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Forced degradation study and validation of a RP-HPLC method for simultaneous estimation for drug content and release of Levodopa, Carbidopa and Entacapone in combined dosage form

Alka N Choudhary, Ajay Chaudhary and Kamlesh K Dutta

Abstract
Objective: The present study was designed to conduct forced degradation study and validate a RP-HPLC method which is capable of estimating the content and release of Levodopa, Carbidopa and Entacapone simultaneously in combined dosage form.
Methods: The developed RP-HPLC method was challenged for all method validation parameters as guided by ICH: Q2(R1) like specificity, linearity, range, accuracy, precision, robustness, system suitability. For determining whether the analytical method is stability indicating, the analytes were exposed under various stress conditions like light, heat, oxidation, hydrolysis to conduct forced degradation study.
Results: The method was found linear in a range of 32 -320, 8 -80 and 32 -320 µg/ml with correlation coefficient of 0.9999, 0.9999 and 0.9995 respectively for Levodopa, Carbidopa and Entacapone. LOD and LOQ of Levodopa, Carbidopa and Entacapone was found to be 2.10, 1.34 & 9.56 µg/ml and 6.35, 4.05 & 28.96 µg/ml respectively. Method precision was found to be within the %RSD ≤ 1. Average accuracy of the method was found to be 100.33, 99.79, 100.12% for Levodopa, Carbidopa and Entacapone respectively. The method was shown to be selective and robust. The method was successfully validated to be stability indicating. Finally, the method was successfully applied for simultaneous estimation of content and release of Levodopa, Carbidopa and Entacapone in commercial available brand of combined dosage form.
Conclusion: The method is found to be simple, economical, specific, precise, accurate, sensitive, robust, rapid & stability indicating and can be transferred to laboratory for routine analysis of the combined dosage form.

Keywords: Levodopa, Carbidopa, Entacapone, RP-HPLC, method validation, forced degradation study, stability indicating, ICH: Q2(R1)

1. Introduction
Analytical method validation is the process of demonstrating that an analytical procedure is suitable for its intended purpose [1]. Results from method validation can be used to judge the quality, reliability and consistency of analytical results; it is an integral part of any good analytical practice. It is the process of defining an analytical requirement, and confirms that the method under consideration has performance capabilities consistent with what the application requires [2]. Before transferring the developed analytical method to laboratory for routine implementation it is necessary to demonstrate the validity of the method using suitable samples or standards that are similar to the unknown samples to be analysed routinely [3, 4]. Following typically validation characteristics (Table 1) are to be addressed during analytical method validation as per ICH: Q2(R1) [5].
Forced degradation study has one among many applications to validate stability indicating power of analytical procedure. During the stress study, the analytes are intentionally exposed to various stress conditions and targeted to create about 2 – 30 % of degradation. Light, heat, oxidation, hydrolysis with acid and base can be employed to achieve desire degradation of the analytes and the analytical method is challenged whether it is stability indicating i.e able to assess unequivocally the analytes in the presence of common degradants [6, 7, 8].
Once an analytical procedure is successfully validated and implemented, the procedures (lifecycle management) should be followed during the life cycle of the product to continually assure that it remains fit for its intended purpose [1, 4].

Furthermore revalidation of analytical procedure may be necessary in the following circumstances [9],

- Change in analytical procedure
- Change in the route of synthesis of the drug substance
- Change in manufacturing process or formulation

The degree of revalidation depends upon nature of the change [1]. Decision of revalidation of all or parts of the analytical procedure shall be based on risk based evaluation [10].

Table 1: Validation characteristics for analytical method validation as per ICH: Q2(R1) [5].

Validation Characteristics	Type of Analytical Procedure			
	Identification	Testing for Impurities		Assay
		Qauntitative	Limit	-Dissolution -Content/Potency
Accuracy	-	+	-	+
Precision	-	+	-	+
Specificity	+	+	+	+
Detection Limit	-	-	+	-
Quantitation Limit	-	+	-	-
Linearity	-	+	-	+
Range	-	+	-	+

2. Materials and Methods

The research work was carried out at Analytical Development Laboratory and Quality Control Laboratory, Quest Pharmaceuticals Pvt. Ltd. All the required materials like APIs, excipients, reagents, chemicals, instruments and other analytical accessories were facilitiated from the company.

Method development was performed on a modular type Shimadzu HPLC System, Model - UFLCXR, equipped with binary pump, automatic sample injector, column thermostat and PDA detector. Data acquisition and interpretation were performed using LabSolutions software.

2.1. Method and Chromatographic Conditions

Diluent: Phoshate Buffer (KH_2PO_4), pH 5.5

Mobile Phase

A - Phoshate Buffer ($NaH_2PO_4.2H_2O$), pH 3.0

B - Methanol

Mode: Gradient (Table 2)

Table 2: Gradient Profile

Time (Mins)	A – Buffer (%)	B – Methanol (%)
0	98	2
15	10	90
17	98	2
20	98	2

Column: Sunshell Fused Core C18, 150 mm x 4.6 mm x 2.6 µm

Detection Wavelength: 280 nm

Column Temperature: 35 ºC

Flow Rate: 1.0 ml/min

Injection Volume: 5 µL

Preparation of Phosphate Buffer, pH 5.5: 6.8 g/L of Monobasic Potassium Phosphate, KH_2PO_4 in water, adjust pH to 5.5 with 1M Sodium Hydroxide.

Preparation of 0.01M Monobasic Sodium Phosphate ($NaH_2PO_4.2H_2O$) Buffer, pH 3.00:1.56 g/L Monobasic Sodium Phosphate, $NaH_2PO_4.2H_2O$ in water, adjust pH to 3.0 with 10% Ortho-Phosphoric Acid.

Preparation of Stock Solution

100 mg of Levodopa, 27 mg of Carbidopa equivalent to 25 mg of Anhydrous Carbidopa and 100 mg of Entacapone was accurately weighed and dissolved to 250 ml of diluents (Phosphate Buffer pH 5.5) to get stock solution containing 400 µg/ml of Levodopa, 100 µg/ml of Anhydrous Carbidopa and 400 µg/ml of Entacapone.

Further, the stock solution was diluted to get test solutions of different concentrations as required.

It is recommended to sonicate the stock solution for 15 minutes in sufficient solvent prior to volume make up and finally filter the final solution through 0.2 µm membrane filter prior to injection.

2.2. Method Validation

2.2.1. Linearity

Linearity study was performed with all three analytes in combined sample solution with minimum of 5 concentrations covering all expected sample concentration range for assay and dissolution study [5].

Acceptance Criteria: The regression line must have correlation coefficient ≥0.999 [12].

2.2.2. Range

Range study was designed to cover 60% of the test concentration expected from lowest strength and 130% of the test concentration expected from highest strength of dose available in the market.

Acceptance Criteria: The following minimum range must be achieved

- For assay: 80 – 120% of the test concentration [5]
- For dissolution: ±20% over the specified range [5]

2.2.3. Limit of Detection (LOD) and Limit of Quantitation (LOQ)

LOD was calculated based on the Standard Deviation of the Response and the Slope of the calibration curve

$LOD = 3.3\sigma/S$ [5]

$LOQ = 10\sigma/S$ [5]

Where σ = the standard deviation of the response

S = the slope of the calibration curve

2.2.4. Specificity
Specificity study was demonstrated by obtaining and comparing chromatograms for blank sample and another sample with all three analyte and common excipients in expected concentration [13].
Acceptance Criteria: [13]

- No interference from blank i.e. no peak in the blank and placebo should coelute (i.e. have same retention time) with any of the analyte peak.
- All analyte peaks should be well separated from its adjacent peak (i.e Resoluiton ≥2).
- All analyte peaks should pass the peak purity criteria to show that the analyte chromatographic peak is not attributable to more than one component.

2.2.5. Precision
Precision study was demonstrated in three levels as follows

a. Repeatability
Repeatability was assessed using 6 determinations at 100% of the test concentration on same day [5].
Acceptance Criteria: %RSD ≤ 2% [13]

b. Intermediate Precision
Intermediate Precision was assessed using 6 determinations at 100% of the test concentration on different days and using different instruments [5].
Acceptance Criteria: %RSD ≤ 2% [13]

c. Reproducibility
Reproducibility was assessed using 6 determinations at 100% of the test concentration in two different laboratories [5].
Acceptance Criteria: %RSD ≤ 2% [13]

2.2.6. Accuracy/Recovery
Accuracy/Recovery was assessed using 9 determinations over 3 concentration levels (e.g., 3 concentrations/3 replicates each) covering the specified range [5].
Acceptance Criteria: Percent Recovery of known amount added should be 95 - 105% [13].

2.2.7. Sample Solution Stability
Stability of the analyte in solution (test preparation) was investigated at normal laboratory condition over 24 hours at 0, 6, 12, 18, 24 hours [12].
Acceptance Criteria: %RSD ≤ 1% [13]

2.2.8. Robustness
Robustness was demonstrated with small but deliberate variations in analytical condition as follows; [5]

- Flow Rate: ± 0.2 ml/min
- Column Temperature: ± 5 ºC
- pH of Buffer: ± 0.2
- Detection Wavelength: ± 5 nm
- Gradient Start Point of Buffer: ± 2%
- Cloumn: Different lots/suppliers
- Column Length: 150 and 100 mm(Different Lot)

Acceptance Criteria: System Suitability Parameters like Resolution (R), Tailing Factor (A_s), Number of Theoretical Plates (NTP) should comply for all analyte peaks [12].

2.2.9. System Suitability
System Suitability was demonstrated by injecting 5 replicate injections of the standard solution [12].
Acceptance Criteria: System Suitability Parameters like Resolution, R (≥ 2), Tailing Factor, A_s (≤ 2), Number of Theoretical Plates, NTP (≥ 2000) should comply for all analyte peaks and % RSD ≤ 1% [12].

2.3. Forced Degradation Study [6, 7, 8]
Force degradation study was attempted and targeted to create 2 – 30 % of degradation. Following stress conditions were attempted;

- Light Degradation
- Heat Degradation
- Oxidation Degradation (Using H_2O_2)
- Acid Degradation (Using HCl)
- Base Degradation (Using NaOH)

Acceptance Criteria

- All analyte peaks should be well separated from its adjacent peak (i.e Resoluiton ≥2).
- All analyte peaks should pass the peak purity criteria to show that the analyte chromatographic peak is not attributable to more than one component.

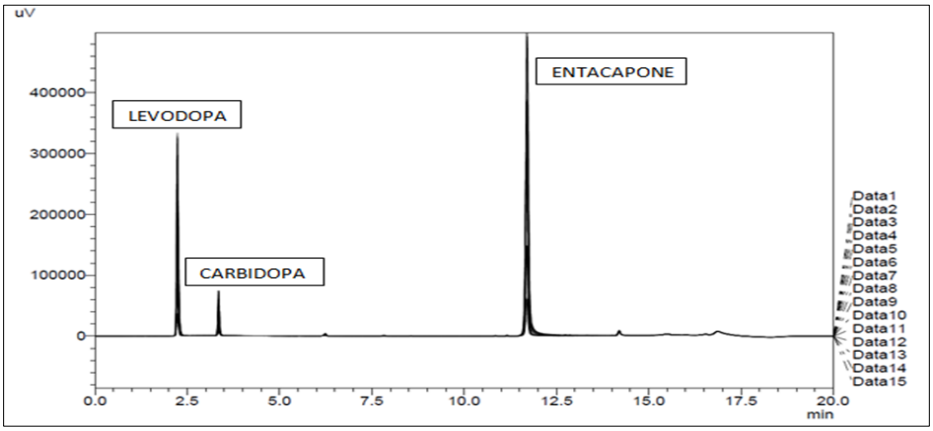
2.3.1. Estimation of Target Analytes in Commercial Brand
The analytical method was applied for analysis of the available commercial brand of Levodopa, Carbidopa and Entacapone Tablets. A commercial sample, **Entacom Plus 100** was analysed for the assay and dissolution.

Entacom Plus 100
Levodopa, Carbidopa & Entacapone Tablets
Each film coated tablet contains:
Levodopa IP 100 mg
Carbidopa IP (Anhydrous) 25 mg
Entacapone IP 200 mg
Colours: Yellow Oxide of Iron, Red Oxide of Iron & Titanium Dioxide
Batch No.: W55041
Mfg. Date: July-2017
Exp. Date: June-2020
Manufacturer: Intas Pharmaceuticals Ltd, India

Acceptance Criteria

- Assay: 90 – 110 % of the labeled amounts of Levodopa ($C_9H_{11}NO_4$), Anhydrous Carbidopa ($C_{10}H_{14}N_2O_4$) and Entacapone ($C_{14}H_{15}N_3O_5$) [14, 15].
- Dissolution: NLT 80 % (Q) of the labeled amounts of Levodopa ($C_9H_{11}NO_4$), Anhydrous Carbidopa ($C_{10}H_{14}N_2O_4$) and Entacapone ($C_{14}H_{15}N_3O_5$) [14, 15].

3. Results and Discussion
3.1. Linearity
Linearity study was conducted over 5 concentrations of analytes as Levodopa – 32 to 320 µg/ml, Carbidopa (Anhydrous) – 8 to 80 µg/ml and Entacapone - 32 to 320 µg/ml (Figure 1, Table 3).



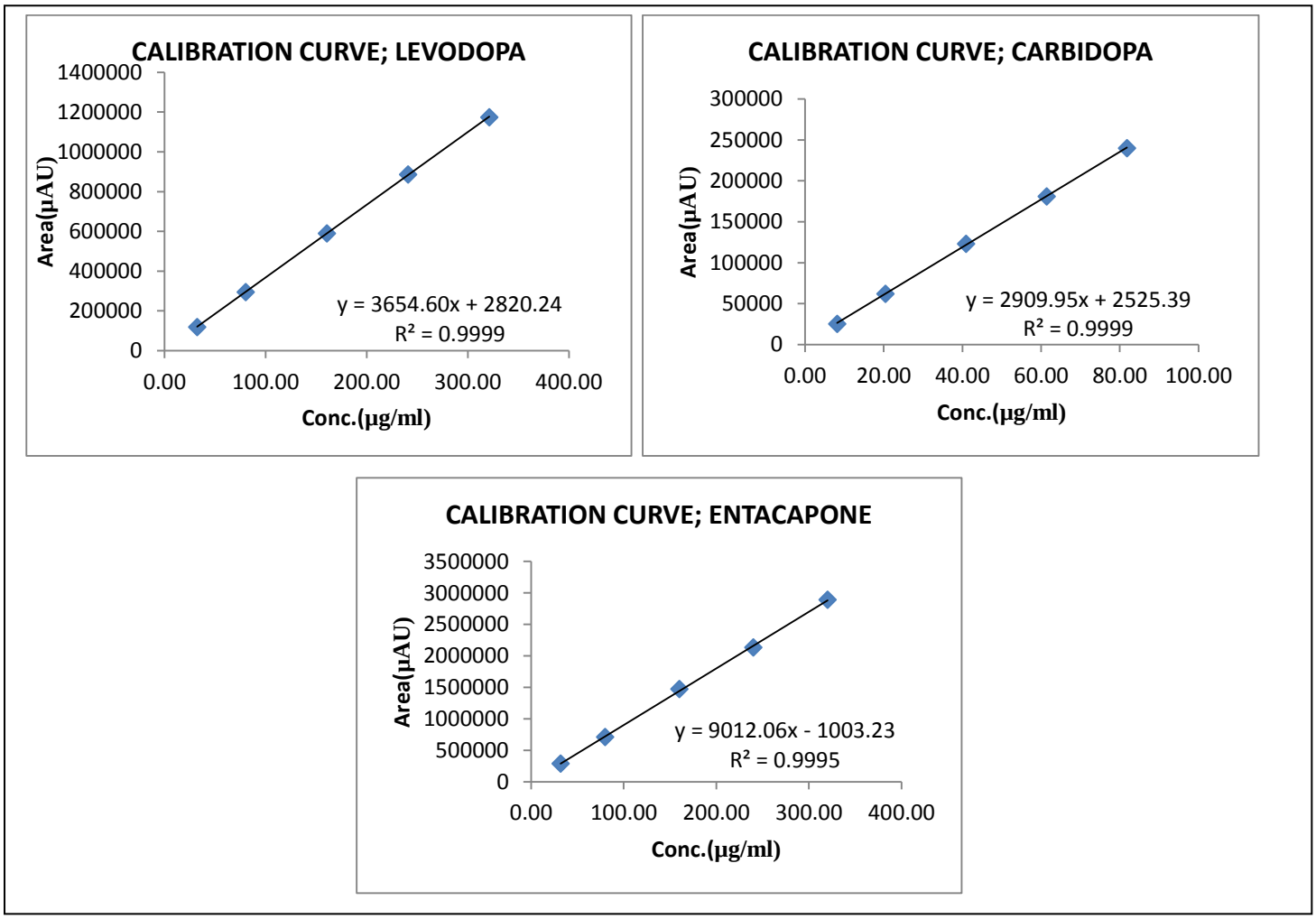


Fig 1: Calibration Curve of Levodopa, Carbidopa and Entacapone.

Table 3: Linearity data of Levodopa, Carbidopa and Entacapone

S. No	Levodopa		Carbidopa		Entacapone	
	Conc. of Solutions(µg/ml)	Avg. Area	Conc. of Solutions(µg/ml)	Avg. Area	Conc. of Solutions(µg/ml)	Avg. Area
1	32.13	119791	8.18	25497	32.00	287225
2	80.32	295517	20.45	61941	80.00	709569
3	160.64	590405	40.90	123297	160.00	1471745
4	240.96	886532	61.36	180881	240.00	2131998
5	321.28	1174644	81.81	239960	320.00	2892480
STEYX	2322.42		1178.45		26102.63	
SLOPE	3654.60		2909.95		9012.06	
LOD(µg/ml)	2.10		1.34		9.56	
LOQ(µg/ml)	6.35		4.05		28.96	

3.2. Limit of Detection (LOD) and Limit of Quantitation (LOQ)

LOD and LOQ were determined from the data obtained from linearity study. LOD of Levodopa, Carbidopa and Entacapone for the method were calculated and found as 2.10, 1.34, and 9.56 µg/ml respectively. LOQ of Levodopa, Carbidopa and Entacapone for the method were calculated and found as 6.35, 4.05, and 28.96 µg/ml respectively.

3.3. Precision

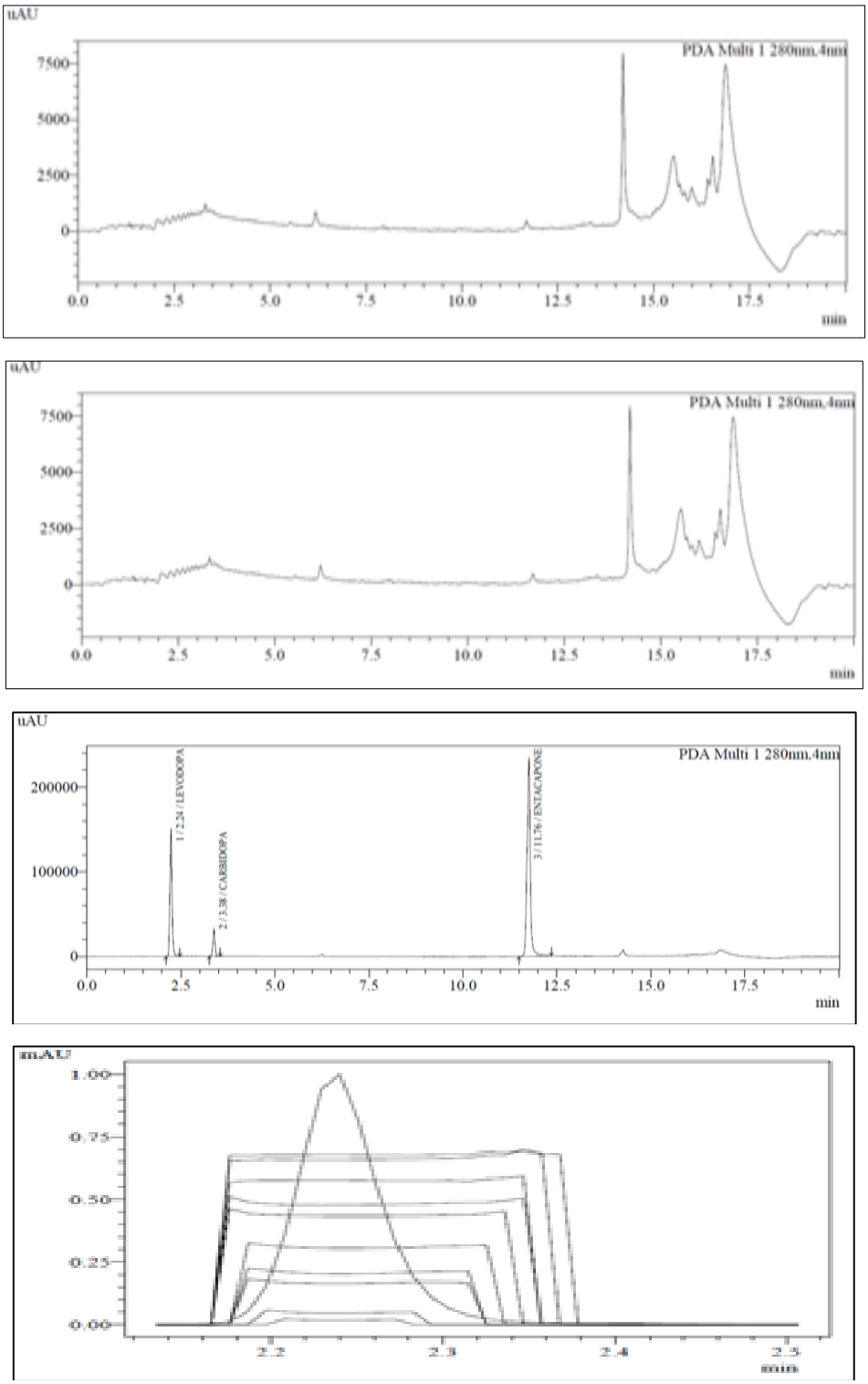
Precision study was performed at three levels.

- Repeatability
- Intermediate Precision
- Reproducibility

Precision study was conducted with 6 replicate determinations at single analyte concentration as Levodopa - 160 µg/ml, Carbidopa (Anhydrous) - 40 µg/ml and Entacapone - 160 µg/ml (Table 5).

3.4. Specificity

Specificity was performed at analyte concentration as Levodopa - 160 µg/ml, Carbidopa(Anhydrous) - 40 µg/ml and Entacapone – 160 µg/ml. Chromatograms obtained from Blank, Placebo and Test Solutions were compared for any interference and peak purity (Figure 2). Placebo solution was prepared by excluding the analytes. Diluent was used as blank solution.



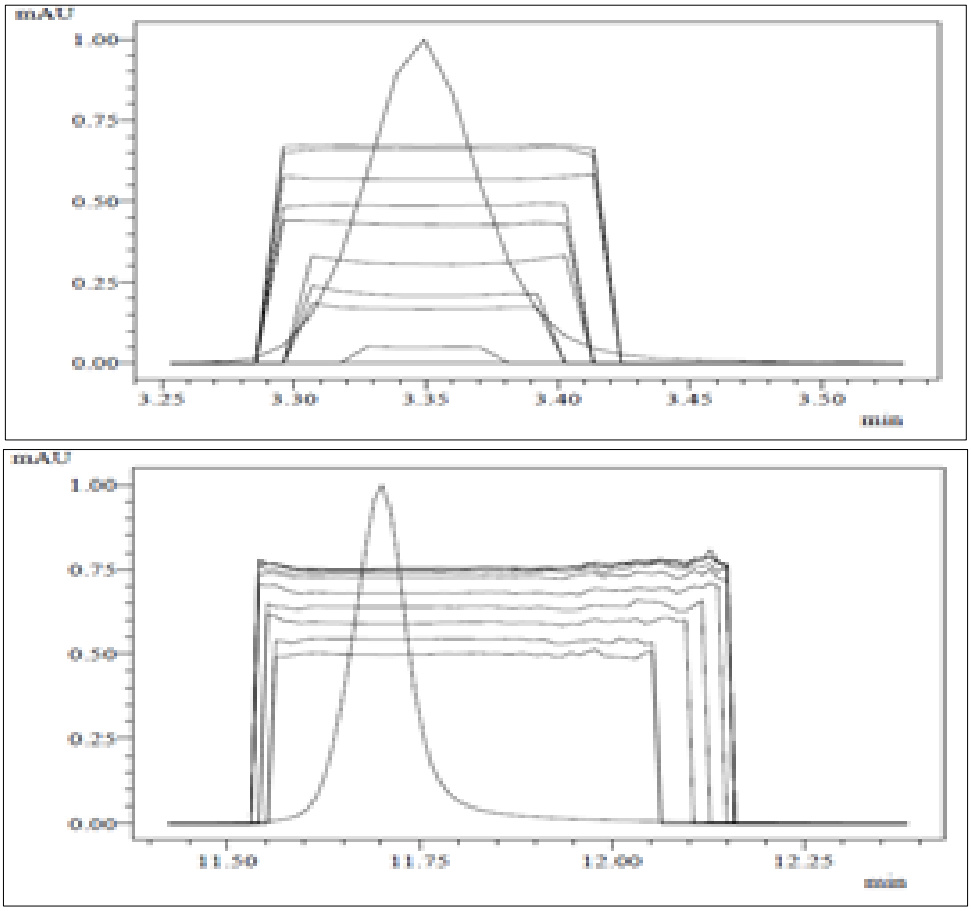


Fig 2: Chromatograms and Ratiograms (Peak Purity Test) for Specificity Study

3.5. Accuracy
It was targeted to perform accuracy/recovery study with 9 determinations, 3 concentrations/ 3 replicates each as Levodopa - 32, 160 and 320 µg/ml, Carbidopa(Anhydrous) - 8, 40 and 80 µg/ml and Entacapone – 32, 160 and 320 µg/ml (Table 5).

3.6. Range
Range of the analytical method was inferred from study of linearity, precision, specificity and accuracy. Method is found to be accurate, precise and specific through the range of 32 to 320 µg/ml, 8 to 80 µg/ml and 32 to 320 µg/ml for Levodopa, Carbidopa and Entacapone respectively.

3.7. Sample Solution Stability
Sample Solution Stability was performed on test solution containing; Levodopa - 160 µg/ml, Carbidopa(Anhydrous) - 40 µg/ml and Entacapone - 160 µg/ml.
The test preparations were stored at normal laboratory condition for 24 hours and its stability was evaluated at 0, 6, 12, 18, 24 hours (Table 5).

3.8. System Suitability
System Suitability was perform by injecting 5 replicate injections of a standard solution containing; Levodopa - 160 µg/ml, Carbidopa (Anhydrous) - 40 µg/ml and Entacapone - 160 µg/ml (Table 5).

3.9. Robustness
Robustness study was performed on test solution containing; Levodopa - 160 µg/ml, Carbidopa (Anhydrous) - 40 µg/ml and Entacapone - 160 µg/ml (Table 4).

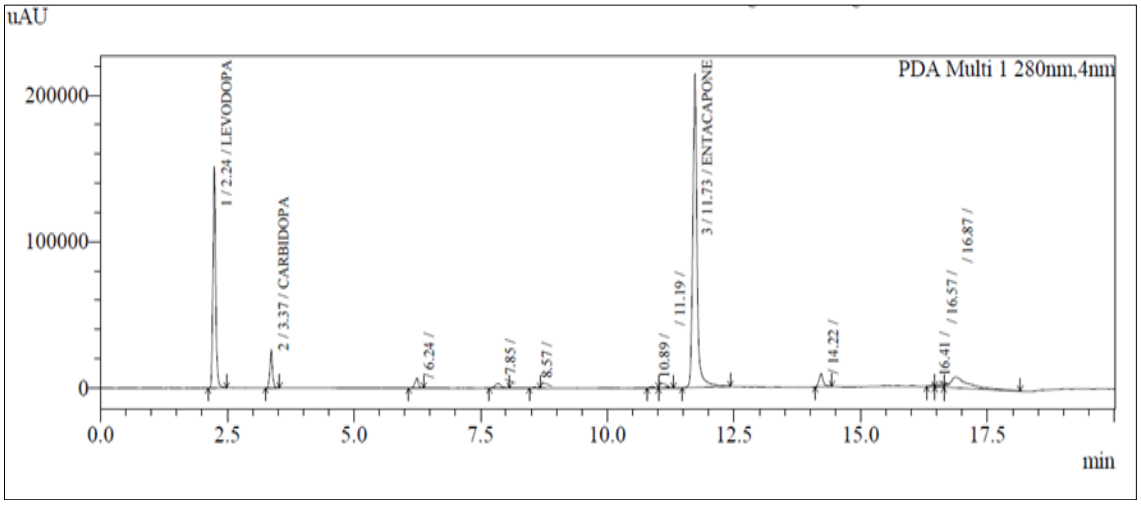
Table 4: Robustness data of Levodopa, Carbidopa and Entacapone

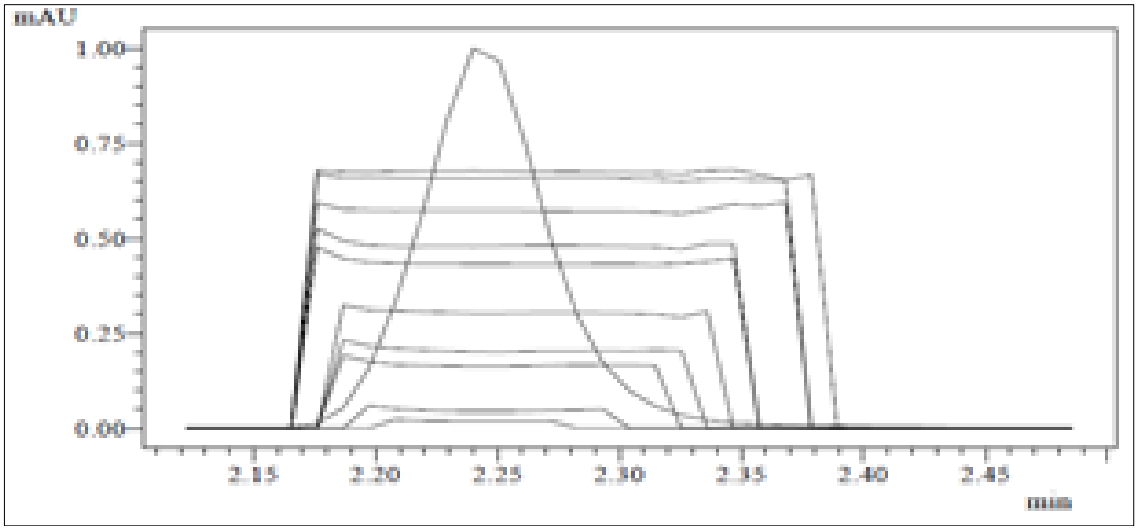
Variations		Levodopa				Carbidopa				Entacapone			
		Rt. (Min)	R	A_s	NTP	Rt. (Min)	R	A_s	NTP	Rt. (Min)	R	A_s	NTP
Optimum Condition		2.230	-	2.221	7307	3.340	10.94	1.124	18332	11.694	67.38	1.154	98209
Flow Rate	0.8 ml/min	2.714	-	1.252	2.714	3.921	10.48	1.106	20016	12.445	61.20	1.144	88118
	1.2 ml/min	1.868	-	1.265	1.868	2.907	11.17	1.146	17139	11.146	73.47	1.117	108292
Column Temp.	30 ºC	2.332	-	1.244	2.332	3.504	11.57	1.102	19543	11.914	64.16	1.107	86668
	40 ºC	2.133	-	1.218	2.133	3.179	9.90	1.112	16530	11.480	67.56	1.208	97818
Buffer pH	2.8	2.269	-	1.200	2.269	3.417	11.04	1.090	17436	11.745	63.78	1.124	88812
	3.2	2.077	-	1.026	2.077	3.146	7.17	0.906	6653	11.669	52.88	1.140	77072
Gradient Start Point	96% Buffer	2.073	-	1.182	2.073	3.100	8.83	1.055	12634	11.558	61.09	1.115	77065
	100% Buffer	2.426	-	1.110	2.426	3.590	10.80	1.062	17946	11.862	59.56	1.134	77374
Detection Wavelength	275 nm	2.230	-	1.220	2.230	3.340	10.95	1.117	18357	11.694	39.72	1.156	98121
	285 nm	2.230	-	1.219	2.230	3.340	10.94	1.117	18358	11.694	38.67	1.156	98209
Different Column	150 mm Diff. Lot	2.228	-	1.130	2.228	3.340	9.44	1.045	13587	11.708	58.44	1.122	74338
	100 mm	1.482	-	1.499	1.482	2.447	9.88	1.351	11278	10.609	60.68	1.641	57565

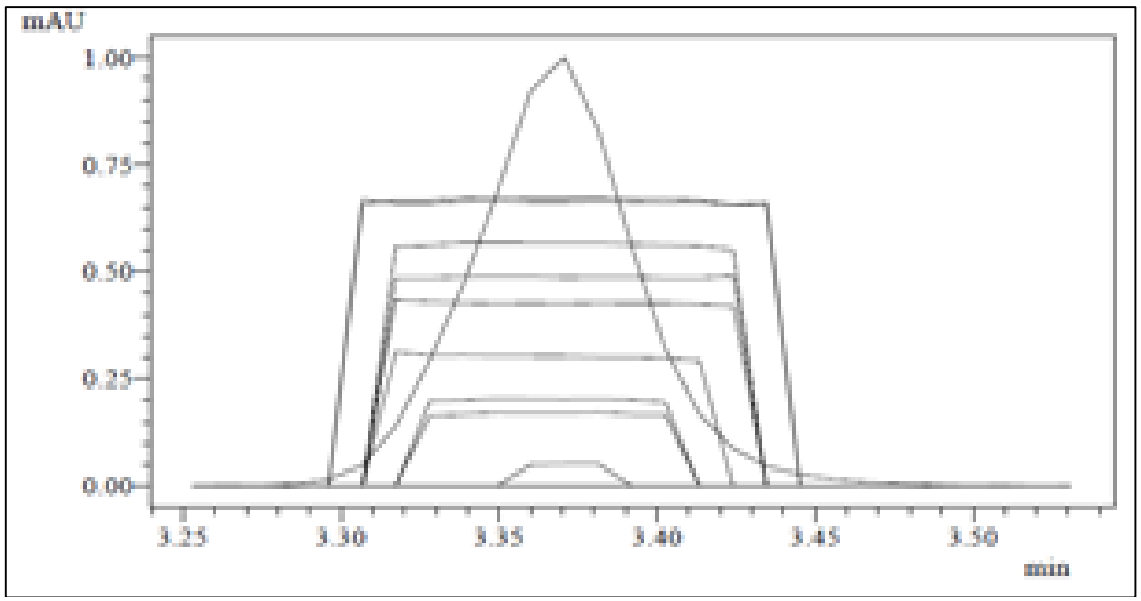
3.10. Forced Degradation Study

a. Light Degradation

The pure drugs (Levodopa, Carbidopa and Entacapone) were exposed to sunlight for 5 days and the same drugs were used for further study (Figure 3, Table 5).







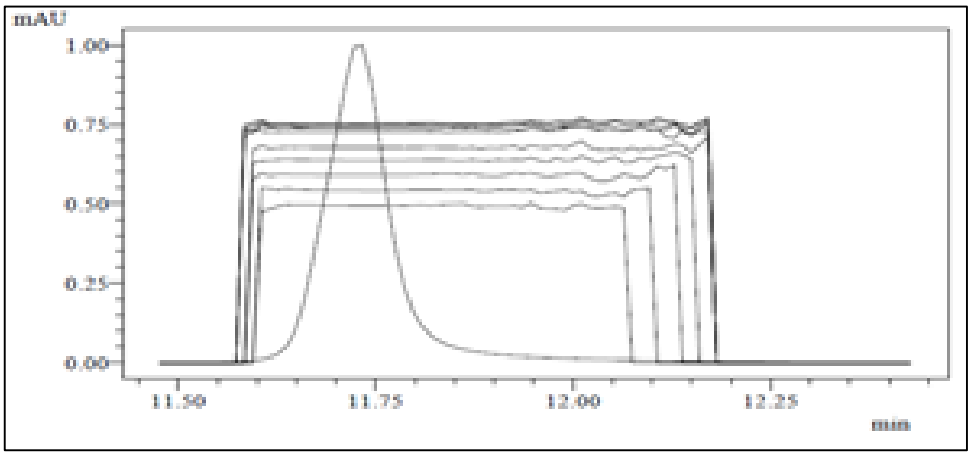
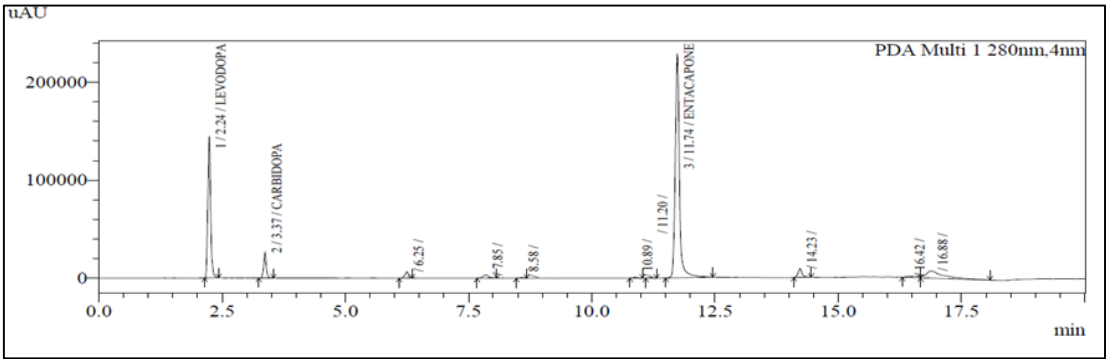
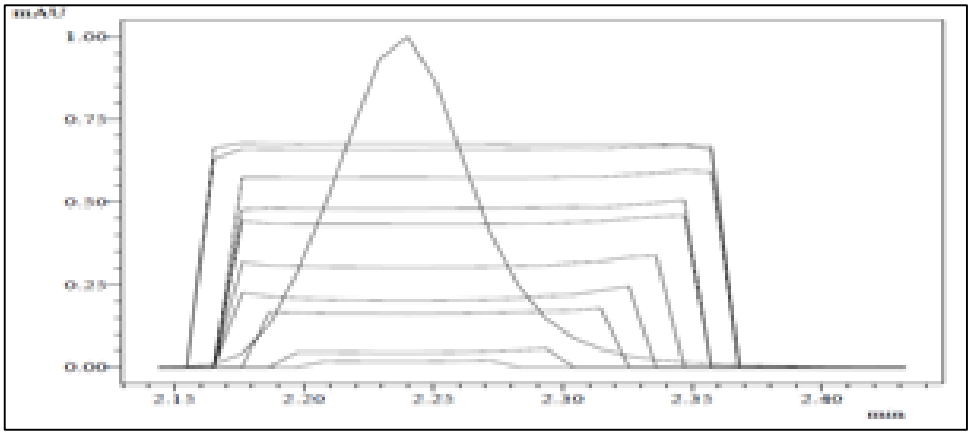


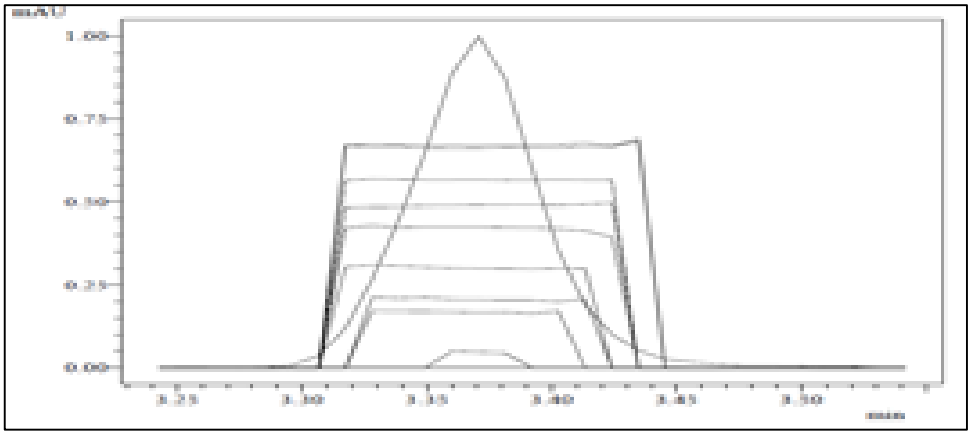
Fig 3: Chromatograms and Ratiograms (Peak Purity Test) for Light Degradation Study

b. Heat Degradation

The pure drugs (Levodopa, Carbidopa and Entacapone) were placed in an oven at 80 ºC for 72 hours and the same drugs were used for further study (Figure 4, Table 5).







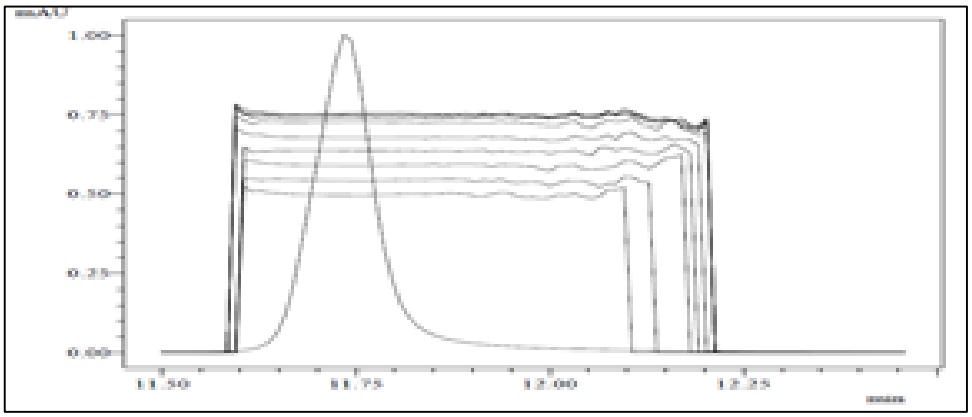
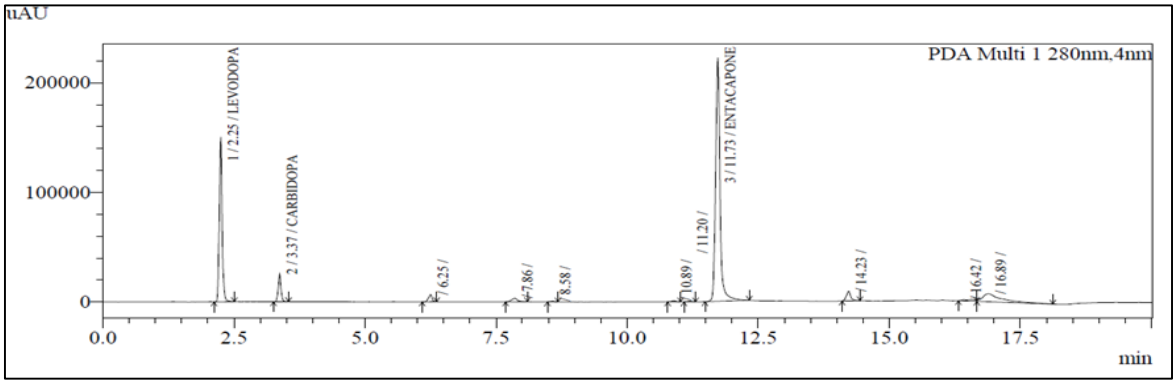
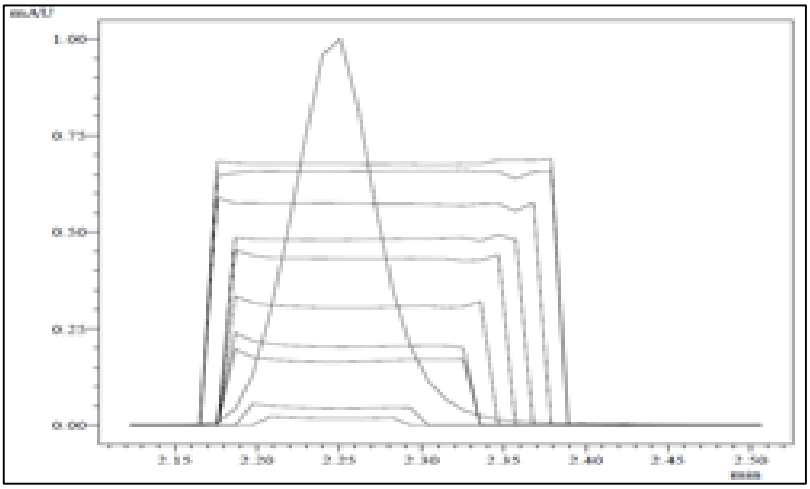


Fig 4: Chromatograms and Ratiograms (Peak Purity Test) for Heat Degradation Study

c. Oxidation Degradation

Oxidation degradation was carried out with 20% H_2O_2 on water bath for 2 hours at 60 ºC (Figure 5, Table 5).





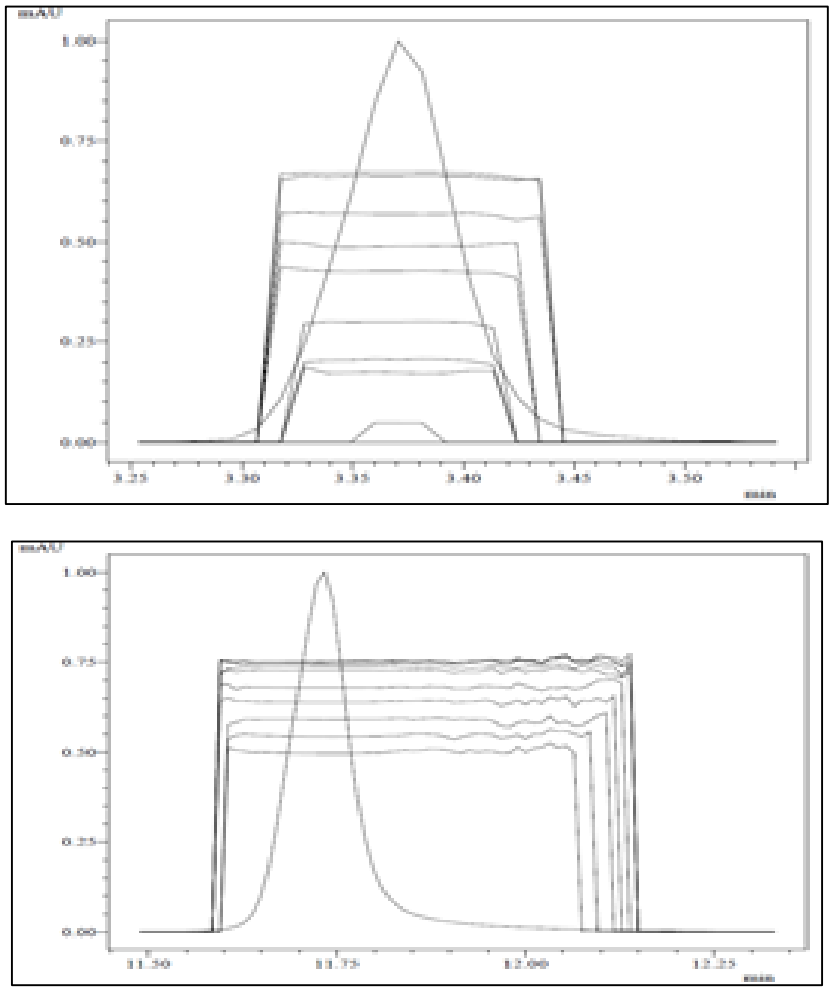
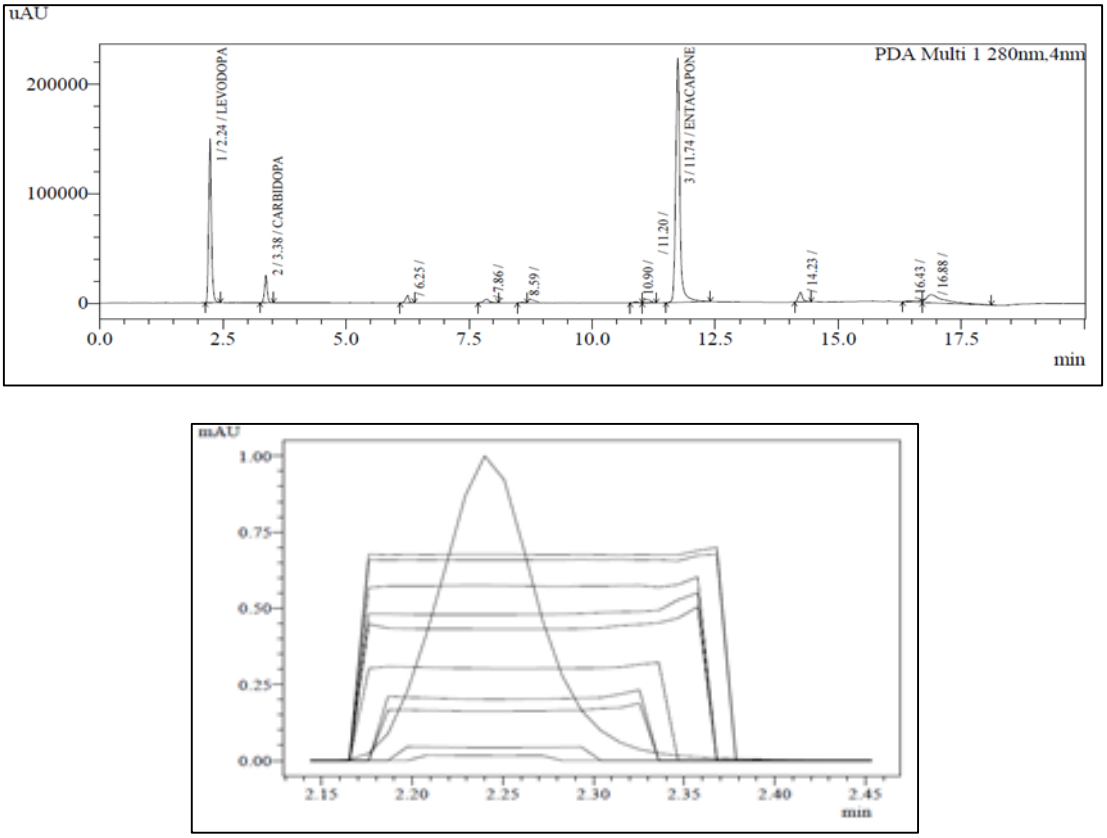


Fig 5: Chromatograms and Ratiograms (Peak Purity Test) for Oxidation Degradation Study

d. Acid Degradation

Acid degradation was carried out with 1N HCl on water bath for 2 hours at 60 ºC (Figure 6, Table 5).



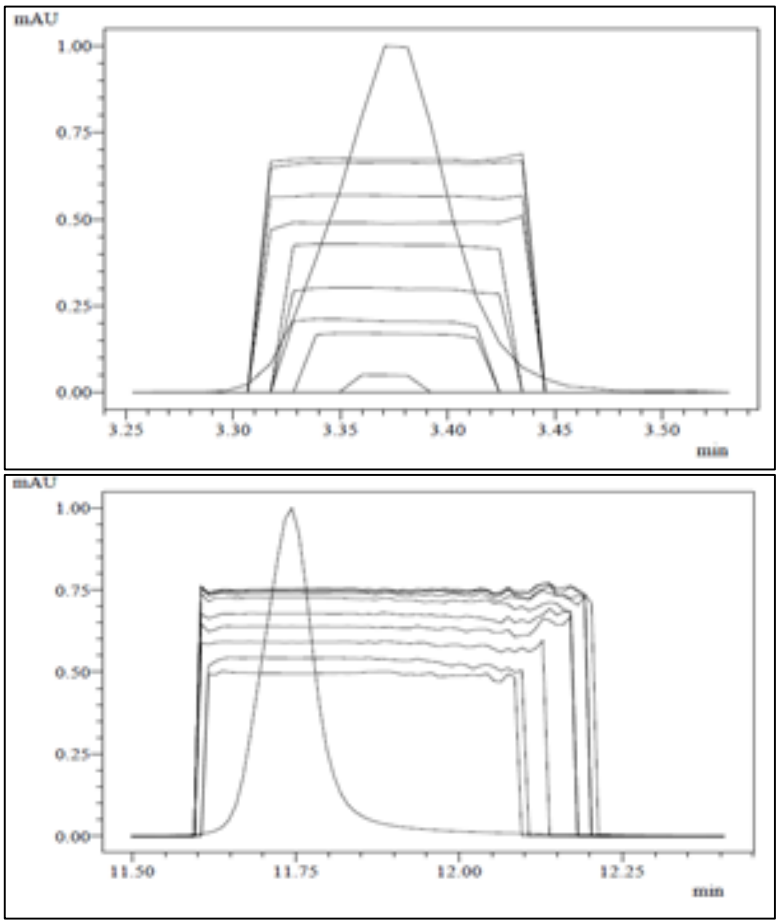
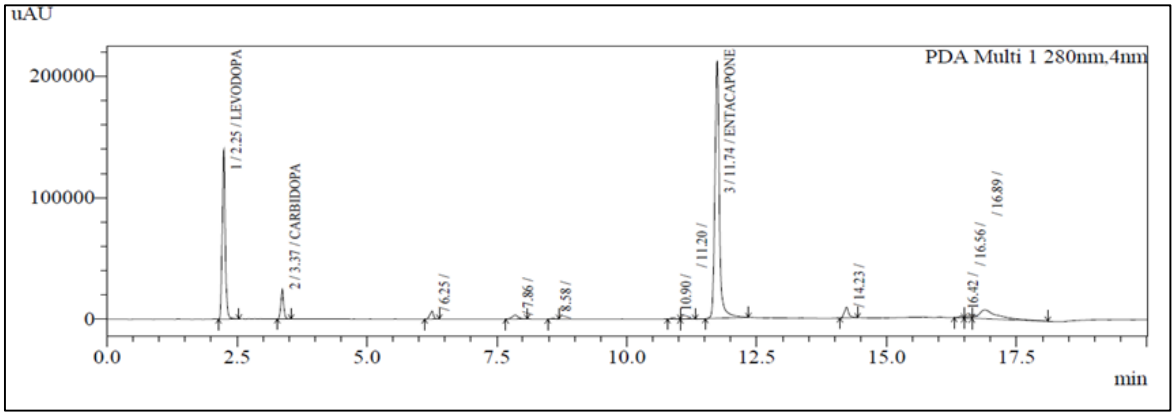


Fig 6: Chromatograms and Ratiograms (Peak Purity Test) for Acid Degradation Study

e. Base Degradation

Base degradation was carried out with 1N NaOH on water bath for 2 hours at 60 ºC (Figure 7, Table 5).



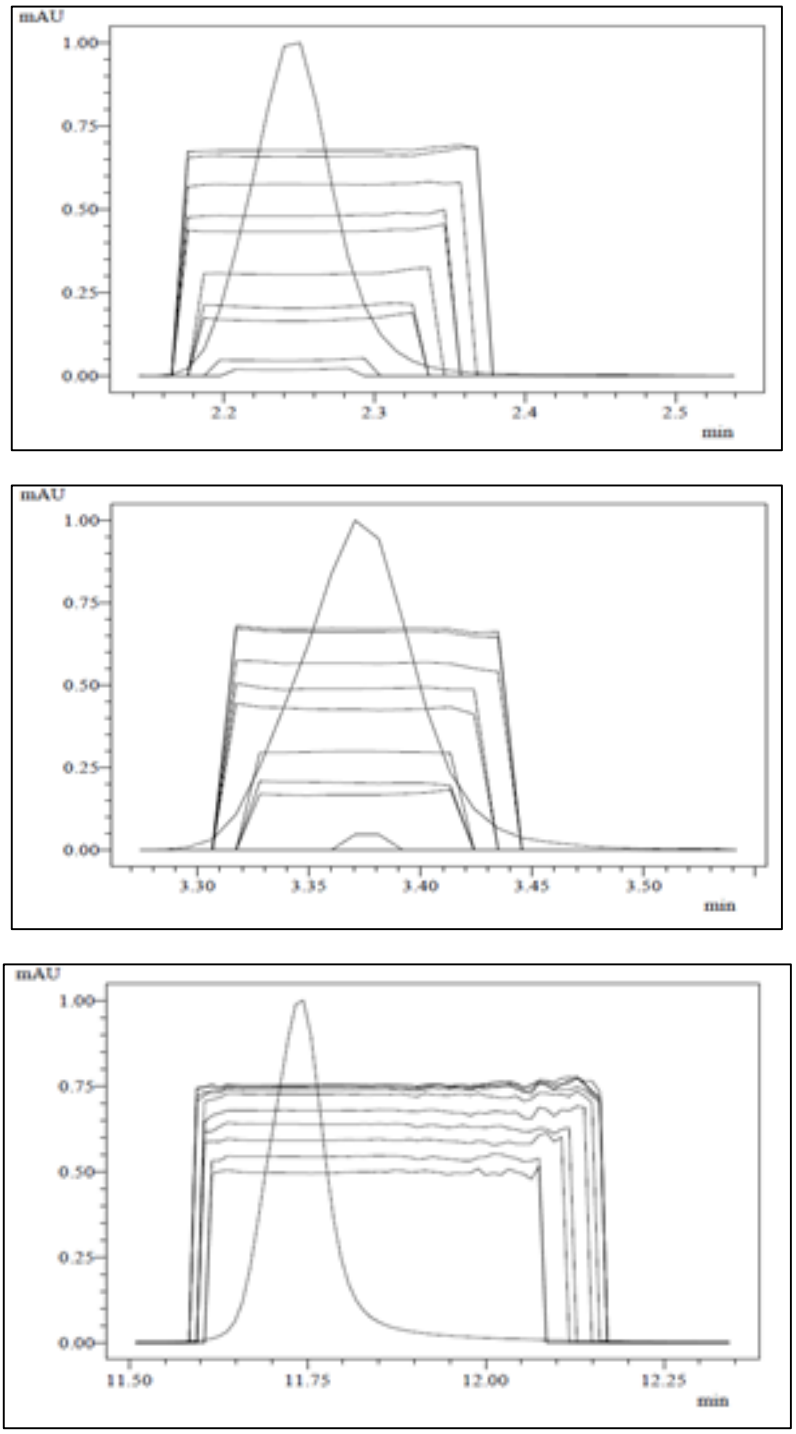


Fig 7: Chromatograms and Ratiograms (Peak Purity Test) for Base Degradation Study

Table 5: Summary of Results

Parameters	**Compounds**		
	Levodopa	**Carbidopa**	**Entacapone**
Linearity Equation	y = 3654.60x + 2820.24	y = 3654.60x + 2820.24	y = 3654.60x + 2820.24
Correlation Coefficient(R^2)	0.9999	0.9999	0.9995
Precision	(Mean%±SD) %RSD	(Mean%±SD) %RSD	(Mean%±SD) %RSD
• Intra-Day Repeatability	(100.27±0.39) 0.3910	(100.03±0.33) 0.3333	(100.18±0.36) 0.3555
• Inter-Day Repeatability	(99.76±0.20) 0.1989	(99.69±0.47) 0.4690	(100.59±0.52) 0.5159
• Intermediate Precision	(100.56±0.34) 0.3429	(100.34±0.46) 0.4628	(100.27±0.45) 0.4453
• Reproducibility	(100.79±0.36) 0.3544	(99.68±0.39) 0.3869	(100.37±0.53) 0.5302
Specificity	No Interference Peak Purity Complies	No Interference Peak Purity Complies	No Interference Peak Purity Complies
Accuracy (% Recovery)	(Mean%±SD) %RSD	(Mean%±SD) %RSD	(Mean%±SD) %RSD
	(100.33±0.73) 0.7265 Min = 99.06	(99.79±1.45) 1.4548 Min = 98.17	(100.12±1.04) 1.0419 Min = 98.17

	Max = 101.71	Max = 101.73	Max = 101.73
Range	32 – 320 µg/ml	8 – 80 µg/ml	32 – 320 µg/ml
System Suitability N = 5 Injections	%RSD	%RSD	%RSD
	0.3767	0.5925	0.0357
Sample Solution Stability (0, 6, 12, 18 & 24 Hrs)	(Mean%±SD) %RSD	(Mean%±SD) %RSD	(Mean%±SD) %RSD
	(100.15±0.43) 0.4318	(99.14±0.57) 0.5759	(99.73±0.49) 0.4935
Robustness • Flow Rate(1±0.2 ml/min) • Column Temp.(35±5ºC) • Buffer pH(3.0±0.2) • Gradient Start Point (98±2 %) • Detection Wavelength (280±5 nm) • Column Length (150 mm) • Different Lots (100 mm)	System Suitability Parameters like Resolution, Tailing Factor, Number of Theoretical Plates(NTP) complies for all analyte peaks at all variations.		
Forced Degradation (%Degradation)	Peak Purity Complies	Peak Purity Complies	Peak Purity Complies
• Light (Sunlight, 5 Days)	3.27	23.23	13.44
• Heat (80ºC, 72 Hrs)	4.50	22.41	6.36
• Oxidation (20% H_2O_2, 2 Hrs, 60 ºC)	2.24	22.80	10.00
• Acid (1 N HCl, 2 Hrs, 60 ºC)	3.16	21.95	9.63
• Base (1 N NaOH, 2 Hrs, 60 ºC)	4.21	24.39	12.95
Assay (% of labeled amount)	100.94	100.12	99.92
Dissolution (% of labeled amount) Dissolution Condition: [14,15,16,17] • Medium: Phos. Buffer, pH 5.5, 900 ml • Apparatus: Paddle, 50 rpm • Time: 30 minutes	100.77, 100.79, 101.67, 100.83, 101.37, 100.61	98.88, 98.52, 98.15, 98.25, 97.34, 97.20	91.22, 94.35, 95.00, 91.53, 94.66, 95.02

4. Conclusion

The method was validated as per the ICH: Q2(R1) and found to be linear, specific, precise, accurate, robust in the range of 32 -320 µg/ml, 8 -80 µg/ml and 32 -320 µg/ml with correlation coefficient (R^2) of 0.9999, 0.9999 and 0.9995 for Levodopa, Carbidopa and Entacapone respectively. The linearity equation was observed as y = 3654.60x + 2820.24, y = 3654.60x + 2820.24 and y = 3654.60x + 2820.24 respectively for Levodopa, Carbidopa and Entacapone. LOD and LOQ of Levodopa, Carbidopa and Entacapone was found to be 2.10, 1.34 & 9.56 µg/ml and 6.35, 4.05 & 28.96 µg/ml respectively. The method has precision within %RSD of about 0.50% and accuracy between 98 - 102%. The sample solution was validated to be stable for 24 hours at normal laboratory conditions. Further, forced degradation studies was conducted to prove the stability indicating capability of the method. The method was found capable to assess unequivocally the analytes in the presence of common degradants. The developed method has significance of being simple, economical, rapid, and is validated with stability indicating power. Hence, the method can be used for routine analysis in the laboratory.

5. Conflict of interest

The authors declare no conflict of interest, financial or otherwise.

6. Acknowledgements

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7. List of abbreviations

RP-HPLC: Reverse Phase High Performance Liquid Chromatography
HPLC: High Performance Liquid Chromatography
SQ: Special Quality
UV: Ultraviolet
PDA: Photo Diode Array
Conc: Concentration
RSD: Relative Standard Deviation
STDEV: Standard Deviation
Avg: Average
Rpm: Revolution Per Minute
LOD: Limit of Detection
LOQ: Limit of Quantitation
API: Active Pharmaceutical Ingredient
ICH: International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use

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