STABILITY INDICATING RP-HPLC METHOD DEVELOPMENT AND METHOD VALIDATION FOR CONTENT ESTIMATION OF ENTACAPONE IN ENTACAPONE TABLETS

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ABSTRACT: A novel, simple, precise, sensitive, and reproducible RP-HPLC method for the Quantitative estimation of Entacapone in bulk and pharmaceutical formulation was developed and validated. The separation was carried out on X – Terra Phenyl (250mm x 4.6 mm), 5µm column with Solvent A: Phosphate buffer and Solvent B: mixture of methanol : Tetrahydrofuran in the ratio of 48 : 2 (v/v) in the ratio of 45:55 %v/v (pH: 2.1 ± 0.05) as the mobile phase at the flow rate of 1.2 ml/min. The eluent detection was carried out using a UV-Visible detector at 300 nm. The retention time of Entacapone was 5.390 min. Linearity was observed Entacapone in the concentration range of 251.67-755.01 µg/ml. The % mean recovery of Entacapone was found to be 100.60%. The present study demonstrates the applicability of chromatographic method to develop a new, sensitive, single RP-HPLC method for the quantitative determination of Entacapone in a bulk form and marketed pharmaceutical dosage forms. Hence, this method can be conveniently adopted for routine analysis in quality control laboratories.

Key Words: Entacapone, RP-HPLC, Method Development, Validation, Accuracy, Precision.

I. INTRODUCTION

Entacapone is a selective, reversible catechol-O-methyl transferase (COMT) inhibitor for the treatment of Parkinson's disease. It is a member of the class of nitrocatechols. When administered concomitantly with levodopa and a decarboxylase inhibitor (e.g., carbidopa), increased and more sustained plasma levodopa concentrations are reached as compared to the administration of levodopa and a decarboxylase inhibitor. Entacapone[1] is a nitrocatechol compound with anti-parkinsonian property. Entacapone is a selective and reversible inhibitor of catechol-O-methyl transferase (COMT), which catalyzes the transfer of the methyl group of S-adenosyl-L-methionine to the phenolic group of substrates that contain a catechol structure including dihydroxy phenylalanine (DOPA), catecholamines (dopamine, norepinephrine, and epinephrine) and their hydroxylated metabolites. When administered in conjunction with dopaminergic agents such as L-DOPA, Entacapone[2] prevents the metabolism and inactivation of adjunct drugs, thereby increasing the bioavailability of these compounds by facilitating their passage across the blood-brain barrier. Entacapone is a catechol-O-methyl transferase inhibitor used in the therapy of Parkinson disease as adjunctive therapy in combination with levodopa and carbidopa. Entacapone[3] has been associated with a low rate of serum enzyme elevations during treatment, but has yet to be implicated in cases of clinically apparent acute liver injury with jaundice. The IUPAC Name of Entacapone is (E)-2-cyano-3-(3, 4-dihydroxy-5-nitro phenyl)-N, N-diethyl prop-2-enamide. The Chemical Structure of Entacapone is follows

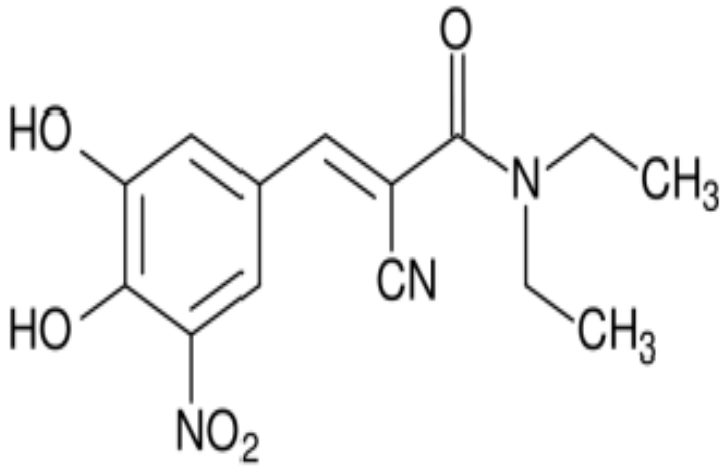


Fig.1. Chemical Structure of Entacapone

II. EXPERIMENTAL

Table-1: List of Instruments Used

S.No.	Name of the instrument	Make
1	HPLC	Waters
2	Analytical Balance	Sartorius
3	pH meter	Thermo
4	Sonicator	PCI Analytic
5	Centrifuge machine	Remi
6	Vacuum Oven	Cintex
7	Orbital shaking incubator	Remi

Table-2: List of Chemicals Used

S.No.	Name of the Reagents/Solvents/Filters	Grade	Make
1	Water	HPLC	MilliQ
2	Monobasic Sodium Phosphate	AR	SRL
3	Orthophosphoric acid	HPLC	Merck
4	Tetrahydrofuran	ACS	Merck
5	Methanol	HPLC	Merck
6	Hydrogen peroxide	Emparta	Merck
7	Sodium hydroxide pellets	Emplura	Merck
8	Hydrochloric acid	Emparta	Merck
9	0.45 µ Membrane filter	NA	Millipore

Preparation of Mobile Phase A:
Weigh and transfer 2.1g of monobasic sodium phosphate in 1000ml of water and sonicate to dissolve. Then adjust the pH to 2.1 ± 0.05 with ortho phosphoric acid. Filter it through the 0.45µ Millipore filter and degas[4].

Preparation of Mobile Phase B:
Prepare a mixture of methanol: Tetrahydrofuran in the ratio of 48: 2 (v/v)

Preparation of Diluent:
Prepare a mixture of methanol: Tetrahydrofuran in the ratio of 70: 30 (v/v)

Preparation of Standard Solution:
Accurately weigh and transfer about 25mg of Entacapone working standard in to 50ml volumetric flask, add about 30ml of Diluent and sonicate to dissolve, make up the volume with diluent and mix well.

Preparation of Sample Solution:
Weighed 20 tablets and average weight were determined. Crushed the tablets into fine powder. Weighed and transferred equivalent to 125mg of Entacapone into 250mL volumetric flask. Then added about 75mL of Tetrahydrofuran and sonicated for 3min. Thereafter added 75mL of methanol and shaken for 5min in Orbital Shaker. Diluted it with methanol upto the mark and centrifuged for 5min at 5000rpm. Supernatant solution is used.

Method Validation
The objective of the method validation is to demonstrate that the method is suitable for its intended purpose as it is stated in ICH guidelines[30]. The method was validated for linearity, precision, accuracy, robustness and system suitability.

System Suitability:
System suitability test was carried out to verify that the analytical system is working properly and can give accurate and precise results. The overall system suitability[5,6] was evaluated for the system suitability of the proposed method. Data from five injections (500µg/mL) were utilized for calculating parameters like theoretical plates, resolution, tailing factor and %RSD of 5 injections.

Linearity:
To establish the linearity[7] of the method, calibration solutions were prepared from the stock solution at five concentration levels from 251.67µg/ml to 755.01µg/ml of analyte concentration. The correlation coefficient, Y-intercept and slope of the calibration curve[8] were calculated.

Precision:
The precision[9] of an analytical method expresses the closeness of agreement (degree of scatter) between a series of

measurements obtained from multiple sampling of the same homogeneous sample over the prescribed conditions. Intra-day and inter-day Precision[10] were determined through repeatability analysis. The precision for drug was checked by injecting six individual preparations. The % RSD of Entacapone was calculated.

Accuracy: The accuracy[11] of the assay method was evaluated in triplicate at three concentration levels i.e., 50, 80, 100, 120, 150µg/mL-1 (50, 80, 100, 120 & 150% of the normal assay concentration) for bulk drug sample. The %recoveries[12] were calculated. The study was carried out in triplicate (n=3). The solutions were injected into HPLC system and the mean peak area of analyte (Entacapone) peak was calculated for assays. Assay[13] (%w/w) of test solution was determined against three injections (n=3) of qualified Entacapone reference or working standard.

Limit of detection (LOD) and Limit of quantification (LOQ): LOD[14] and LOQ for Entacapone was calculated as suggested by ICH guidelines using equations LOD = 3.3 σ/s and LOQ[15] = 10 σ/s, respectively. Where, σ is the SD of the response and S is the slope of the calibration curve.

Robustness: To determine the robustness[16] of the method, system suitability parameters were verified by making deliberated changes in the chromatographic conditions, viz, changing flow rate by 0.2 units from 1.0 to 1.4 mL-1. The effect of pH variation was studied by varying from 2 to 2.4 in 0.2 pH units. The effect of column oven temperature on resolution was studied at 35 to 45^{0}C. In all the above varied conditions, the components of the mobile phase[17] were held constant. To study the effect of change in mobile phase composition by changing the organic ratio, the organic component was changed by 10% from 90 to 110% keeping the buffer ratio constant.

Stability Studies: Selectivity[18] was assessed by performing forced degradation studies. The ICH stress testing of the drug substance can help to demonstrate the basic stability[19] of the molecule and validate the stability – indicating power of the analytical procedures used.

III. RESULTS AND DISCUSSION

Development of Method

Final Optimized Chromatographic Conditions:

HPLC Column: X – Terra Phenyl (250mm x 4.6mm), 5µm

Mobile Phase: A - 100% Buffer

B – Methanol: Tetrahydrofuran (48:2)

Isocratic Elution:

Flow	%A	%B
1.2	45%	55%

Flow rate: 1.2ml/min

Column Temperature: 30°C

Sample Temperature: 25°C

Injection Volume: 10µL

Wavelength: 300nm

Run time: 15 min

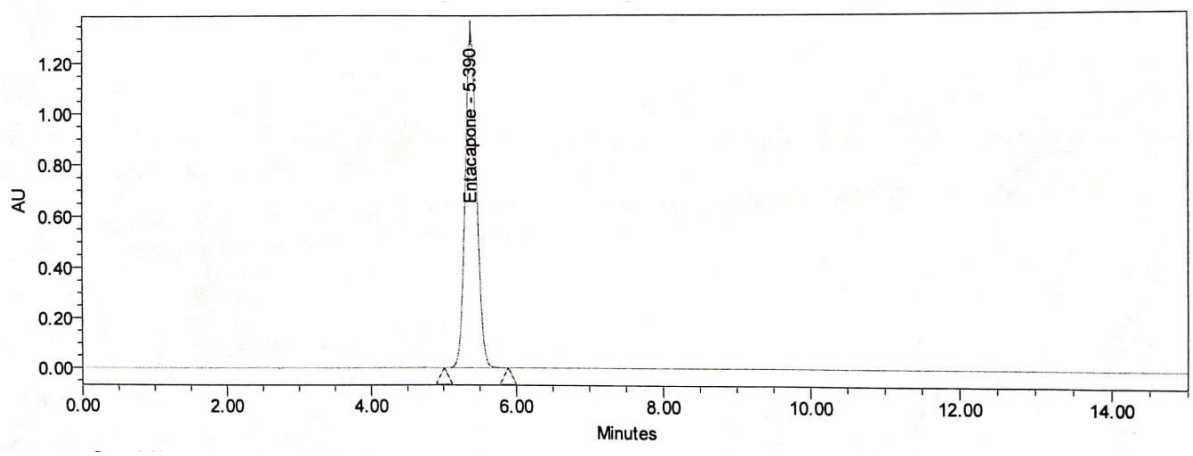


Fig.2. Optimized Chromatographic Condition

Method Validation

The proposed method was validated according to the ICH guidelines for system suitability, specificity, recovery, precision[20], linearity, and robustness, limit of detection (LOD) and limit of quantification (LOQ). Under the

validation study, the following parameters were studied.

System Suitability:

The system suitability solution and standard solution were prepared and analysed as per test method to evaluate the system suitability parameters and the results were found to be within the limits. The standard solution was injected five times to evaluate system precision and the result is found to be within the limits.

In order to assess the system suitability parameters, the standard solution and system suitability solution were prepared and analysed in accordance with the test procedure. The finding was found to be with in the acceptable range[21]. Five injections of the standard solution were made to test the system's precision, and the results were determined to be within acceptable limits.

Table-3: Results of System Suitability

System Suitability Parameters	Observed Value	Acceptance Criteria
The tailing factor for Entacapone peak from the chromatogram of standard solution.	1.0	NMT 2.0
% Relative standard deviation for Entacapone peak area from five replicate Injections of standard solution.	0.2	NMT 2.0
Plate Count	5780	NLT 2000

Table-4: System Precision

Injection Number	Peak Area	Acceptance Criteria
1	14544478	The % RSD for peak area of an Entacapone from five replicate injections of standard solution should be not more than 2.0
2	14586595	
3	14609350	
4	14630944	
5	14654750	
Average	**14605223**	
SD	**42333.45**	
%RSD	**0.29**	

Method Precision

The method precision[22] was performed by analysing the sample solution of Entacapone tablets at working concentration six times (six replicate sample preparations). Table shows Percentage relative standard deviation of Entacapone assay values of six replicate sample preparations.

Table-5: Results of Method Precision

Sample No	% Assay
1	102.5
2	102.5
3	102.4
4	102.2
5	101.9
6	102.3
Mean	**102.3**
SD	**0.228035**
% RSD	**0.2**

Intermediate Precision

The ruggedness of method was demonstrated by conducting the precision study by different analyst. Assay was performed for six individual test preparations as per test method**.** The % RSD for assay[23] results from six individual test preparations is found to be within the limit. The overall %RSD for the assay results obtained from both method precision and intermediate precision[24] is found to be within the limit. The system suitability results

were evaluated as per the test method and results are found to be within the limits.

Table-6: Results of Intermediate Precision

Sample No	% Assay
1	101.2
2	100.6
3	100.1
4	99.5
5	99.8
6	100.8
Average	**100.3**
SD	**0.643946**
% RSD	**0.6**

Accuracy

Entacapone recovery was tested at levels ranging from 50% to 150% of the initial assay concentration. Sample solutions were made in triplicate for each level and were then analysed in accordance with test method. According to the calculations, the individual % recovery, % average recovery and %RSD for recovery[25] at each level were all within the acceptable ranges.

Table-7: Results of Accuracy

% Level Spiked	Sample No.	% Recovery	% Recovery Mean	% RSD
50%	1	101.6	101.2	0.83
	2	101.7		
	3	100.2		
80%	1	100.3	100.1	0.21
	2	100.0		
	3	99.9		
100%	1	100.4	101.0	0.65
	2	100.9		
	3	101.7		
120%	1	99.8	100.7	0.80
	2	101.2		
	3	101.2		
150%	1	100.3	99.8	0.44
	2	99.6		
	3	99.5		
Overall			**100.6**	**0.76**

Specificity

Prepared Blank, Placebo and standard preparations are injected into the system.

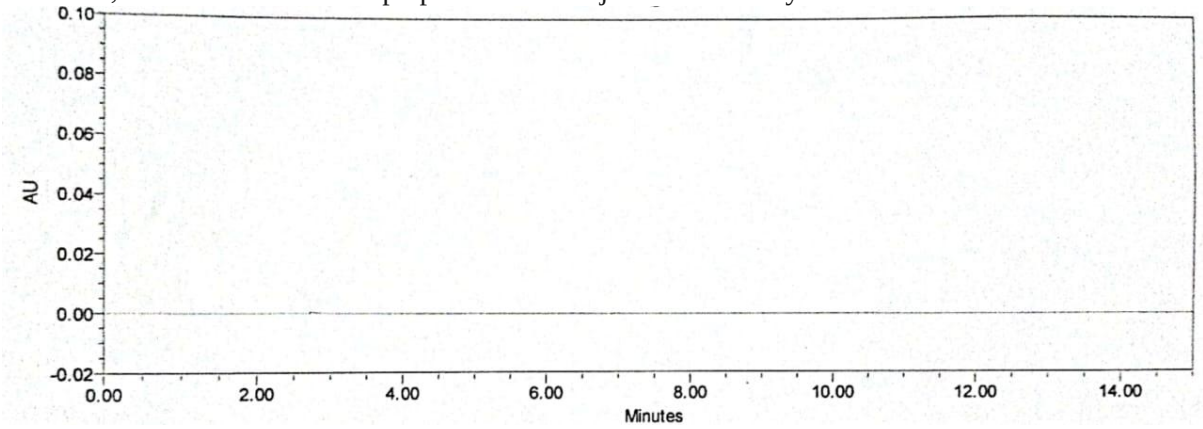


Fig.3. Blank Chromatogram

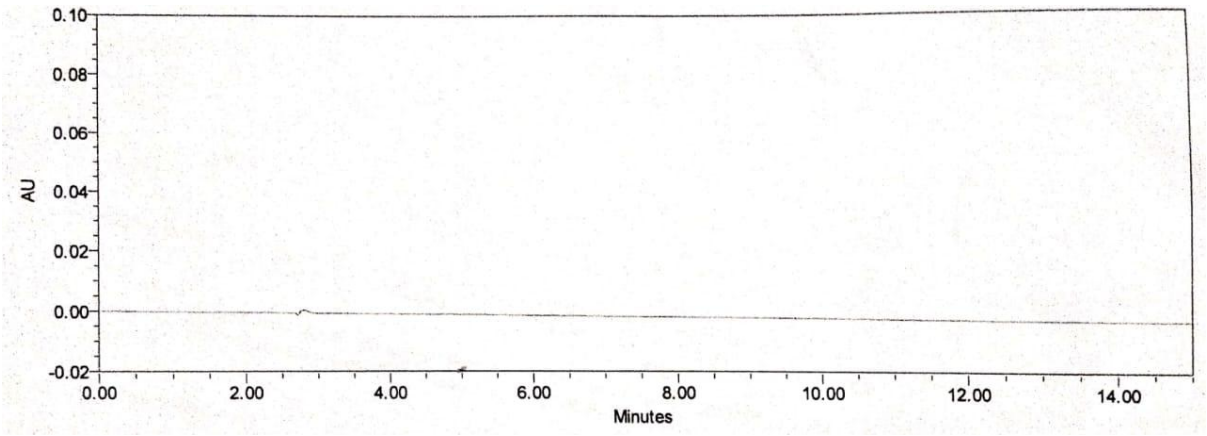


Fig.4. Placebo Chromatogram

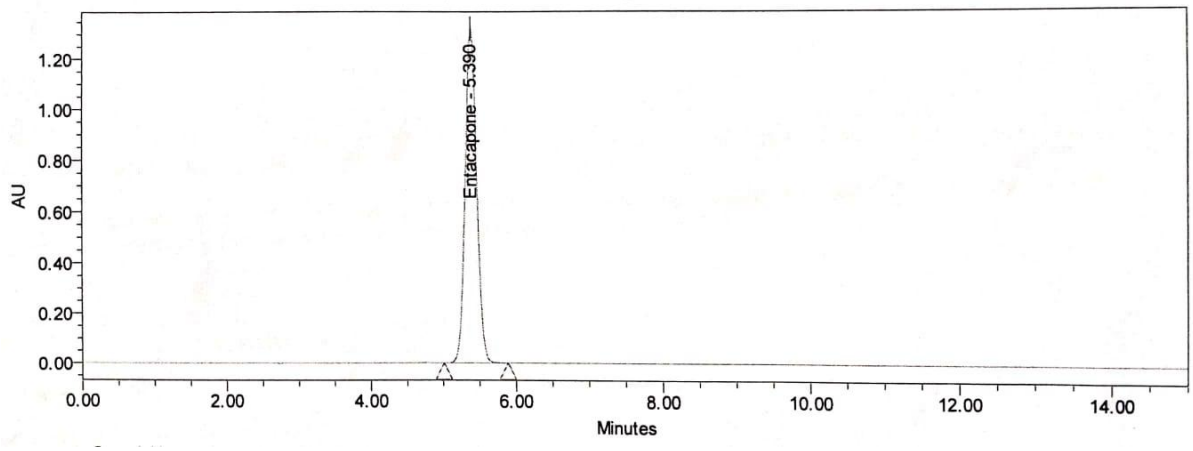


Fig.5. Standard Chromatogram

Forced Degradation:

Forced degradation study was carried out with acidic (HCl), basic (NaOH), oxidation (H2O2), stress condition in solution state and thermal, humidity and photo degradation[26] in solid state using Entacapone tablets.

Acidic Degradation:

Weighed the sample equivalent to 125mg from an average weight of 10 tablets in 250mL volumetric flask. Optimum degradation has been found at a condition of 5mL of 5N HCl for 3hrs at room temperature. Added 5mL of 5N HCl to the above weighed sample in volumetric flask and kept it aside for 3hrs. After 3hrs 5mL of 5N NaOH was added and shaken for neutralization step to take place. Thereafter, 75mL of Tetrahydrofuran was added and sonicated for 3min. Then added 75mL of methanol and shaken for 5min in orbital shaker. It was then made upto mark with methanol and centrifuged for 5min. The supernatant solution was used.

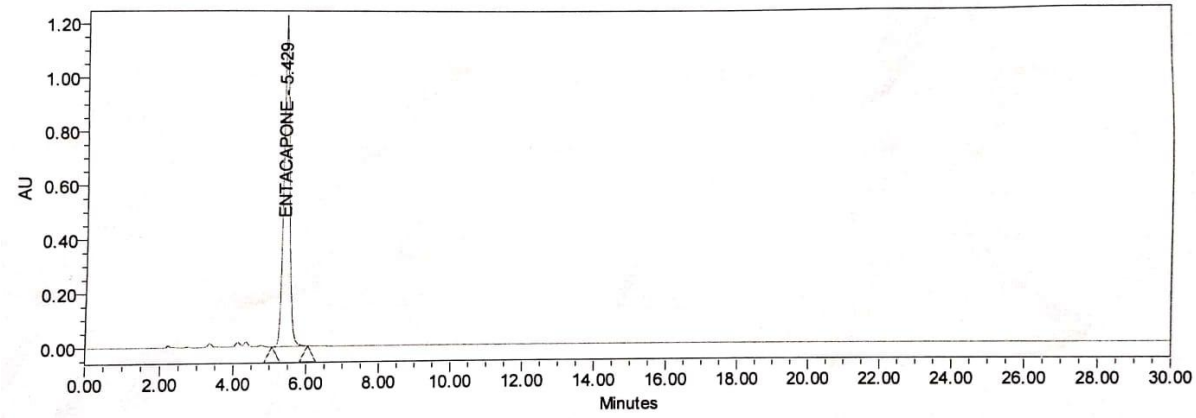
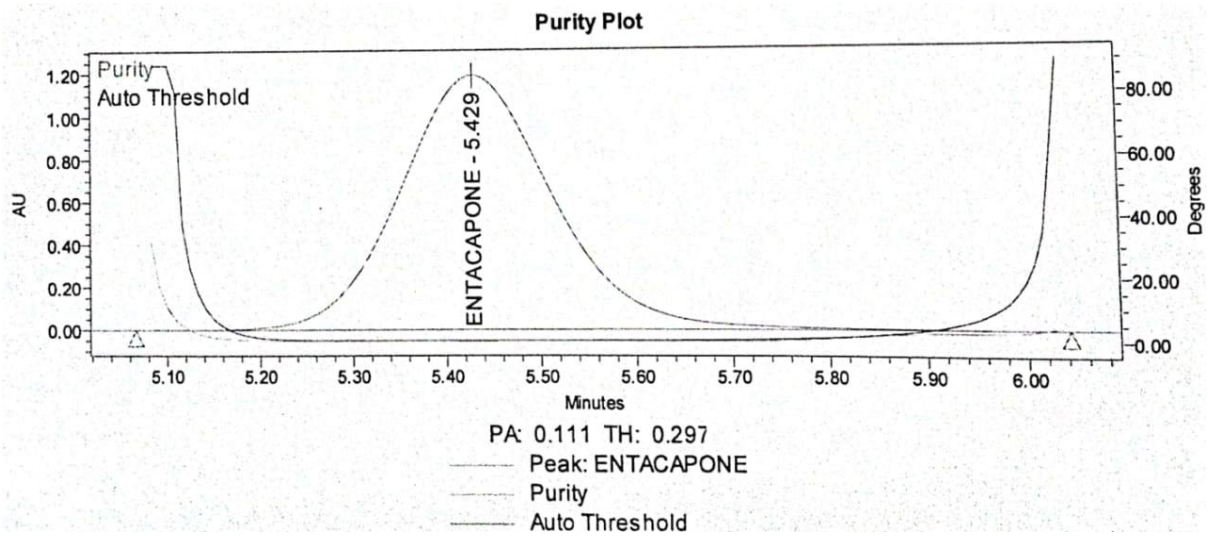


Fig.6. Peak Purity for Acid Stressed Sample



Basic Degradation:

Weighed the sample equivalent to 125mg from an average weight of 10 tablets in 250mL volumetric flask. Optimum degradation[27] has been found at a condition of 5mL of 2N NaOH for 15min at room temperature. Added 5ml of 2N NaOH to the weighed sample in volumetric flask and kept it aside for 15min.Added to it 5mL of 2N HCl and shaken for neutralization step to take place. Thereafter, 75mL of Tetrahydrofuran was added and sonicated for 3min. Then added 75mL of methanol and shaken for 5min in orbital shaker. It was then made upto mark with Methanol and centrifuged for 5min. The supernatant solution is used.

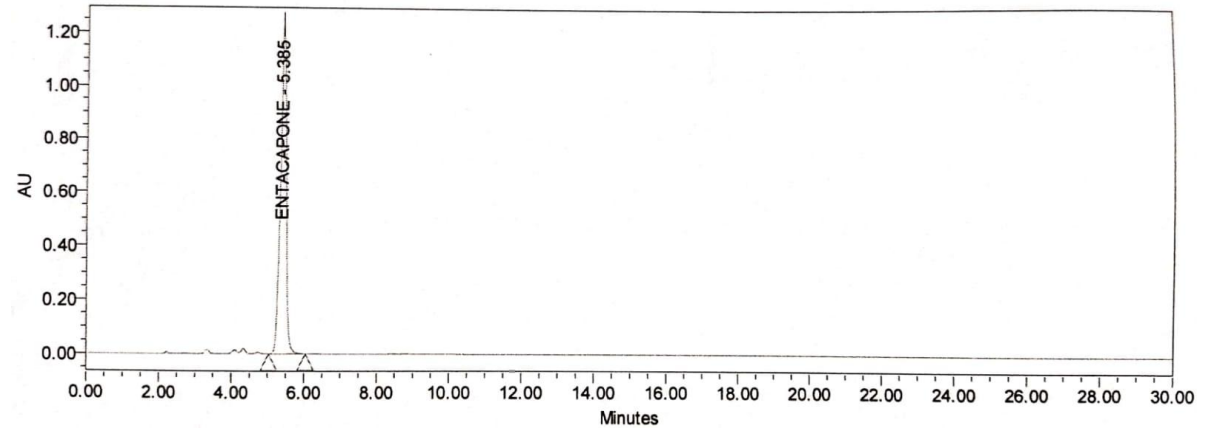
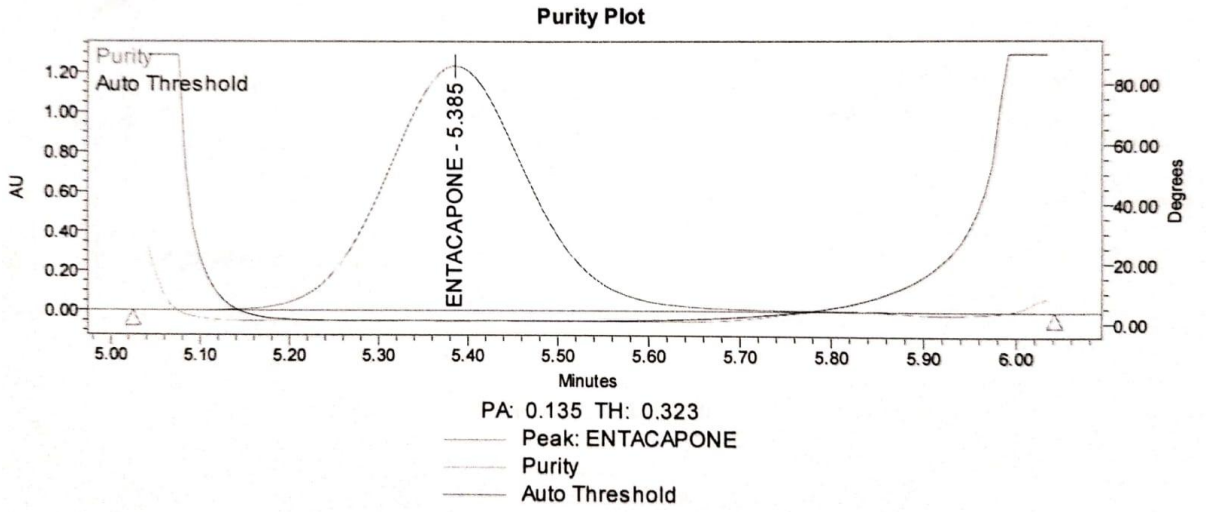


Fig.7. Peak Purity for Base Stressed Sample



Oxidative Degradation

Weighed the sample equivalent to 125mg from an average of 10 tablets in 250mL volumetric flask. Optimum degradation has been found at a condition of 5mL of 30% H_2O_2 for 3hrs at room temperature. Added 5mL of 30% H_2O_2 to the weighed sample in volumetric flask and kept aside for 3hrs. Therefore 75mL of Tetrahydrofuran was

added and sonicated[28] for 3min. Then added 75mL of Methanol and shaken for 5min in Orbital shaker. It was then made upto the mark with Methanol and centrifuged for 5min. The supernatant solution was used.

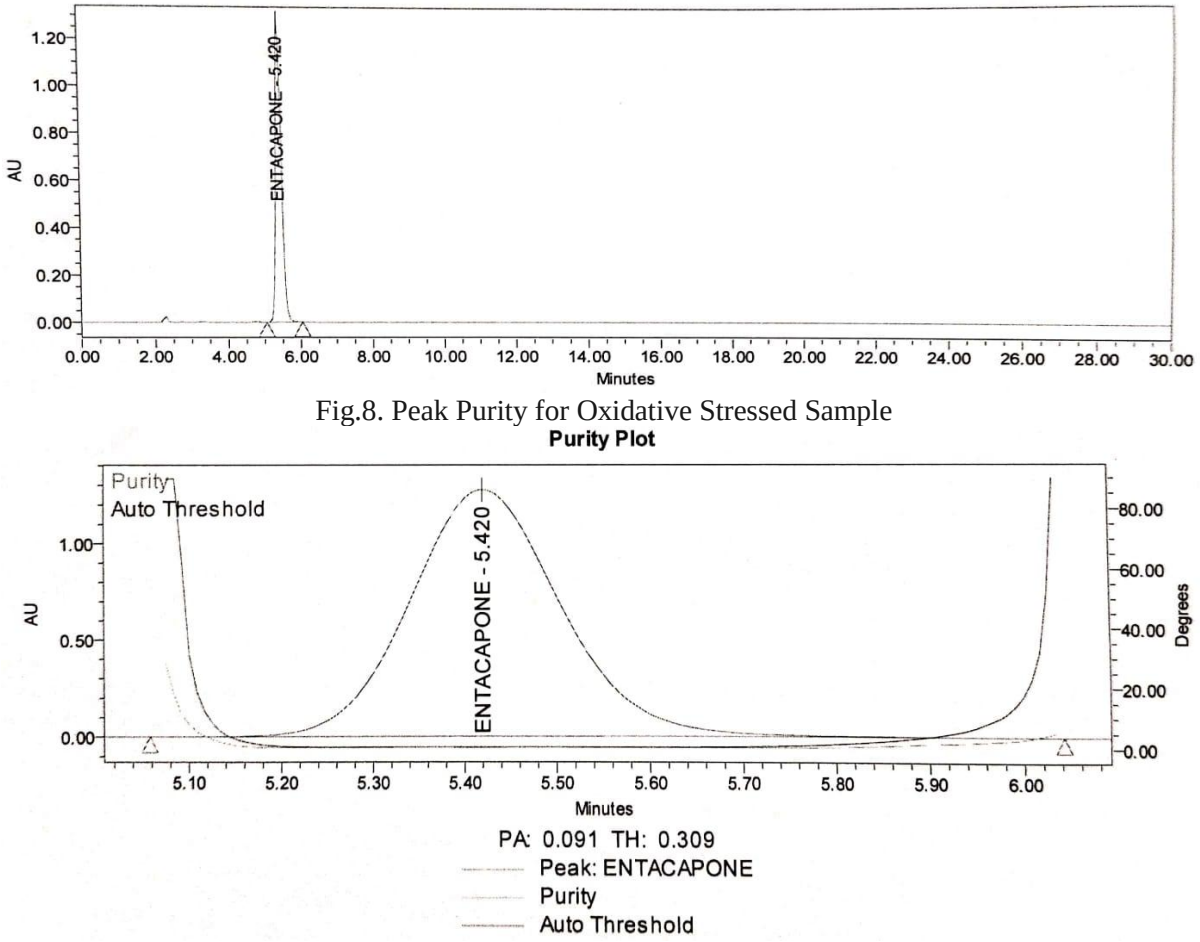


Fig.8. Peak Purity for Oxidative Stressed Sample

Thermal Degradation

Weighed the sample equivalent to 125mg from an average of 10 tablets in 250mL volumetric flask. Weighed accurately sample equivalent to 125mg from an average of 10 tablets exposed to 105°C for 24hrs in volumetric flask. Thereafter, 75mL of Tetrahydrofuran was added and sonicated for 3min. Then added 75mL of Methanol and shaken in orbital shaker for 5min. It was then made upto mark with Methanol and centrifuged for 5min. Use the supernatant solution.

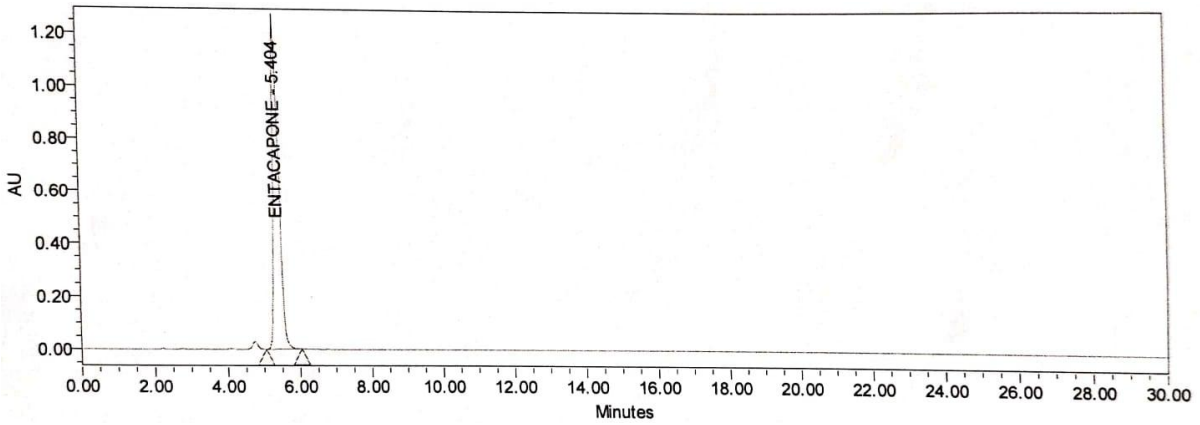


Fig.9. Peak Purity for Thermal Stressed Sample

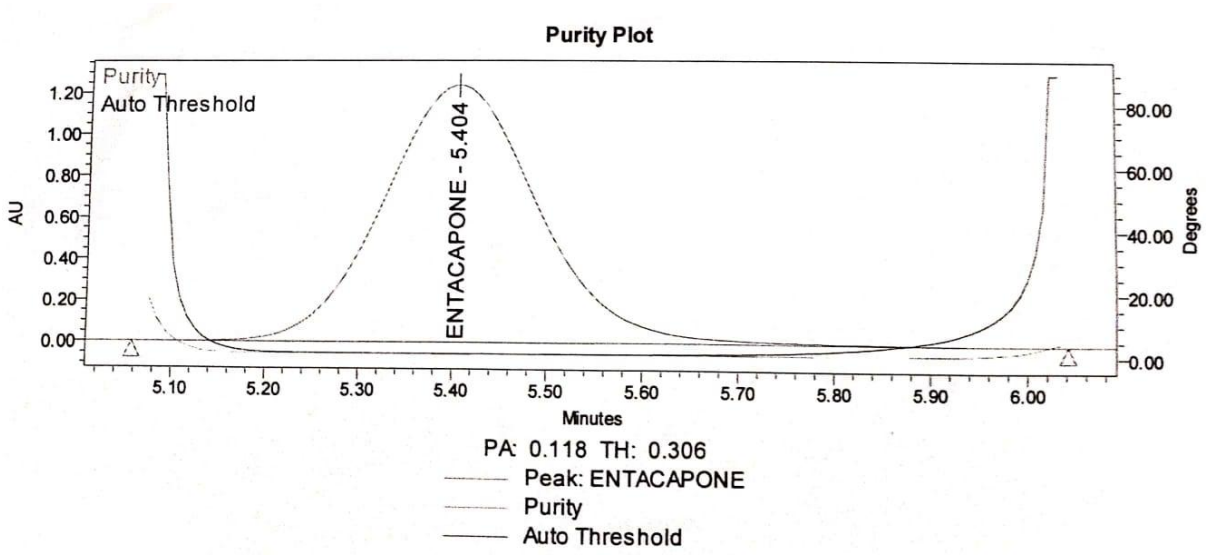


Table-8: Stress Degradation studies

S.No.	Condition		% Assay	% Degradation	Purity Angle	Purity Threshold	Purity Flag
1	As Such	_	102.42	_	0.081	0.312	No
2	Acid	5mL of 5N HCl at room temperature for 3hrs	93.18	9.02	0.111	0.297	No
3	Base	5mL of 2N NaOH at room temperature for 15min	94.79	7.45	0.135	0.323	No
4	Oxidative	5mL of 30%H_2O_2 at room temperature for 3hrs	100.91	1.47	0.091	0.309	No
5	Thermal	105°C for 24hrs	97.95	4.36	0.118	0.306	No

Linearity

A graph between concentrations and area was drawn to establish the linearity of the detector response. Entacapone standard solutions were made in a range of 50% to 150%, and then they were tested according to the test procedure. We Calculated the Correlation Coefficient[29] by plotting the concentration in µg/mL on X-axis against the response on Y-axis. The outcomes are found to be within the acceptable limit.

Linearity Level Preparation

Linearity levels were prepared as per the dilutions mentioned in the following table

Table-9: Linearity Data for Entacapone

SXGT	Weight (mg)		50.74	
	Potency		99.2	
Levels	V1	V2	V3	Conc.(ppm)
50%	50	2.5	10	251.67
80%	50	4	10	402.67
100%	50	5	10	503.34
120%	50	6	10	604.00
150%	50	7.5	10	755.01

Table-10: Results of Linearity

S.No.	Concentration (µg/mL)	Peak Area
1	251.67	7345672
2	402.67	11561631

3	503.34	14494954
4	604.00	17355289
5	755.01	21799481

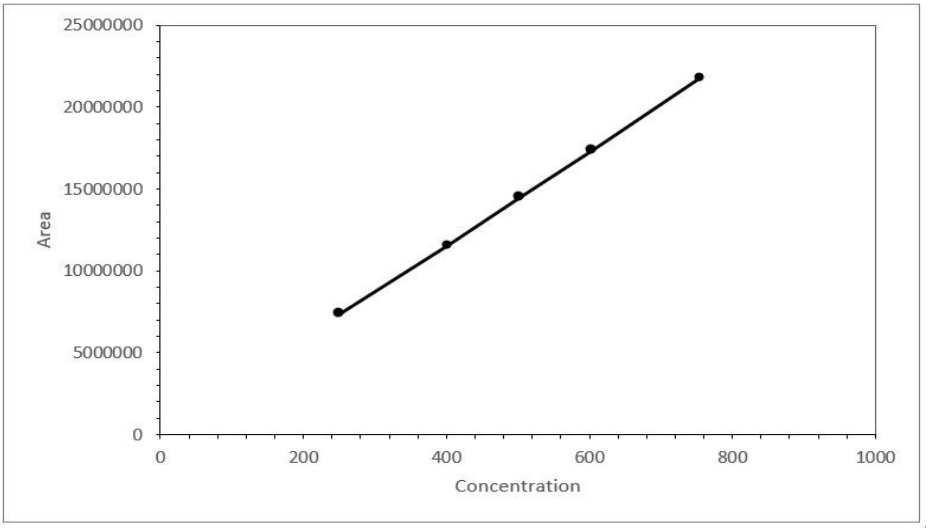


Fig.10. Linearity Graph of Entacapone

Filter Validation:

Prepare standard solution (single preparation) and test solution of Entacapone tablets 200mg strength as per the test method. Centrifuge some portion of the test solution and also filter remaining portion of the test solution through 0.45u PVDF, PTFE, and Nylon.

Inject unfiltered standard solution, filtered standard solution, filtered test solution and centrifuged test solutions in duplicate.

Table-11: Filters Used

Filter Description	Filters		
	PVDF	PTFE	Nylon
Manufacturers Name	Millipore	Millipore	Millipore
Size	0.45 µm	0.45 µm	0.45 µm

Table-12: Results of Filter Interference

Sample No.	% Assay				Difference between Centrifuged and filtered sample		
	Centrifuged	PVDF	PTFE	Nylon	PVDF	PTFE	Nylon
1	101.68	102.06	101.68	101.27	0.37	0.00	0.40

Solution Stability:

Solution stability was performed by analysing standard and sample preparation using Entacapone tablets 200mg periodically into HPLC system at room temperature i.e.; 25°C

Table-13: Results of Stability of Test Preparations

Time (Hours)	%Assay of Test Preparation	Difference from Initial
Initial	100.15	NA
After 24 Hrs	101.52	1.37

Robustness

Effect of Variation in Flow rate:

To ascertain the impact of change in flow rate, robustness testing was done. At flow rates of 1.0 mL/min and 1.4 mL/min, the characteristics of the system suitability were assessed. The results of the system suitability tests showed that they were both within the acceptable limits for higher and lower flow rates. It can be inferred from this that the range of acceptable flow rate variation is 1.0 to 1.4 mL/min.

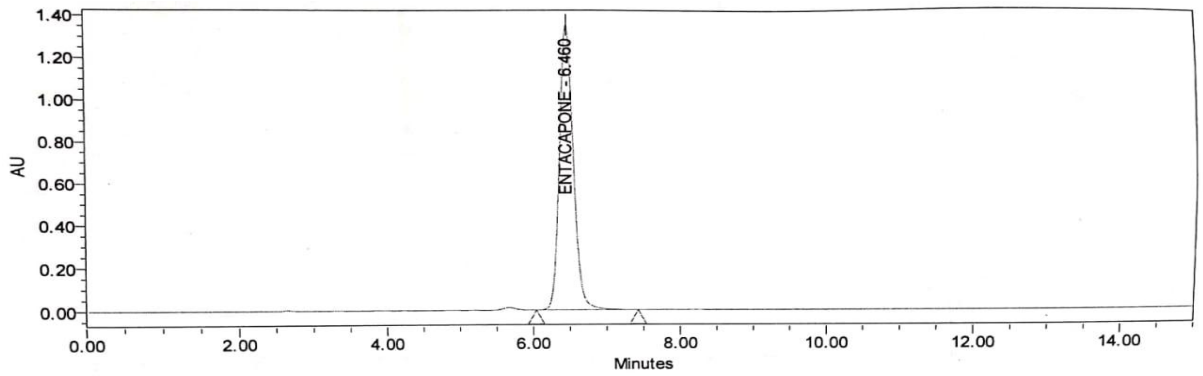


Fig.11. Chromatogram for Less Flow

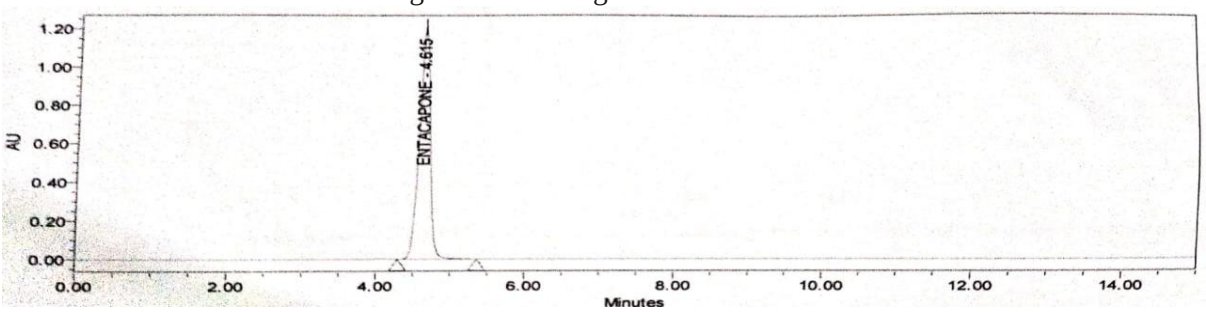


Fig.12. Chromatogram for More Flow

Effect of Variation in Column Oven Temperature

To ascertain the impact of change in temperature, robustness testing was done. At temperatures of 25°C and 35°C, the characteristics of system suitability were assessed. The results of system suitability tests showed that they were both within the acceptable limits for higher and lower temperatures. It can be inferred from this that the range of acceptable temperature variation id 25°C to 35°C.

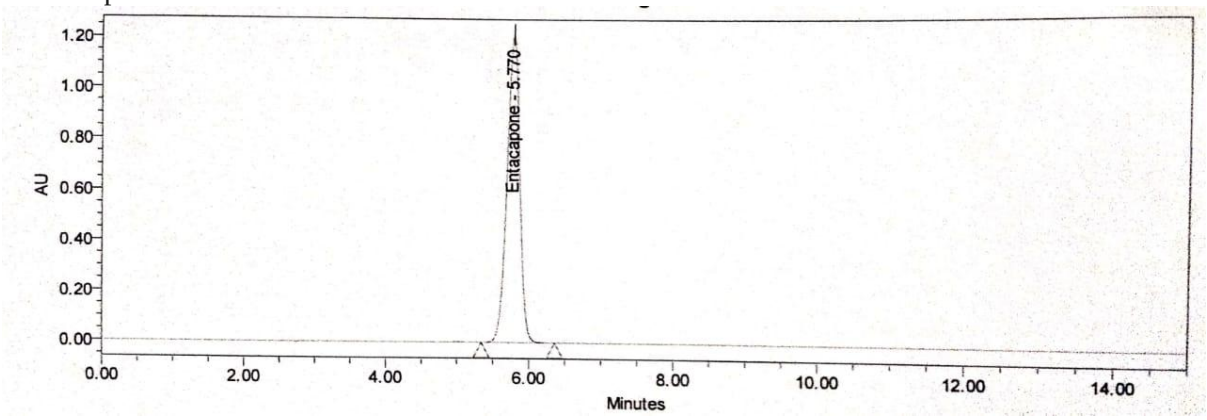


Fig.13. Chromatogram for Low Temperature

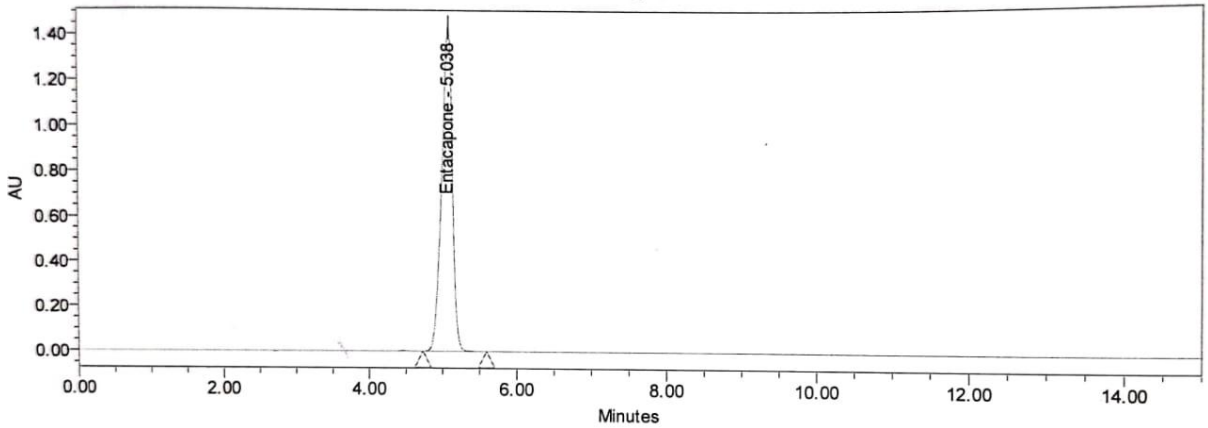


Fig.14. Chromatogram for High Temperature

Effect of Variation in Wavelength

To ascertain the impact of change in wavelength, robustness testing was done. At wavelength of 298nm and 302nm, the characteristics of system suitability were assessed. The results of the system suitability tests showed that they were both within the acceptable limits for both higher and lower wavelength. It may be inferred from this that range of acceptable wavelength variation is 298nm to 300nm.

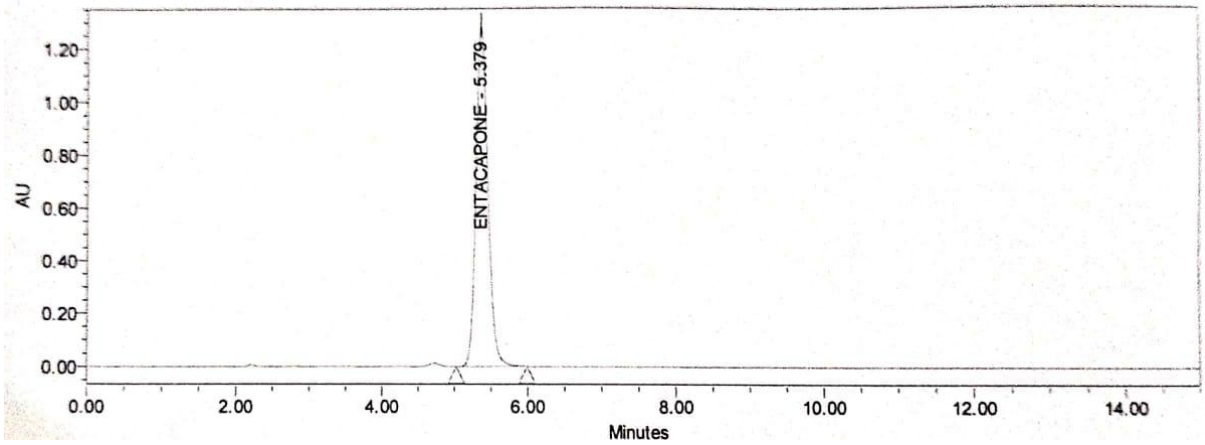


Fig.15. Chromatogram for Low Wavelength

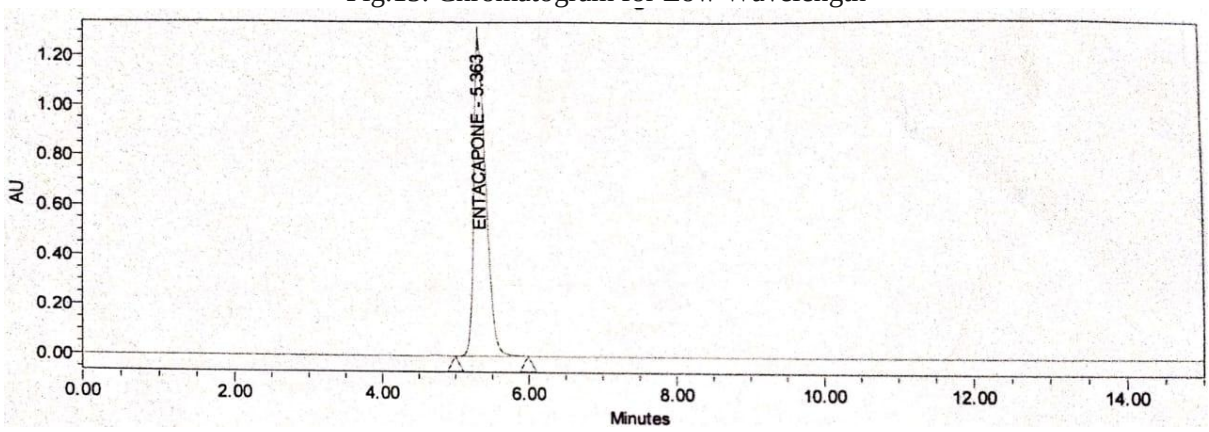


Fig.16. Chromatogram for High Wavelength

Effect of Variation in pH

To ascertain the impact of change in pH, robustness testing was done. At pH of 1.9 and 2.3, the characteristics of the system suitability were assessed. The results of the system suitability tests showed that they were both within the acceptable limits for both higher and lower pH. It may be inferred from this that range of acceptable pH variation is 1.9 to 2.3.

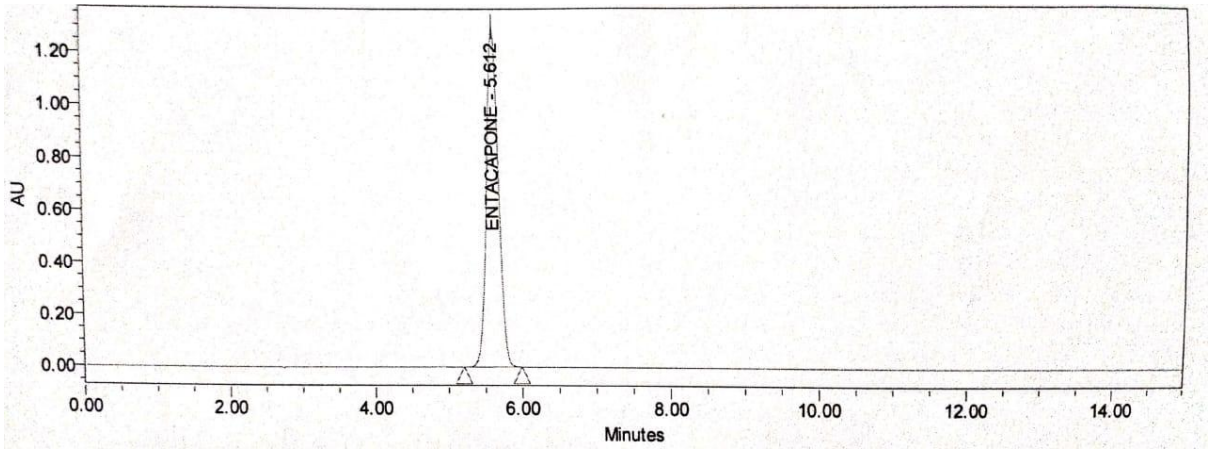


Fig.17. Chromatogram for pH-1.9

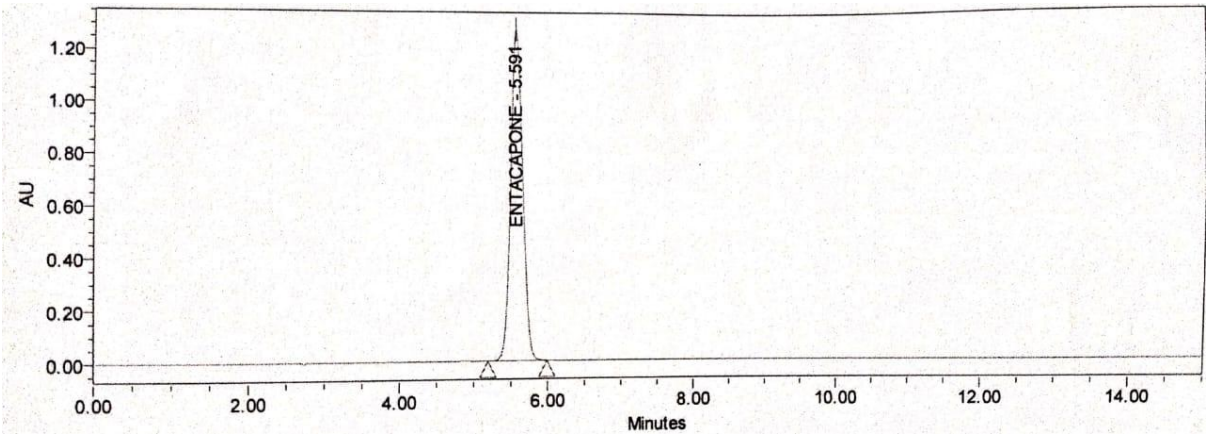


Fig.18. Chromatogram for pH-2.3

IV. CONCLUSION

A New analytical RP-HPLC method for the estimation of Entacapone in bulk form and their marketed pharmaceutical dosage form was developed and validated as per the ICH guidelines. Linearity was observed in the concentration range from 251.67µg/ml to 755.01µg/ml for Entacapone with correlation coefficients (r2 = 0.999). The percentage recoveries of Entacapone were in the range of 98.0% - 102% which was with in the acceptance criteria. The percentage RSD was NMT 2% which proved the precision of the developed method. The developed method is simple, sensitive, rapid, linear, precise, rugged, accurate, specific, and robust. Hence, the RP-HPLC method can be applied for the routine analysis of Entacapone in bulk and pharmaceutical dosage forms.

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