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A VALIDATED STABILITY IMPLYING RP-HPLC METHOD FOR PHARMACEUTICAL FORMULATIONS TO ESTIMATE LENALIDOMIDE

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ABSTRACT

A new, simple and precise RP-HPLC method was developed in this study for the quantitative analysis of lenalidomide (LLM) in pharmaceutical formulations using an analytical quality by design approach. Inertsil C8 mobile phase analytical columns containing a phosphate buffer were used for this purpose in the estimation of LLM and its degradation products present in the mixture of buffer, acetonitrile and methanol in the ratio 80:8:12 (v v/v). The 1.0 ml / minute flow rate is maintained and all degradation tests are conducted using the UV/VIS detector at 254 nm. Method Validation is carried out in compliance with the guidelines and criteria of the International Council for Harmonization (ICH); precision, accuracy, specificity, stability, robustness, linearity, quantization limit 0.25 µgml-1 (LOQ) and detection limit 0.75µgml-1 (LOD) were successfully evaluated. The current RP-HPLC method indicates that the peaks' purity angle is less than their threshold angle, suggesting that it is ideal for stability studies. For the effective separation of LLM and its impurities in the pharmaceutical dosage formulations, the established method may therefore be used.

Keywords: RP-HPLC; Lenalidomide, Forced Degradation, ICH guidelines, Method validation.

INTRODUCTION

Lenalidomide (3-(4-amino-1,3-dihydro-1-oxo-2H-isoindol-2-yl)-2,6-piperidinedione) is a thalidomide derivative and was originally intended as a therapy for multiple myeloma, where thalidomide is an approved therapeutic modality, but has also been shown to be effective in hematological disorders known as myelodysplastic syndromes [1-3]. The molecular formula and molar mass of this derivative are $C_{13}H_{13}N_3O_3$ and 259.261 g/mol, respectively. and 259.261 g/mol respectively. While this compound is structurally related to thalidomide, it has an improved profile of toxicity and more potent immunomodulatory function [4,5]. In Phase II trials for chronic lymphocytic leukemia, non-lymphoma, Hodgkin's amyloidosis and myelofibrosis with myeloid metaplasia, LLM has proven to be a promising medication [6,-9]. The strong evidence-based clinical success of LLM in patients has led to its recent US-FDA approval by Celgene Corporation under the trade name Revlimid Capsules [10]. Clinical trials have also been used to treat advanced cancers such as blood cancers: Hodgkin's lymphoma and non-lymphoma, Hodgkin's bone marrow cancer: chronic lymphocytic leukaemia, and solid tumor cancer: carcinoma and pancreas [9,10]. Basically, LLM increases the T-cells' functional potential and inhibits in vitro angiogenesis in human systems [11]. The recommended dosage of LLM is 10.0 mg a day, but if there is neutropenia or thrombocytopenia, it can be reduced to 5.0 mg any other day. The demand for suitable analytical technologies to ensure the consistency of LLM formulations is therefore growing.

Few methods have been reported for the determination of LLM in bulk material and capsules involving spectrophotometric methods and others [12]. In addition, for the analysis of LLM bulk material and its related impurities, two HPLC methods and capsules have been reported [13,14] Several chromatographic methods including liquid chromatography – UV Detector (LC–UV) were also used foe LLM quantification[15]. In the recent past, various analytical techniques in pharmaceutical research have been developed with different detectors [16]. In the literature for evaluating LLM and impurities using HPLC, HPLC assay and LC-MS methods, few analytical methods are reported [17,18]. To the best of our knowledge, there is no stability-indicating research method for quantification of lenalidomide available in the literature. This paper describes the relevance of a validated quantitative determination method for lenalidomide stability studies.

ANALYTICAL METHOD VALIDATION

Instruments, Reagents, Standards & Samples Used

HPLC- Waters - Alliance 510 with UV- 484 Data Ace software (Instrument I.D: AL-011) and HPLC - Agilent 1100 Series with Chromeleon software (Instrument I.D: AL-013), HPLC Analytical column Inertsil ODS-C8 – 25cm x 4.6mm x 5µm, Analytical weighing balance - Mettler Toledo B204S, Millipore membrane 0.2µm, Laboratory accessories. Lenalidomide working standard, Revlimid (Lenalidomide) Capsules 25 mg, DiSodium Hydrogen Phosphate-AR Grade, Phosphoric Acid – AR Grade, Hydrochloric Acid– AR Grade, Sodium Hydroxide– AR Grade, Hydrogen Peroxide– AR Grade, Acetonitrile - HPLC Grade, Methanol- HPLC Grade, all the chemicals were procured from Bross Scientific Pvt. Ltd., A.P., INDIA.

The quantitative determination is carried out by HPLC system equipped with UV/VIS detector.

Chromatographic conditions:

Column	:	Inertsil ODS-C_8 – 25cm x 4.6mm x 5µm
Mobile Phase	:	Prepared a phosphate buffer solution by adding 35.8 g/l solution of disodium hydrogen phosphate to pH 3.3 with dilute phosphoric acid. Dilute 100.0 ml of the solution to 2000.0 ml with water. Prepare a mixture of buffer, acetonitrile and methanol in the ratio 80:8:12
Wavelength	:	254 nm
Flow Rate	:	1.0 ml / minute
Injection volume	:	20 µl
Run time	:	15 minutes
Blank solution	:	Use Diluent as blank
Diluent	:	Use Mobile phase as diluent

Preparation of Lenalidomide Standard Solution:

For this, we initially accurately weighed about 20 mg of the working standard of Lenalidomide and transferred it to a volumetric flask of 20 ml. Later added 10 ml of diluent and allowed to sonicate to dissolve and then diluted to volume with diluent. Transferred 1 ml of the solution to 10ml volumetric flask and diluted and mixed to filter through 0.2µm nylon membrane (Dilution scheme: 20mg → 20.0 ml / 1.0 ml → 10.0 ml).

Preparation of Test Solution:

To determine the average weight of 10 tablets, tablets were Powdered and its Weigh was accurately weighed. From this 240 mg of Lenalidomide sample powder was transferred to a 20 ml volumetric flask. To this added 10 ml of diluent and sonicate to dissolve and then diluted to volume with diluent. From this transferred 1 ml of the solution to 10ml volumetric flask and diluted and mixed thoroughly. Later filtered the solution through 0.2µm nylon membrane filter (Dilution scheme: 240mg → 20.0 ml / 1.0 ml → 10.0 ml).

Procedure:

Same blank volumes injected separately, five replication device suitability solution injections (Lenalidomide working standard solution). Then they injected two test solution injections and registered the chromatograms. Disregard every peak in the test solution due to blankness. Calculated the percentage of RSD for five replicate injections of system suitability solution (Lenalidomide standard working solution). Later, the tailing factor and theoretical peak plates in the chromatogram obtained with the 5th injection of the device suitability solution were tested (Lenalidomide working standard solution).

The limits are as below,

1) Theoretical plates should be not less than 2000.

2) Tailing factor should be less than 2.0.

3) % RSD should be not more than 2.0%.

VALIDATION PARAMETERS

Specificity / Selectivity

By injecting the diluent blank solution, excipient combination, device suitability solution, test solution, selectivity was carried out. **Acceptance criteria:** From any other peak and from each other, the Lenalidomide peak should be well resolved. At the retention time of the Lenalidomide, the diluent null, excipient blend solution does not display any peak. As per the analytical process, the outcomes of system suitability criteria were found to follow the pre-established acceptance criteria (Table 1).

Table - 1: System suitability - Selectivity

Sr. No.	Area of Lenalidomide
1	2176.57
2	2171.20
3	2161.23
4	2166.80
5	2175.46
Mean	**2170.25**
Standard Deviation (±)	**6.35**
(%) Relative Standard Deviation	**0.29**

At the wavelength given in the method, all of the injections were processed. No interference was detected from the diluent blank solution, with Lenalidomide peak excipient blend solution.

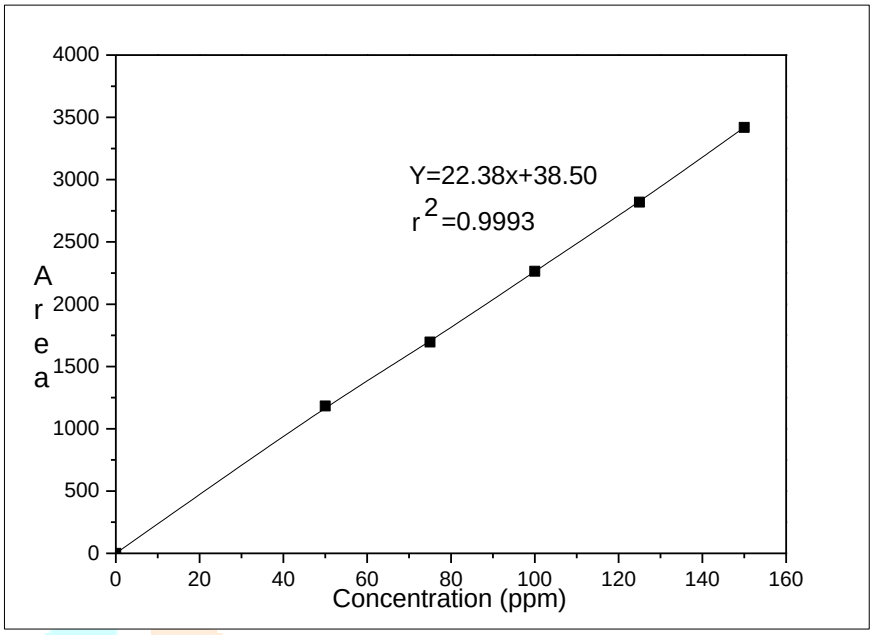
Linearity

Linearity and Range for standard:

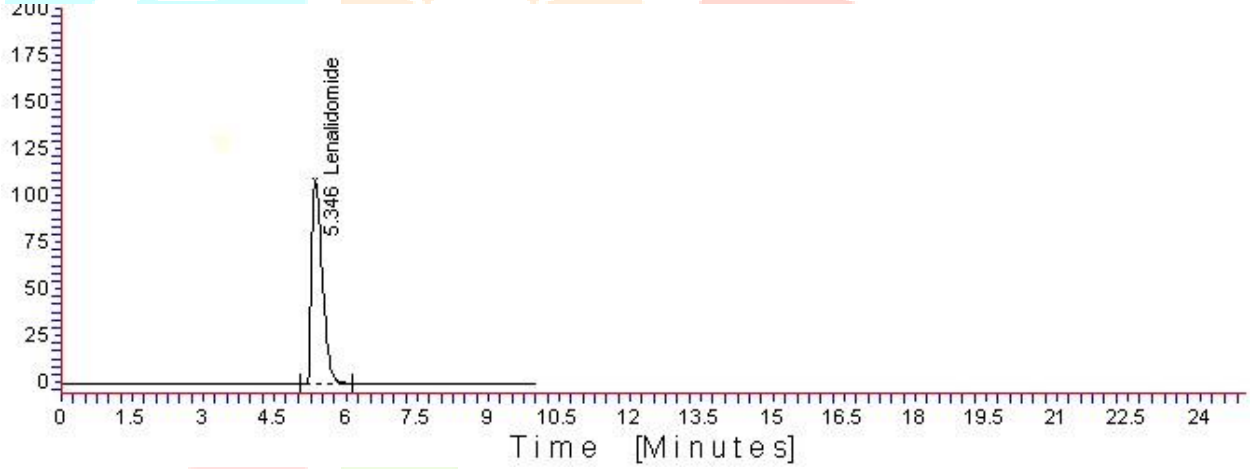
Five standard solutions of Lenalidomide were prepared for the linearity analysis from a range starting from 50% to 150% of the theoretical concentration of assay preparation. As per the protocol, the device suitability solution and the linearity solutions were injected. The concentration graph of linearity against peak response was plotted and the coefficient of correlation was calculated and the results were summarized in Table 2. **Acceptance criteria:** Correlation coefficient should be greater than or equal to 0.999.

Table -2 Results of linearity of standard

Linearity Level	Sample Concentration (in %)	Sample Concentration (in ppm)	Peak Area	Correlation Coefficient
Level – 1	50	50	1183.35	**0.999**
Level – 2	75	75	1696.44	
Level – 3	100	100	2265.07	
Level – 4	125	125	2819.41	
Level – 5	150	150	3419.73	



Figer-2 Linearity graph of Lenalidomide



Peak No	Retn.Time	Area	Height	Area %	Height %	Width@50%
1	5.347	1675.283	109.931	100	100	0.25
Total		**1675.283**	**109.931**	**100**	**100**	

Fig-3 Chromatogram of Lenalidomide Sample

Precision

Method Precision

Procedure

Six test solutions of Lenalidomide in Revlimid (Lenalidomide) Capsules 25 mg and were prepared as per the analytical method. The % RSD of % assay of six test solutions was calculated. **Acceptance criteria:** % RSD of the results of six test solutions should not be more than 2.0%. As per the analytical process, the results of the device suitability criterion have been found to meet the pre-established acceptance criteria. Table 3 summarizes the findings of the assay obtained from six preparations for test solutions.

Table – 3 The Results of method precision

Test Solution	% Assay of Lenalidomide
1	100.65
2	100.33
3	100.18
4	100.45
5	100.73
6	102.29
Mean	**100.77**
Standard Deviation (±)	**0.77**
(%) Relative Standard Deviation	**0.76**

Intermediate Precision

Procedure

Six test solutions of Revlimid (Lenalidomide) Capsules 25 mg were prepared as per the analytical method on different day. These test solutions were analyzed by a different analyst using different HPLC column of same make but having different serial number and different HPLC system. The % RSD of % assay results of twelve test solutions (six samples from method precision and six samples from intermediate precision) was calculated. **Acceptance criteria:** % RSD of the results of twelve test solutions (six of method precision and six of intermediate precision) should not be more than 2.0%. criteria were found to follow the pre-established acceptance criteria. Table - 4 shows the results of an assay collected from six test solutions. Table 5 presents the percent RSD of assay results from process accuracy and intermediate accuracy.

Table - 4: Results of intermediate precision

Test Solution	% Assay of Lenalidomide
1	100.91
2	102.54
3	102.00
4	101.32
5	101.55
6	101.81
Mean	**101.69**
Standard Deviation (±)	**0.57**
(%) Relative Standard Deviation	**0.56**

Table - 5: Results of twelve test solutions of Lenalidomide in

(six of method precision & six of intermediate precision)

Analysis performed during method precision study **By Analyst 1 on system 1 and on column 1 on day 1**	
Same column	**% Assay of Lenalidomide**
1	100.65
2	100.33
3	100.18
4	100.45
5	100.73
6	102.29
Analysis performed during intermediate precision study **By Analyst 2 on system 2 and on column 2 on day 2**	
Column sr. no.	**015337030136 01**
Test Solution	**% Assay of Lenalidomide**
7	100.91
8	102.54
9	102.00
10	101.32
11	101.55
12	101.81
Mean of twelve samples	**101.23**
Standard Deviation (±)	**0.80**
(%) Relative Standard Deviation	**0.79**

Accuracy (% Recovery)

Procedure:

Accuracy study was performed by analyzing Lenalidomide test solutions which were prepared by mixing Lenalidomide API with excipient blend. These test solutions were prepared by adding a quantity of Lenalidomide API to excipient blend to produce three different concentration solutions equivalent to 50%, 75%, 100%, 125% and 150% of test concentration. **Acceptance criteria:** Mean recovery at each concentration level should be between 98.0% and 102.0%. The r**esults of the** system suitability criteria were found to meet the pre-established acceptance criteria as per the analytical method. The results of accuracy study obtained are presented in Table-6.

Table – 6 Accuracy (%Recovery) – results

Level of addition	**Amount of Lenalidomide added**	**Amount of Lenalidomide found in**	**Recovery (%)**
First Level (Rec-50 %)	10.90	10.73	98.44
Second Level (Rec-75 %)	15.20	15.00	98.68
Third Level (Rec-100 %)	19.90	19.98	100.40
Fourth Level (Rec-125	24.90	24.76	99.44
Fifth Level (Rec-150 %)	30.10	29.83	99.10
Mean			**99.21**
Standard Deviation (±)			**0.77**
(%) Relative Standard Deviation			**0.77**

The percentage recovery for Lenalidomide at each level lies between 98.0% and 102.0%. % RSD at each recovery level is less than 2.0%. The analytical method meets the pre-established acceptance criteria for recovery study as per protocol. Hence, it is concluded that the method is accurate.

Forced Degradation

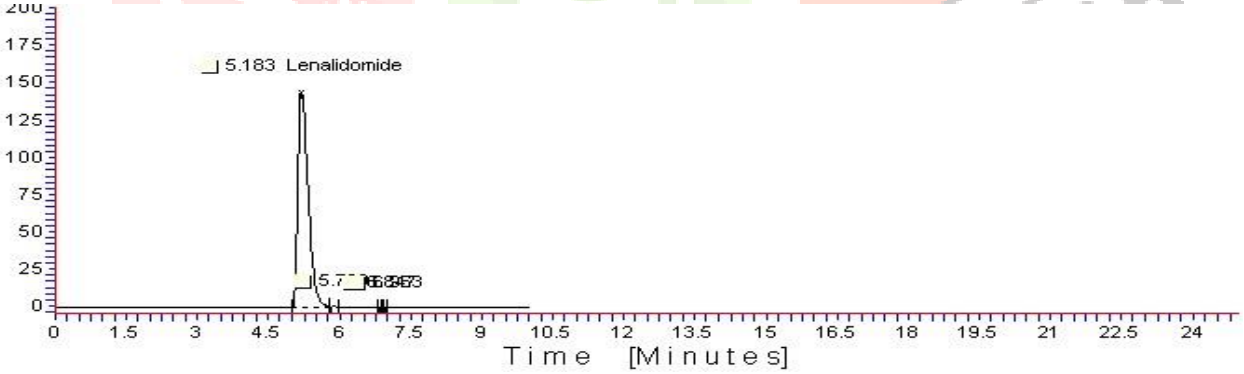
Forced degradation experiments are carried out in order to assess the stability of the assay system and to observe any degraded compounds. 5N HCl, 5N NaOH, thermal degradation and UV degradation are subject to stress in the lenalidomide sample. All of the solutions listed above are chromatographed and the chromatograms are recorded. The following stress conditions are followed for degradation.

Table – 7 Conditions – Forced Degradation

Sample stress condition	Description of stress condition
Acid degradation	5N HCl heated at about 60°C for 10 min on a water bath.
Alkali degradation	5N NaOH heated at about 60°C for 10 min on a water bath.
Thermal degradation	105ºC for 12 hours
UV degradation	expose to UV-radiation for 7 days

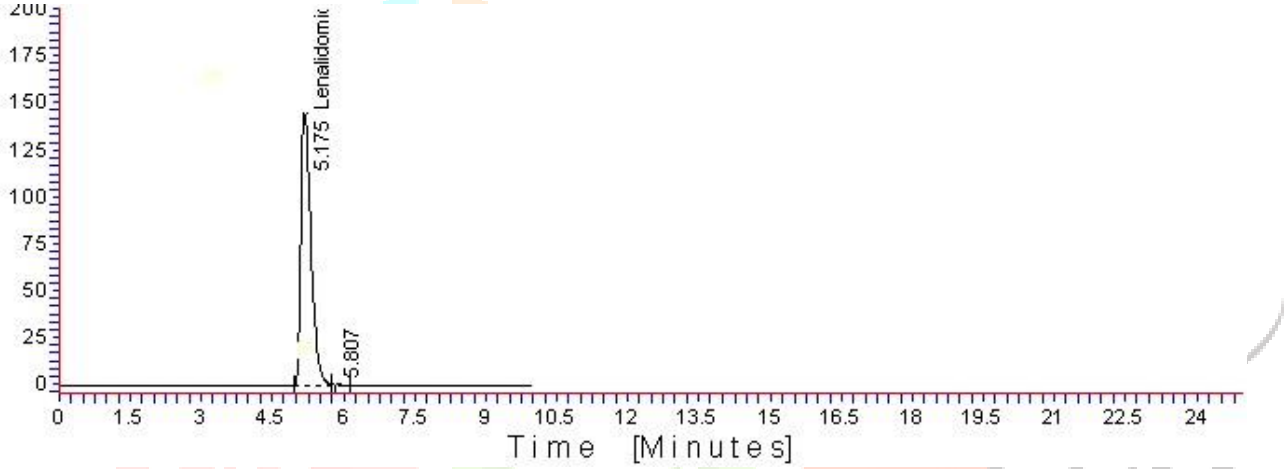
Table- 8 Percentrage of degradation by applying different conditions

Acid Stress	**% Degradation**
Standard	0.147
Sample	0.094
Alkali Stress	**% Degradation**
Standard	0.140
Sample	0.155
Thermal Stress	**% Degradation**
Standard	0.186
Sample	0.220
UV Stress	**% Degradation**
Standard	0.101
Sample	0.139



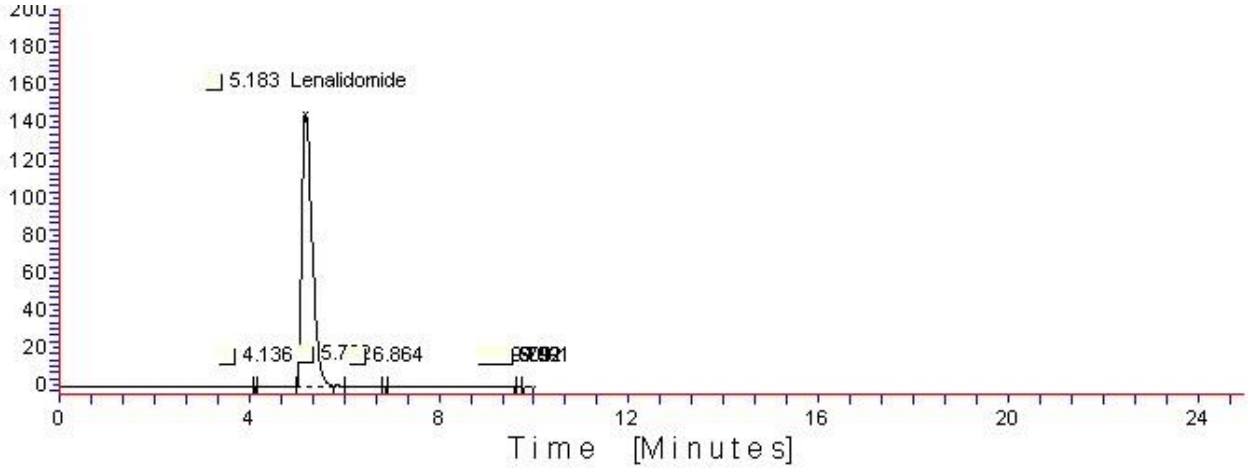
Peak No	Retn.Time	Area	Height	Area %	Height %	Width@50%
1	5.184	2169.028	144.674	99.906	99.718	0.25
2	5.799	1.716	0.31	0.079	0.214	0.1
3	6.847	0.081	0.033	0.004	0.023	0.05
4	6.963	0.233	0.067	0.011	0.046	0.067
Total		**2171.058**	**145.084**	**100**	**100**	

Fig – 4 Chromatogram of Acid degradation



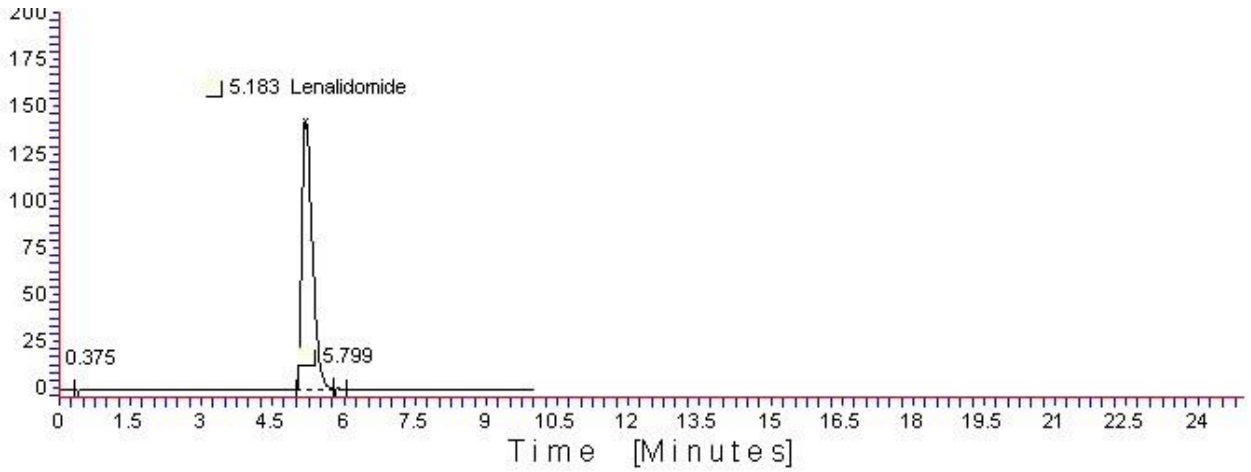
Peak No	Retn.Time	Area	Height	Area %	Height %	Width@50%
1	5.175	2166.896	144.39	99.845	99.779	0.25
2	5.807	3.373	0.319	0.155	0.221	0.183
Total		**2170.269**	**144.709**	**100**	**100**	

Fig-5 Chromatogram of Alkali degradation



Result-A Table						
Peak No	**Retn.Time**	**Area**	**Height**	**Area %**	**Height %**	**Width@50%**
1	4.136	0.091	0.06	0.004	0.041	0.033
2	5.184	2107.328	146.109	99.78	99.425	0.233
3	5.782	3.845	0.521	0.182	0.354	0.15
4	6.864	0.437	0.087	0.021	0.059	0.1
5	9.609	0.08	0.053	0.004	0.036	0.033
6	9.792	0.112	0.074	0.005	0.05	0.033
7	9.991	0.075	0.05	0.004	0.034	0.033
Total		**2111.968**	**146.954**	**100**	**100**	

Fig-6 Chromatogram of Thermal degradation



Peak No	Retn.Time	Area	Height	Area %	Height %	Width@50%
1	0.375	0.005	0	0	0	0.033
2	5.183	2168.249	143.638	99.861	99.751	0.25
3	5.799	3.014	0.358	0.139	0.249	0.15
Total		**2171.268**	**143.996**	**100**	**100**	

Fig-7 Chromatogram of UV degradation

Acceptance Criteria:

The degradation peaks should be well separated from each other. The peak purity for Lenalidomide peak should pass. **Conclusion:** There is no interference between the peaks obtained for the chromatograms of degradation preparations. The degradation peaks under forced degradation are well separated from each other. The peak purity for Lenalidomide peak is passing. Hence, the method is very precise, selective and specific to the estimation of Assay of Lenalidomide in Revlimid (Lenalidomide) Capsules 25 mg by HPLC and the same method is stability indicating, as the degraded products are well separated from Lenalidomide and as well from each adjacent peaks.

Robustness:

Prepare two test solutions of the same lot (as used in 7.0.a and 7.0.b) of Lenalidomide in Revlimid (Lenalidomide) Capsules 25 mgas per analytical method. Inject this solution along with diluent blank solution and system suitability solution along different chromatographic conditions as shown below:

- Change in Column Lot
- Change in flow rate (± 0.2 ml/minute)
- Change in wavelength (± 2 nm)
- Change in mobile phase composition (± 0.2)

Change in Column Lot

(Normal Experimental Condition: Inertsil ODS-C_8 – 25cm x 4.6mm x 5µm**)**

The system suitability criteria were found to meet the pre-established acceptance criteria as per the analytical method. The assay results obtained with different flow rate conditions are as given in Table - 9.

Table – 9 Results for change in column

Flow rate →	**Same column**	**Different column**
Sample	**% Assay**	
Test solution	100.65	100.17
Average assay result from method precision	100.77	100.77
Mean	100.71	100.47
Standard Deviation (±)	0.08	0.42
(%) Relative Standard Deviation	0.08	0.42

Change in Flow Rate (± 0.2 mL/minute):

(Normal Experimental Condition: 1.0ml/minute)

The system suitability criteria were found to meet the pre-established acceptance criteria as per the analytical method. The assay results obtained with different flow rate conditions are as given in Table - 10.

Table - 10: Results for change in flow rate

Flow rate →	**0.8 mL/minute**	**1.2 mL/minute**
Sample	**% Assay**	
Test solution	99.90	100.16
Average assay result from method precision	100.77	100.77
Mean	**100.34**	**100.47**
Standard Deviation (±)	**0.62**	**0.43**
(%) Relative Standard Deviation	**0.61**	**0.43**

Change in Wavelength (± 2 nm):

(Normal Experimental Condition: 254nm)

The system suitability criteria were found to meet the pre-established acceptance criteria as per the analytical method. The assay results obtained with different wavelength conditions are as given in Table - 11.

Table - 11 Results for change in wavelength

Wavelength →	**252 nm**	**256 nm**
Sample	**% Assay**	
Test solution	101.58	101.19
Average assay result from method precision	100.77	100.77
Mean	**101.18**	**100.98**
Standard Deviation (±)	**0.57**	**0.30**
(%) Relative Standard Deviation	**0.57**	**0.29**

Change in change in composition of mobile phase:

(Normal Experimental Condition: Buffer: Acetonitrile: Methanol = 80:8:12**)**

The system suitability criteria were found to meet the pre-established acceptance criteria as per the analytical method. The assay results obtained with change in mobile phase composition are as given in Table - 12.

Table - 12 Results for Change in change in composition of mobile phase

Mobile phase composition	**78 B : 9ACN : MeOH 13**	**82 B : 7ACN : MeOH 11**
Sample	**% Assay**	
Test solution	101.72	101.53
Average assay result from method precision	100.77	100.77
Mean	**101.25**	**101.15**
Standard Deviation (±)	**0.67**	**0.54**
(%) Relative Standard Deviation	**0.66**	**0.53**

The analysis of the same lot of Revlimid (Lenalidomide) Capsules 25 mg was carried out at different conditions of column lot, flow rate, wavelength, and composition of mobile phase. The system suitability was found to meet the pre-established criteria at all the conditions and the % RSD between results obtained with changed

condition and average result of method precision is not more than 2.0%. The analytical method meets the pre-established acceptance criteria for robustness study as per protocol. Thus, the method is robust.

Stability of Analytical Solution:

Procedure:

System suitability solution and test solution of Revlimid (Lenalidomide) Capsules 25 mgwere prepared on 0^{th},12^{th}, 24^{th}, 36^{th} and 48^{th} hour of experiment and stored these solutions at room temperature for every time interval up to 48 hrs and analyzed these solutions on 48 hrs with freshly prepared test solution. The system suitability solution was prepared freshly at the time of analysis. The assay of Revlimid (Lenalidomide) Capsules 25 mgin the sample was calculated. **Acceptance criteria:** The analyte is considered stable if there is no significant change in % assay. The assay results obtained during solution stability experiment are as given in Table- 13

Table - 13 Results for solution stability

% Assay results calculated against the freshly prepared system suitability standard	
Sample	**% Assay of Lenalidomide**
0^{th} hr	100.20
12^{th} hr	100.27
24 hr	100.26
36 hr	100.50
48 hr	100.59
Mean	**100.36**
Standard Deviation (±)	**0.17**
(%) Relative Standard Deviation	**0.17**

The system suitability was found to meet the pre-established criteria and the % RSD between assay results obtained for freshly prepared test solution and the stored test solutions is less than 2.0%. There is no significant change in assay level observed up to 48Hrs for test solution at room temperature. Thus, it can be concluded that the solution is stable up to 48Hrs at room temperature.

Table – 14 Summaries and Conclusion

S.No	Parameter	Result	Acceptance Criteria
1	**Specificity: Selectivity**	The Lenalidomide peak in test solution was found to be well resolved from peaks due to diluent blank solution and excipient blend solution. The diluent blank and excipient blend solution do not show any peak at the retention time of the Lenalidomide.	The Lenalidomide peak all should be well resolved from any other peak and from each other. The diluent blank solution and excipient blend solution should not show any peak at the retention time of the Lenalidomide.
	Specificity: Forced Degradation – **Standard** **Sample**	The peaks due to degradation products are found to be well separated from the peak. The peak purity criteria of Lenalidomide were found to pass at each condition of degradation. The peaks due to degradation products are found to be well separated from the peak. The peak purity criteria of Lenalidomide were found to pass at each condition of degradation.	The Lenalidomide peak should be well resolved from any other peak. The peak purity will be demonstrated by a Photo Diode Array (PDA) detector.
2	**Linearity and Range of Standard**	Correlation coefficient = 0.999 Range = 50 ppm to 150 ppm	Correlation coefficient should be greater than or equal to 0.999.
3	**Linearity and Range of standard in presence of placebo**	Correlation coefficient = 0.999 Range = 50 ppm to 150 ppm	Correlation coefficient should be greater than or equal to 0.999.
4	**Method precision**	% RSD = 0.76	% RSD of the results of six test solutions should not be more than 2.0%.

5	**Intermediate precision**	% RSD = 0.56	% RSD of the results of twelve test solutions (six of Method Precision and six of Intermediate Precision) should not be more than 2.0%.
6	**Accuracy (%Recovery)**	Level 1 at 50% Recovery = 98.44% Level 2 at 75% Recovery = 98.68% Level 3 at 100% Recovery= 100.40% Level4 at 125% Recovery = 99.44% Level 5 at 150% Recovery= 99.10%	Mean recovery at each concentration level should be between 98.0% and 102.0%.
7	**Robustness**	1] System suitability criteria are found to meet the pre-established acceptance criteria. 2] % RSD between results obtained with changed condition and average result of method precision, are found less than 2.0%.	System suitability criteria should pass as per analytical method and the % RSD between results obtained with changed condition and average result of method precision, should not be more than 2.0%.
8	**Stability of analytical solution**	No significant change is observed in the % assay up to 48 Hrs. Hence the solution is found to be stable up to 48 Hours at room temperature.	The analyte is considered stable if there is no significant change in % assay.

CONCLUSION

The above summary and validation data summarized in this document demonstrate that the lenalidomide assay method for Revlimidomide is analytical (Lenalidomide). HPLC capsules of 25 mg are found to be acceptable, selective, specific, reliable, linear, precise and robust. At room temperature, the analytical solution is found to be stable up to 48 Hrs. It is therefore assumed that the method of analysis is validated and can be used for routine analysis and stability research.

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