plates	USP Tailing
1	Temp-1	5.470	1333917	11616	0.96
2	Temp-2	4.468	1070968	10314	0.97
3	Flow-1	5.418	1319088	11580	0.93
4	Flow-2	4.486	1063748	10555	0.99

Table 8: Robustness for carbidopa

Sample. No	Sample Name	RT	Area	Theoretical plates	USP Tailing
1	Temp-1	7.753	2876120	8937	1.12
2	Temp-2	6.369	2377002	7508	1.11
3	Flow-1	7.716	2909218	8306	1.14
4	Flow-2	6.381	2344351	7564	1.13

Table 9: Robustness for entacapone

Sample.No	Sample Name	RT	Area	Theoretical plates	USP Tailing
1	Temp-1	11.685	5405599	11994	1.06
2	Temp-2	9.625	4357708	10662	1.05
3	Flow-1	11.686	5419316	12158	1.06
4	Flow-2	9.642	4359570	11103	1.05

LOD & LOQ

Limit of quantification and detection were predicted by plotting linearity curve for different nominal concentrations of Levodopa, Carbidopa and Entacapone. Relative standard deviation (σ) method was applied, the LOQ and LOD values were predicted using following formulas (a) and (b). Precision was established at these predicted levels.

(a) LOQ = 10 σ / S, (b) LOD = 3.3 σ / S

Where σ = residual standard deviation of response; s = slope of the calibration curve.

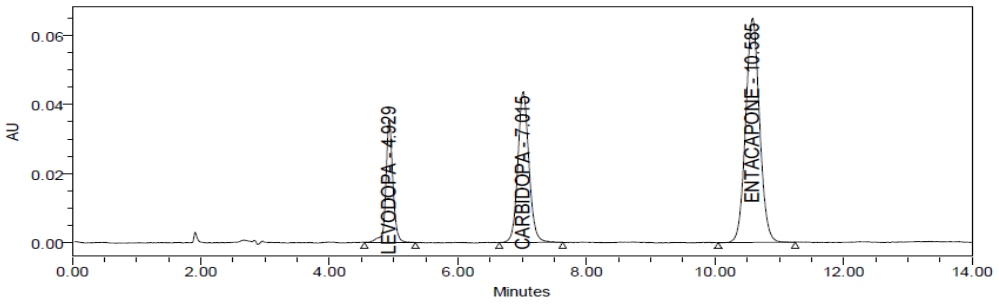

Figure 10: Chromatogram for LOD

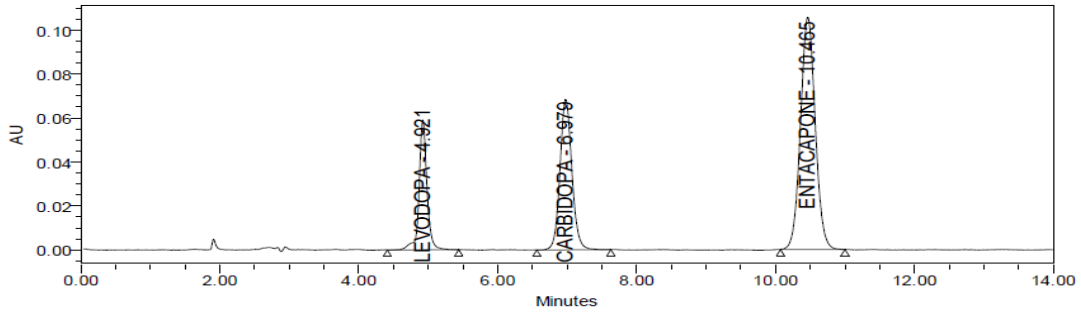

Figure 11: Chromatogram for LOQ

Table 10: LOD and LOQ for levodopa , carbidopa and entacapone

Sample No	Sample Type	Sample Name	RT	Area	Value (µg/ml)
1	LOD	Levodopa	4.929	267232	0.926
2	LOQ	Levodopa	4.921	467859	3.088
1	LOD	Carbidopa	7.015	522169	0.191
2	LOQ	Carbidopa	6.979	815801	0.638
1	LOD	Entacapone	10.585	974895	2.068
2	LOQ	Entacapone	10.465	1563210	6.893

Over All Summary of the Method

System suitability results were given in by Table 1 and system suitability parameters are retention time, resolution, tailing and plate count were shown uniformity and %RSD was less than 1. Hence the system is suitable for analysis of the selected drugs. The result given in Table 6 concluded that the method precision passed for levodopa, carbidopa and entacapone studies. The method accuracy was evaluated by recovery studies. The percent recover of levodopa, carbidopa and entacapone was found to be 99%, 99% & 100%, respectively. The percent recovery values are as per the acceptance criteria of ICH (97% - 103%). The low percentage indicated that the method is accurate. The results are shown in Tables 3, 4 and 5. Linearity calibration curve was given below Figures 4, 5 and 6. The calibration graph was plotted by taking concentration versus area to construct the linear regression equation and to calculate the value of correlation co-efficient. Linear correlation was found to be Y = 43363 (R^2=0.999) for levodopa, Y = 25862 (R^2=1) for carbidopa and Y = 43363 (R^2=1) for entacapone Method robustness results were given by Tables 7, 8 & 9. LOQ and LOD results are given by Table 10.

CONCLUSION

The proposed HPLC method was found to be simple, precise, accurate and sensitive for the simultaneous estimation of levodopa, carbidopa and entacapone in pure and pharmaceutical dosage forms. Hence, this method can easily and conveniently adopt for routine quality control analysis of levodopa, carbidopa and entacapone pure and its pharmaceutical dosage forms.

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