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Novel Method Development and Validation for The Quantitative Estimation of Lenalidomide in Api Form and Marketed Capsule Dosage Form by Using RP-HPLC

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Abstract

A new, simple, rapid, precise, accurate and reproducible RP-HPLC method for estimation of Lenalidomide in bulk form and marketed formulation. Separation of Lenalidomide was successfully achieved on a Develosil ODS HG-5 RP C_{18}, 5µm, 15cmx4.6mm i.d. column in an isocratic mode of separation utilizing Methanol: Phosphate buffer (0.02M, pH-3.6) in the ratio of 45:55% v/v at a flow rate of 1.0 mL/min and the detection was carried out at 255nm. The method was validated according to ICH guidelines for linearity, sensitivity, accuracy, precision, specificity and robustness. The response was found to be linear in the drug concentration range of 12-28mcg/mL for Lenalidomide. The correlation coefficient was found to be 0.9995 for Lenalidomide. The LOD and LOQ for Lenalidomide were found to be 5.004µg/mL and 15.164µg/mL respectively. The proposed method was found to be good percentage recovery for Lenalidomide, which indicates that the proposed method is highly accurate. The specificity of the method shows good correlation between retention times of standard solution with the sample solution. Therefore, the proposed method specifically determines the analyte in the sample without interference from excipients of pharmaceutical dosage forms.

Keywords

Lenalidomide, RP-HPLC, Accuracy, Precision, Robustness, ICH Guidelines.

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INTRODUCTION

Lenalidomide is a dicarboximide that consists of 1-oxoisoindoline bearing an amino substituent at position 4 and a 2,6-dioxopiperidin-3-yl group at position 2. Inhibits the secretion of TNF-alpha. It has a role as an angiogenesis inhibitor, an antineoplastic agent and an immunomodulator. It is a member of isoindoles, a dicarboximide, a member of piperidones and an aromatic amine. Lenalidomide[1] (previously referred to as CC-5013) is an immunomodulatory drug with potent antineoplastic, anti-angiogenic, and anti-inflammatory properties. It is a 4-amino-glutamyl analogue of [thalidomide] and like thalidomide, lenalidomide exists as a racemic mixture of the active S (-) and R(+) forms. However, lenalidomide is much safer and potent than thalidomide, with fewer adverse effects and toxicities. Thalidomide and its analogues, including lenalidomide, are referred to as immunomodulatory imide drugs (also known as cereblon modulators),

which are a class of immunomodulatory drugs that contain an imide group. Lenalidomide[2] works through various mechanisms of actions that promote malignant cell death and enhance host immunity. Available as oral capsules, lenalidomide is approved by the FDA and EU for the treatment of multiple myeloma, myelodysplastic syndromes, mantle cell lymphoma, follicular lymphoma, and marginal zone lymphoma in selected patients. Due to severe teratogenicity, pregnancy must be excluded before the start of treatment and patients must enroll in the lenalidomide Risk Evaluation and Mitigation Strategy (REMS) program to ensure contraception adherence. Lenalidomide[3] is a Thalidomide Analog. The IUPAC Name of Lenalidomide is 3-(7-amino-3-oxo-1H-isoindol-2-yl) piperidine-2, 6-dione. The Chemical Structure of Lenalidomide is shown in Fig 1.

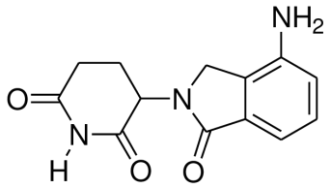

Fig-1: The Chemical Structure of Lenalidomide

Only limited methods have been reported in the literature survey[31-34]. The aim of the present work was to develop and validate a simple, fast, and reliable isocratic RP-HPLC method for the determination of Lenalidomide in bulk and pharmaceutical dosage forms. The important features and novelty of the proposed method included simple with sonication of small amount of powder sample at ambient temperature and dilution; short elution time with good separation eluted prior to Lenalidomide; good precision (R.S.D. less than 2%) and high recovery (greater than 98%-102%). Confirmation of the applicability of the developed method was validated according to the International Conference on Harmonisation[30] (ICH), to determine the use of Lenalidomide in bulk and marketed pharmaceutical dosage forms.

EXPERIMENTAL

Instruments Used

Table-1: Instruments Used

Instruments and Glass wares	Model
HPLC	WATERS Alliance 2695 separation module, Software: Empower 2, 996 PDA detector.
pH meter	LabIndia
Weighing machine	Sartorius
Volumetric flasks	Borosil
Pipettes and Burettes	Borosil
Beakers	Borosil
Digital ultra sonicator	Labman

Chemicals Used:

Table-2: Chemicals Used

Chemical	Brand names
Lenalidomide	Synpharma Research Lab, Dilsuknagar
Water and Methanol for HPLC	LICHROSOLV (MERCK)
Acetonitrile for HPLC	Merck
Ethanol	Sd fine-Chem ltd; Mumbai
DMSO	Sd fine-Chem ltd; Mumbai
DMF	Sd fine-Chem ltd; Mumbai
Orthophosphoric Acid	Sd fine-Chem ltd; Mumbai

HPLC Method Development:

Preparation of Standard Solution:

Accurately weigh and transfer 10 mg of Lenalidomide working standard into a 10ml of clean dry volumetric flasks add about 7ml of Methanol and sonicate to dissolve and removal of air completely and make volume up to the mark with the same Methanol. Further pipette 0.1ml of the above Lenalidomide

stock solutions[4]into a 10ml volumetric flask and dilute up to the mark with Methanol.

Preparation of Sample Solution:

Twenty capsules were taken, and the average weight was calculated as per the method prescribed in I.P. The weighed tablets were finally powdered and triturated well. A quantity of powder of Lenalidomide equivalent to 10mg was transferred to a clean and dry 10 ml volumetric flask and 7 ml of HPLC grade[5] methanol was added and the resulting solution was sonicated for 15 minutes. Make up the volume up to 10 ml with same solvent. Then 1 ml of the above solution was diluted to 10 ml with HPLC grade methanol. One ml (0.1 ml) of the prepared stock solution diluted to 10 ml and was filtered through membrane filter (0.45µm) and finally sonicated to degas.

Procedure:

Inject the samples by changing the chromatographic conditions and record the chromatograms, note the conditions of proper peak elution for performing validation parameters as per ICH guidelines[6].

Mobile Phase Optimization:

Initially the mobile phase tried was Methanol and Methanol: Water with varying proportions. Finally, the mobile phase was optimized to Methanol and Phosphate buffer (0.02M, pH-3.6) in proportion 45:55% v/v.

Optimization of Column:

The method[7]was performed with various C18 columns like, X- bridge column, Xterra, and C18 column. Develosil ODS HG-5 RP C18, 5µm, 15cmx4.6mm i.d.was found to be ideal as it gave good peak shape and resolution at 1.0ml/min flow.

Preparation of Buffer and Mobile Phase:

Preparation of Potassium Dihydrogen Phosphate (KH2PO4) Buffer (0.02M) (pH-3.6):

Dissolve 2.72172g of potassium dihydrogen phosphate in 1000 ml HPLC water and adjust the pH 3.6 with diluted orthophosphoric acid. Filter and sonicate the solution by vacuum filtration and ultra-sonication.

Preparation of Mobile Phase:

Accurately measured 450 ml (45%) of Methanol and 550 ml of Phosphate buffer (55%) were mixed and degassed in digital ultra sonicater for 15 minutes and then filtered through 0.45 µ filter under vacuum filtration.

Diluent Preparation:

The Mobile phase was used as the diluent.

Method Validation Parameters

System Suitability

Accurately weigh and transfer 10 mg of Lenalidomide working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent[8]. (Stock solution)**.** Further pipette 0.1ml of the above Lenalidomide stock solution into a 10ml volumetric flask and dilute up to the mark with diluents.

Procedure:

The standard solution was injected five times and measured the area for all five injections in HPLC. The %RSD[9] for the area of five replicate injections was found to be within the specified limits.

Specificity:

Preparation of Standard Solution:

Accurately weigh and transfer 10 mg of Lenalidomide working standard into a 10ml of clean dry volumetric flasks add about 7ml of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 0.1ml of the above Lenalidomide stock solutions into a 10ml volumetric flask and dilute up to the mark with diluents.

Preparation of Sample Solution:

Weight 10 mg equivalent weight of Lenalidomide sample into a 10mL clean dry volumetric flask and add about 7mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. Further pipette 0.1ml of Lenalidomide above stock solution[10] into a 10ml volumetric flask and dilute up to the mark with diluent.

Procedure:

Inject the three replicate injections of standard and sample solutions and calculate the assay by using formula:

$$\%ASSAY = \frac{\text{Sample area}}{\text{Standard area}} \times \frac{\text{Weight of standard}}{\text{Dilution of standard}} \times \frac{\text{Dilution of sample}}{\text{Weight of sample}} \times \frac{\text{Purity}}{100} \times \frac{\text{Weight of tablet}}{\text{Label claim}} \times 100$$

Linearity and Range:

Accurately weigh and transfer 10 mg of Lenalidomide working standard into a 10ml of clean dry volumetric flasks add about 7ml of Diluents[11] and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Preparation of Level – I (12ppm of Lenalidomide):
Take 0.12ml of stock solution into 10ml volumetric flask and make up the volume up to mark with diluents and sonicate the solution for bubble entrapment using ultrasonicator.
Preparation of Level – II
(16ppm of Lenalidomide):
Take 0.16ml of stock solution into 10ml of volumetric flask and make up the volume up to mark with diluents and sonicate the solution for bubble entrapment using ultrasonicator[12].
Preparation of Level–III
(20ppm of Lenalidomide):
Take 0.2ml of stock solution into 10ml of volumetric flask and make up the volume up to mark with diluents and sonicate the solution for bubble entrapment using ultrasonicator.
Preparation of Level – IV
(24ppm of Lenalidomide):
Take 0.24ml of stock solution into 10ml of volumetric flask and make up the volume up to mark with diluents and sonicate the solution for bubble entrapment using ultrasonicator.
Preparation of Level – V
(28ppm of Lenalidomide):
Take 0.28ml of stock solution into 10ml of volumetric flask and make up the volume up to mark with diluents and sonicate the solution for bubble entrapment using ultrasonicator.
Procedure:
Inject each level into the chromatographic system[13] and measure the peak area.
Plot a graph of peak area versus concentration (on X-axis concentration and on Y-axis Peak area) and calculate the correlation coefficient.
Precision
Repeatability
Preparation of Lenalidomide Product Solution for Precision:
Accurately weigh and transfer 10 mg of Lenalidomide working standard into a 10ml of clean dry volumetric flasks add about 7ml of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 0.1ml of the above Lenalidomide stock solutions into a 10ml volumetric flask and dilute up to the mark with diluents.
Procedure:
The standard solution was injected six times and measured the area for all six injections in HPLC[14]. The %RSD for the area of six replicate injections was found to be within the specified limits.

Intermediate Precision:
To evaluate the intermediate precision (also known as Ruggedness) of the method, Precision was performed on different days by maintaining same conditions.
Procedure:
Analyst 1:
The standard solution was injected six times and measured the area for all six injections in HPLC. The %RSD for the area of six replicate injections was found to be within the specified limits.
Analyst 2:
The standard solution was injected six times and measured the area for all six injections in HPLC. The %RSD for the area of six replicate injections was found to be within the specified limits[15].
Accuracy:
For Preparation of 80% Standard Stock Solution:
Accurately weigh and transfer 10 mg of Lenalidomide working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)**.** Further pipette 0.08ml of the above Lenalidomide stock solution into a 10ml volumetric flask and dilute up to the mark with diluents.
For Preparation of 100% Standard Stock Solution:
Accurately weigh and transfer 10 mg of Lenalidomide working standard into a 10ml of clean dry volumetric flasks[16] add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 0.1ml of the above Lenalidomide stock solution into a 10ml volumetric flask and dilute up to the mark with diluents.
For Preparation of 120% Standard Stock Solution:
Accurately weigh and transfer 10 mg of Lenalidomide working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 0.12ml of the above Lenalidomide stock solution into a 10ml volumetric flask and dilute up to the mark with diluents.
Procedure:
Inject the Three replicate injections of individual concentrations (80%, 100%, 120%) were made under the optimized conditions[17]. Recorded the chromatograms and measured the peak responses. Calculate the Amount found, and Amount added for Lenalidomide and calculate the individual recovery and mean recovery values.

LIMIT OF DETECTION AND LIMIT OF QUANTIFICATION (LOD & LOQ):

Preparation of 5.004µg/ml Solution (For LOD):

Accurately weigh and transfer 10 mg of Lenalidomide working standard into a 10ml of clean dry volumetric flasks add about 7ml of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 0.05004ml of the above Lenalidomide stock solutions into a 10ml volumetric flask and dilute up to the mark with diluents.

Preparation of 15.164µg/ml Solution (For LOQ):

Accurately weigh and transfer 10 mg of Lenalidomide working standard into a 10ml of clean dry volumetric flasks add about 7ml of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 0.15164ml of the above Lenalidomide stock solutions into a 10ml volumetric flask and dilute up to the mark with diluents.

Robustness:

The analysis was performed in different conditions to find the variability of test results. The following conditions are checked for variation of results.

For preparation of Standard Solution:

Accurately weigh and transfer 10 mg of Lenalidomide working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)**.** Further pipette 0.1ml of the above Lenalidomide stock solution into a 10ml volumetric flask and dilute up to the mark with diluents.

Effect of Variation of Flow Conditions:

The sample was analyzed at 0.9 ml/min and 1.1 ml/min instead of 1ml/min, remaining conditions are same. 20µl of the above sample was injected and chromatograms[18]were recorded.

Effect of Variation of Mobile Phase Organic Composition:

The sample was analyzed by variation of mobile phase i.e. Methanol: Phosphate Buffer was taken in the ratio and 50:50, 40:60 instead (45:55), remaining conditions are same. 20µl of the above sample was injected and chromatograms were recorded.

RESULTS AND DISCUSSION

Method Development

Wavelength Detection:

The detection wavelength was selected by dissolving the drug in mobile phase to get a concentrationof10µg/ml for individual and mixed standards. Their solution was scanned in U.V range[19]from200-400nm. The UV spectrum of Lenalidomide was obtained and the Lenalidomide showed absorbance's maxima at 255nm.The UV spectra of drug are follows:

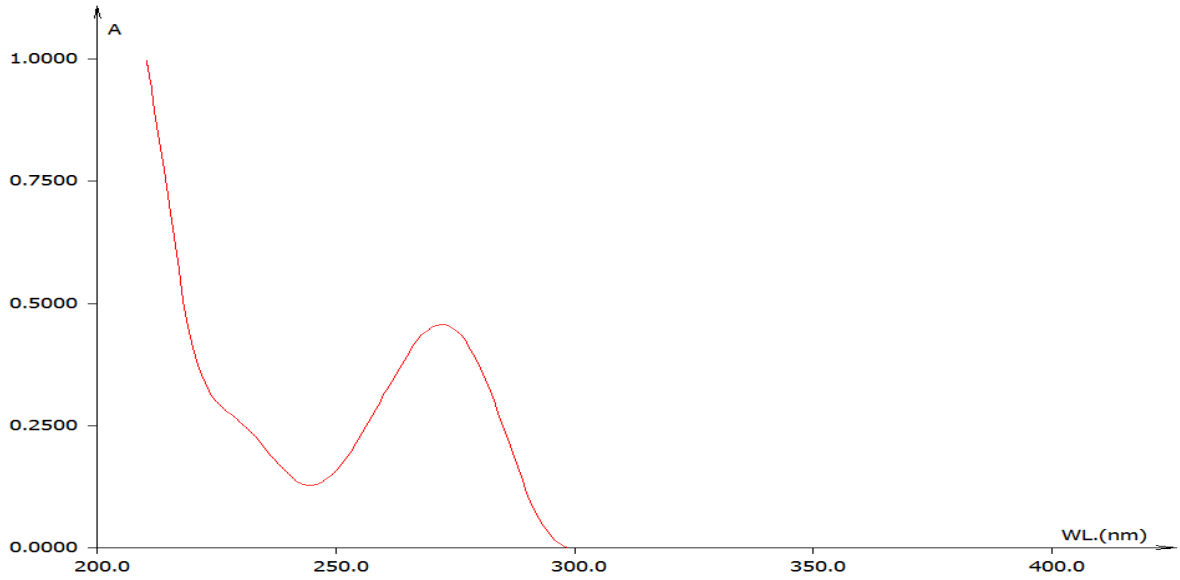

Fig-2: UV Spectrum of Lenalidomide

Observation:

While scanning the Lenalidomide solution we observed the maxima at 255nm. The UV spectrum has been recorded on T60-LAB INDIA make UV-Vis spectrophotometer model UV-2450.

Optimized Chromatographic Method:

Table-3: Optimized Chromatographic Conditions

Mobile phase	Methanol: Phosphate buffer (0.02M, pH-3.6) = 45:55 v/v
Column	Develosil ODS HG-5 RP C_{18}, 5µm, 15cmx4.6mm i.d.
Column Temperature	Ambient
Detection Wavelength	255 nm
Flow rate	1.0 ml/ min.
Run time	07 min.
Temperature of Auto sampler	Ambient
Diluent	Mobile Phase
Injection Volume	20µl
Type of Elution	Isocratic

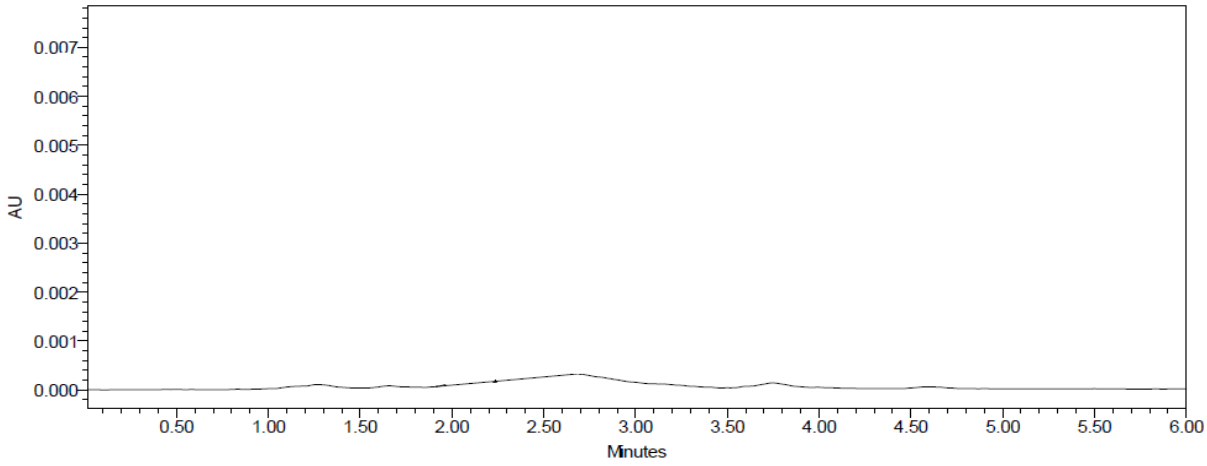

Fig-3: Chromatogram of Blank Solution

Standard Solution:

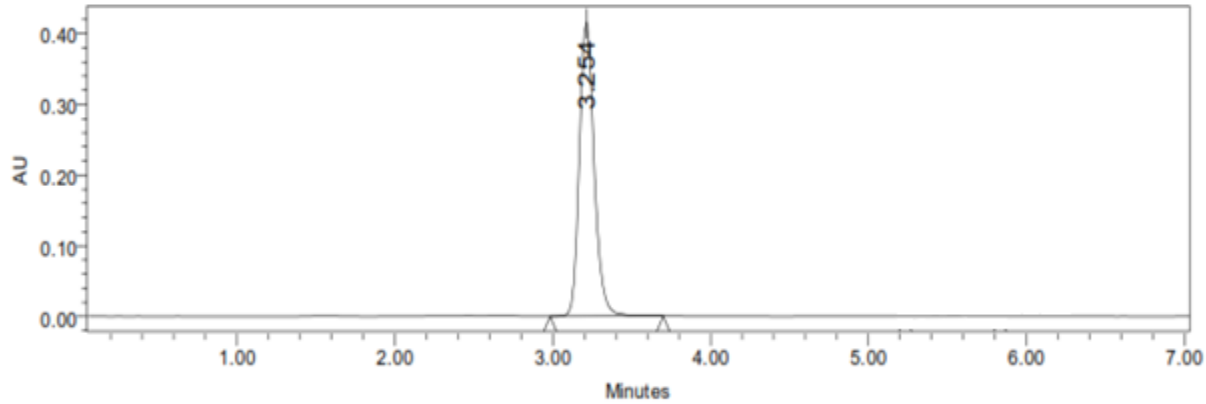

Fig-4: Chromatogram of Lenalidomide in Optimized Chromatographic Condition

Analytical Method Validation

Validation[20-22]was conducted out in accordance with ICH Q2 (R1) guidelines to ensure the analytical method's performance.

System Suitability: System suitability testing is an integral part of many analytical procedures. The tests are based on the concept that the equipment, electronics, analytical operations and samples to be analyzed constitute an integral system[23] that can be evaluated as such. The following system suitability test parameters were established. The data are shown in Table-4 & 5.

Table-4: Data of System Suitability Test

S. No.	Injection No.	RT	Area	USP Plate Count	USP Tailing
1	Injection 1	3.253	284568	7368	1.26
2	Injection 2	3.254	285684	7295	1.25
3	Injection 3	3.215	283659	7346	1.27
4	Injection 4	3.297	284754	7394	1.29
5	Injection 5	3.253	283695	7425	1.25
6	Injection 6	3.213	284578	7385	1.27
Mean			**284489.7**	**7368.833**	**1.265**
S. D			**752.5617**		
%RSD			**0.26453**		

Table-5: System Suitability Results for Lenalidomide (Flowrate)

S.No.	Parameter	Limit	Result
1	Asymmetry	$T \leq 2$	Lenalidomide = 0.12
2	Theoretical plate	$N > 2000$	Lenalidomide = 7258
3	Tailing Factor	(Tf)< 2	Lenalidomide = 1.25

Specificity:

Specificity can be determined by comparing the chromatograms obtained from the drugs with the chromatogram obtained from the blank solution. Blank solution was prepared by mixing the excipients in the mobile phase without drug. Drug solutions were prepared individually and the sample containing three drugs was also prepared. Now these mixtures were filtered by passing through 0.45 µ membrane filter before the analysis. In this observation no excipient peaks were obtained near the drug in the study run time. This indicates that the proposed method[24] was specific.

The chromatograms representing the peaks of blank, Lenalidomide and the sample containing the three drugs were shown in following figures respectively.

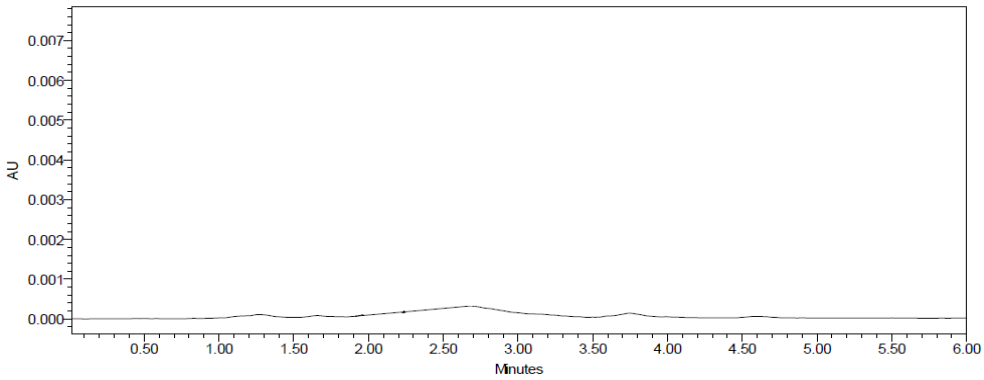

Fig-5: Chromatogram of Blank Solution

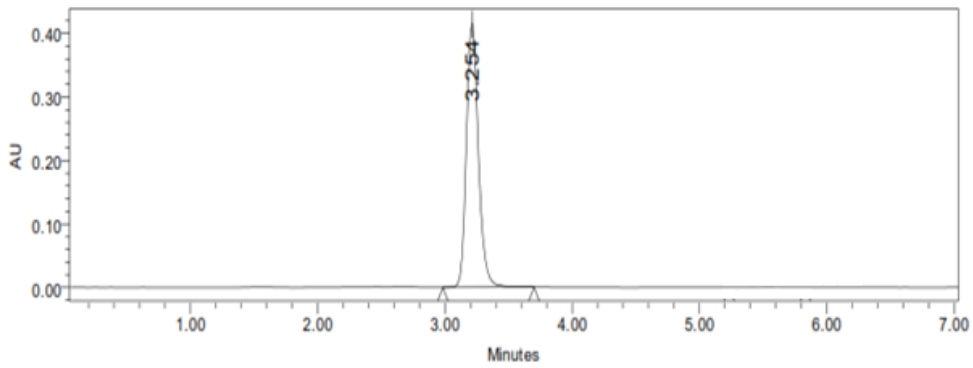

Fig-6: Chromatogram of Lenalidomide Standard Solution

Observation: In this test method blank, standard solutions were analyzed individually to examine the interference. The above chromatograms show that the active ingredient was well separated from blank and their excipients and there was no interference of blank with the principal peak. Hence the method is specific.

Linearity: To evaluate the linearity, serial dilution of analyte was prepared from the stock solution was diluted with mobile phase to get a series of concentration ranging from 0-28µg/ml for Lenalidomide. The prepared solutions were filtered through Whatman filter paper (No.41). From these solutions, 20µl injections of each concentration were injected into the HPLC system and chromatographed under the optimized conditions. Calibration curve[25] was constructed by plotting the mean peak area (Y-axis) against the concentration (X-axis).

Plotting of Calibration Graphs:

Theresultantareasoflinearitypeaksareplottedagainst Concentration.

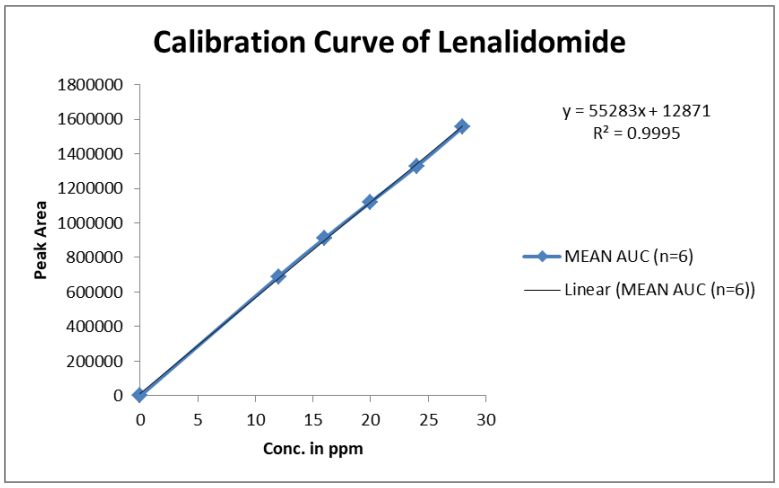

Fig-7: Standard Curve for Lenalidomide

Observation: Linearity range was found to be 0-28µg/ml for Lenalidomide. The correlation coefficient was found to be 0.9995, the slope was found to be 55283and intercept was found to be 12871 for Lenalidomide.

Table-6: Linearity Readings for Lenalidomide

CONC.(µg/ml)	MEAN AUC (n=6)
0	0
12	690316
16	910621
20	1121057
24	1328903
28	1554666

Linearity Plot:

The plot of Concentration (x) versus the Average Peak Area (y) data of Lenalidomide is a straight line.

Y = mx + c

Slope (m) = 55283

Intercept (c) = 12871

Correlation Coefficient (r) = 0.9995

Acceptance/Validation Criteria: The response linearity is verified if the Correlation Coefficient is 0.99 or greater.

Conclusion: Correlation Coefficient (r) is 0.99, and the intercept is 12871. These values meet the validation criteria.

Accuracy:

Inject the three replicate injections of individual concentrations (80%, 100%, 120%) were made under the optimized conditions. Recorded the chromatograms and measured the peak responses. Calculate the Amount found, and Amount added for Lenalidomide and calculate the individual recovery and mean recovery values[26]. Accuracy at different concentrations (80%, 100%, and 120%) was prepared and the % recovery was calculated.

Table-7: Accuracy results of Lenalidomide

Sample ID	Concentration (µg/ml)			%Recovery of Pure drug	Statistical Analysis
	Conc. Found	Conc. Recovered	Peak Area		
S_1: 80 %	8	8.064107	458679	99.867	Mean= 100.4113%
S_2: 80 %	8	7.843532	446485	100.637	S.D. = 0.473694346
S_3: 80 %	8	8.19449	465887	100.73	% R.S.D.= 0.471753
S_4: 100 %	10	9.892661	559767	99.41	Mean= 100.6646667%
S_5: 100 %	10	9.978655	564521	100.868	S.D. = 1.166369295R.S.D.=
S_6: 100 %	10	10.19623	576549	101.716	1.158667
S_7: 120 %	12	11.85907	668476	99.878	Mean= 100.4637%
S_8: 120 %	12	12.16785	685546	100.69	S.D. = 0.51154309
S_9: 120 %	12	12.18644	686574	100.823	% R.S.D. = 0.509181

Observation: The mean recoveries were found to be 100.411, 100.664 and 100.463% for Lenalidomide. The limit for mean % recovery is 98-102% and as both the values are within the limit, hence it can be said that the proposed method was accurate.

Precision: The precision of each method was ascertained separately from the peak areas obtained by actual determination of six replicates of a fixed amount of drug Lenalidomide. The percentage relative standard deviations [27] were calculated for Lenalidomide are presented in Table-8.

i) Repeatability

Obtained Six (6) replicates of 100% accuracy solution as per experimental conditions. Recorded the peak areas and calculated % RSD.

Table-8: Repeatability Results of Lenalidomide

HPLC Injection Replicates	AUC for Lenalidomide
Replicate – 1	285479
Replicate – 2	284571
Replicate – 3	286954
Replicate – 4	283261
Replicate – 5	285964
Replicate – 6	284259
Average	**285081.3**
Standard Deviation	**1318.666**
% RSD	**0.462558**

Observation: The repeatability study which was conducted on the solution having the concentration of about 20µg/ml for Lenalidomide (n=6) showed a RSD of 0.462558% for Lenalidomide. It was concluded that the analytical technique showed good repeatability.

ii)Intermediate Precision/Ruggedness

To evaluate the intermediate precision (also known as Ruggedness) of the method, Precision was performed on different days by maintaining same conditions.

Procedure:

Analyst 1: The standard solution was injected for six times and measured the area for all six injections in HPLC. The %RSD for the area of six replicate injections was found to be within the specified limits.

Analyst 2:

The standard solution was injected six times and measured the area for all six injections in HPLC. The %RSD for the area of six replicate injections was found to be within the specified limits.

Intra Day (Day-1)/Analyst-1:

Table-9: Results of Ruggedness for Lenalidomide (Analyst-1)

S. No.	Peak Name	RT	Peak Area	Theoretical Plates	Tailing Factor
1	Lenalidomide	3.253	284568	7368	1.26
2	Lenalidomide	3.254	285684	7295	1.25
3	Lenalidomide	3.215	283659	7346	1.27
4	Lenalidomide	3.204	286598	7457	1.22
5	Lenalidomide	3.202	287965	7635	1.29
6	Lenalidomide	3.297	285698	7459	1.28
Mean			**285695.3**		
Std. Dev.			**1508.898**		
%RSD			**0.528149**		

Inter Day (Day -2/Analyst-2):

Table-10: Results of Ruggedness for Lenalidomide (Analyst-2)

S.No.	PeakName	RT	Peak Area	Theoretical Plates	Tailing Factor
1	Lenalidomide	3.297	294754	7394	1.29
2	Lenalidomide	3.253	293695	7425	1.25
3	Lenalidomide	3.213	294578	7385	1.27
4	Lenalidomide	3.297	296534	7584	1.23
5	Lenalidomide	3.210	296571	7745	1.24
6	Lenalidomide	3.254	298698	7658	1.25
Mean			**295805**		
Std.Dev.			**1819.334**		
%RSD			**0.615045**		

Observation: Intraday and interday studies show that the mean RSD (%) was found to be within acceptance limit (≤2%), so it was concluded that there was no significant difference for the assay, which was tested within day and between days. Hence, the method at selected wavelength was found to be precise.

Robustness: Robustness is defined as the capacity of that method to be unaffected by even small deliberate changes that occur in the method parameters. The evaluation of robustness[28] of a method is done by varying the chromatographic parameters such as pH, temperature, flow rate, mobile phase proportions change, ionic strength etc., and determining any possible effect on the results obtained by that method. The results are shown in Table-11.

Table-11: Result of Method Robustness Test for Lenalidomide

Parameter used for Sample Analysis	Peak Area	Retention Time	Theoretical plates	Tailing factor
Actual Flow rate of 1.0 mL/min	283261	3.254	7258	1.25
Less Flow rate of 0.9 mL/min	315864	3.297	7569	1.29
More Flow rate of 1.1 mL/min	298542	3.212	7841	1.41
Less organic phase	279856	3.253	7965	1.27
More organic phase	306985	3.215	7458	1.28

Observation: Influence of small changes in chromatographic conditions such as change in flow rate (± 0.1ml/min), Temperature (±2^{0}C), Wavelength of detection (±2nm) &organic phase (±5%) studied to determine the robustness of the method are also in favour of (Table-11, % RSD < 2%) the developed RP-HPLC method for the analysis of Lenalidomide (API).

LOD: The limit of detection (LOD) is the lowest concentration of analyte in a sample which can be detected, but not quantitated. LOD is a limit test that

specifies whether an analyte is above or below a certain value. Signal-to-noise ratio of three-to-one is used to determine LOD.

L.O.D. = 3.3(SD/S).
Where, SD = Standard deviation of the response
S = Slope of the calibration curve

Table-12: Results of LOD

	LOD
SD of Intercept	19518.16286
Slope	55283

Observation: The LOD was found to be 1.165µg/ml for Lenalidomide.

LOQ: The Limit of Quantitation (LOQ) is defined as the lowest concentration of an analyte in a sample that can be determined with acceptable precision and accuracy under the stated operational conditions of the method. Signal-to-noise ratio of ten-to-one is used to determine LOQ.

L.O.Q. = 10(SD/S)
Where, SD = Standard deviation of the response
S = Slope of the calibration curve

Table-13: Results of LOQ

	LOQ
SD of Intercept	19518.16286
Slope	55283

Observation: The LOQ was found to be 3.53µg/ml for Lenalidomide.

Assay of Pharmaceutical Dosage form

Twenty tablets/Capsules were taken and the I.P. method was followed to determine the average weight. Finally, the weighed tablets are powdered and triturated well by using mortar and pestle. A quantity of powder which is equivalent to the 100mg of drugs were transferred to a clean and dry 100ml of volumetric flask and add 70 ml of mobile phase and the resulted solution was sonicated for 15 minutes by using ultra sonicator, Then the final volume was make up to the mark with the mobile phase. The final solution was filtered through a selected membrane filter (0.45 µm) and in order to sonicate to degas the mobile phase (Solvent system). From this above stock arrangement (1 ml) was exchanged to five distinctive 10 ml volumetric flagons and volume was made up to 10 ml with same dissolvable framework (Mobile stage). The readied arrangements were infused in five repeats into the HPLC framework, and the perceptions were recorded. A duplicate injection (Blank Solution) of the standard arrangement likewise infused into the HPLC framework and the chromatograms and peak zones were recorded and Figured 14.

Assay % =

$$\frac{AT}{AS} \times \frac{WS}{DS} \times \frac{DT}{WT} \times \frac{P}{100} \times \text{Avg. Wt} = \text{mg/tab}$$

Where:

AT = Peak Area of drug obtained with test preparation
AS = Peak Area of drug obtained with standard preparation
WS = Weight of working standard taken in mg
WT = Weight of sample taken in mg
DS = Dilution of Standard solution
DT = Dilution of sample solution
P = Percentage purity of working standard

The assay[29] was performed as explained in the previous chapter. The results which are obtained are following:

Table-14: Recovery Data for estimation Lenalidomide in Lenmid 10 Capsule

Brand Name of Lenalidomide	Labelled Amount of Drug (mg)	Amount (mg) found by the Proposed Method (n=3)	Assay %
Lenmid 10 Capsule (Cipla)	10mg	9.495 (± 0.298)	99.598% (± 0.478)

RESULTS AND DISCUSSION:

The amount of drug in Lenmid 10 Capsule was found to be 9.495 (± 0.298) mg/tab for Lenalidomide& %Purity was 99.598 (±0.478) %.

SUMMARY AND CONCLUSION

To develop a precise, linear, specific & suitable stability indicating RP-HPLC method for analysis of Lenalidomide, different chromatographic conditions were applied & the results observed are presented in previous chapters. Isocratic elution is simple, requires only one pump & flat baseline separation for easy and reproducible results. So, it was preferred for the current study over gradient elution. In case of RP-HPLC various columns are available, but here Develosil ODS HG-5 RP C_{18}, 5µm, 15cmx4.6mm i.d. column was preferred because using this column peak shape, resolution and absorbance were good. Mobile phase & diluent for preparation of various samples were finalized after studying the solubility of API in different solvents of our disposal (methanol, acetonitrile, water, 0.1N NaOH, 0.1NHCl). Detection wavelength was selected after scanning the standard solution of drug over 200 to 400nm. From the U.V spectrum of Lenalidomide it is evident that most of the HPLC work can be accomplished in the wavelength range of 255 nm conveniently. Further, a flow rate of 1 ml/min & an injection volume of 20µl were found to be the best analysis. The result shows the developed method is yet another suitable method for assay which can help in the analysis of Lenalidomide in different formulations.

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