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Stability Indicating RP-HPLC Method for Quantitative Estimation of Lenalidomide and Its Impurities in Solid Dosage Form

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Abstract: The main aim and objectives of the research are to develop an effective, sensitive, economical, and simple reverse-phase HPLC method developed for determining and quantifying Lenalidomide impurities in Lenalidomide solid dosage formulations. The lack of research work and no compendial methods available for estimating this drug influenced the current research investigation to give a simple, sensitive, rapid, precise, accurate and robust gradient high-performance liquid chromatographic method for the determination and quantification of Lenalidomide and its impurities. Samples are analyzed using reverse phase (RP-HPLC) using stationary phase an Inertsil ODS-3V (150 x 4.6 mm, 3µm), and the mobile phase consists of two channels A and B. channel-A: pH 3.0 phosphate buffer and Channel-B: Acetonitrile: water (90:10 v/v) in the proportion of gradient elution. The flow rate is 1.0 mL/min. The column temperature was maintained at 40°C, and the sample cooler temperature was maintained at 5°C, injection volume of 20 µL, and wavelength of 210 nm. The developed HPLC method was validated concerning specificity, and the chromatograms were recorded for blank, placebo, standard, sample, and spiked sample solutions of Lenalidomide and its related substances. Specificity studies reveal that the peaks are well separated from each other. For precision, the results were found to be within acceptable limits. The limit of detection (LOD) and limit of quantitation (LOQ) for impurity-A 0.1124 µg/mL and 0.0371µg/mL, Impurity-B 0.2247µg/mL and 0.0742µg/mL, respectively. The linearity results for Lenalidomide and all the impurities in the specified concentration range are satisfactory, with a correlation coefficient greater than 0.99. The accuracy studies were shown as % recovery for Lenalidomide and its impurities at the specification level; the results obtained were within limits. Solution stability parameter was established; standard, sample, and spiked sample solutions are stable up to 48 hrs on a bench top at the refrigerator. Filter validation parameters were established, and the filtered spiked sample solutions are compatible with both 0.45 µm PVDF & 0.45 µm Nylon filters.

Keywords: Lenalidomide, determination of related substances, Forced degradation, LOD and LOQ, liquid chromatography.

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1. INTRODUCTION

Lenalidomide (3-(4-amino-1-oxo 1,3-dihydro-2H-isoindol-2-yl) piperidine-2,6-dione) is an orally available thalidomide analog, which is showing both anti-angiogenic and immunomodulatory / anti-inflammatory properties. Lenalidomide, sold under the trade name Revlimid among others, is a medication used to treat multiple myeloma, smoldering myeloma, and myelodysplastic syndromes (MDS)[1-7]. The molecular formula is $C_{13}H_{13}N_3O_3$, and the molecular weight is 259.2606 g/ml. Its chemical structure is shown in Fig. 1. Lenalidomide is indicated for treating adult patients with multiple myeloma (MM) in combination with dexamethasone. It is also indicated as maintenance therapy in multiple myeloma following autologous hematopoietic stem cell transplantation (auto-HSCT). It is indicated for treating adult patients with transfusion-dependent anemia due to low- or intermediate-1-risk myelodysplastic syndromes (MDS) associated with a deletion 5q cytogenetic abnormality with or without additional cytogenetic abnormalities. Lenalidomide is indicated for treating adult patients with mantle cell lymphoma (MCL) whose disease has relapsed or progressed after two prior therapies, including bortezomib. Along with a rituximab product, lenalidomide is indicated for treating adult patients with previously treated follicular lymphoma (FL) or marginal zone lymphoma (MZL). Lenalidomide is a drug with multiple mechanisms of action. Lenalidomide exerts immunomodulating effects by altering cytokine production, regulating T-cell co-stimulation, and enhancing NK cell-mediated cytotoxicity. Lenalidomide directly inhibits the cullin ring E3 ubiquitin ligase complex: upon binding to cereblon, a substrate adaptor of the complex, lenalidomide modulates substrate specificity of the complex to recruit substrate proteins of the ligase, including Ikaros (IKZF1), Aiolos (IKZF3), and CK1α. These substrates are then tagged for ubiquitination and subsequent proteasomal degradation. IKZF1 and IKZF3 are B-cell transcription factors essential for B-cell differentiation and survival of malignant cells. IKZF3 also regulates the expression of interferon regulatory factor 4 (IRF4), a transcription factor that regulates the aberrant myeloma-specific gene. The degradation of IKZF3 can partly explain the immunomodulatory actions of lenalidomide since it is a repressor of the interleukin 2 gene (IL2): as lenalidomide decreases the level of IKZF3, the production of IL-2 increases, thereby increasing the proliferation of natural killer (NK), NKT cells, and CD4+ T cells.6 Lenalidomide inhibits the production of pro-inflammatory cytokines TNF-α, IL-1, IL-6, and IL-12, while elevating the production of anti-inflammatory cytokine IL-10.3 Lenalidomide acts as a T-cell co-stimulatory molecule that promotes CD3 T-cell proliferation and increases the production of IL-2 and IFN-γ in T lymphocytes, which enhances NK cell cytotoxicity and ADCC. It inhibits the expression and function of T-regulatory cells, which are often overabundant in some hematological malignancies. Lenalidomide is not subject to extensive hepatic metabolism involving CYP enzymes, and metabolism contributes to a minor extent to the clearance of lenalidomide in humans. Lenalidomide undergoes hydrolysis in human plasma to form 5-hydroxy-lenalidomide and N-acetyl-lenalidomide. Lenalidomide is rapidly absorbed with high bioavailability. It has a Tmax ranging from 0.5 to six hours. Lenalidomide exhibits a linear pharmacokinetic profile, with its AUC and Cmax increasing proportionally with dose[s] [8-15]. The literature survey reveals no HPLC methods are reported in major pharmacopeias like USP, EP, JP, and BP. Only a few analytical methods have been reported to date for the estimation of Lenalidomideby using spectrophotometric[17], RP-HPLC methods[18-19], and LC-MS methods[20-21]. We aim to develop stability indicating the HPLC method's estimation of Lenalidomide and its impurities in the solid dosage form. The present work describes a simple, stability-indicating HPLC method for determining related substances in Lenalidomide in solid dosage form according to ICH guidelines [22-23].

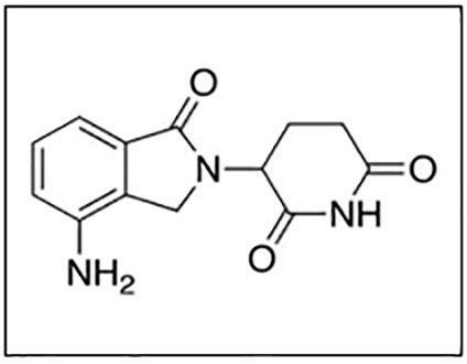

Fig. 1: Chemical structure of Lenalidomide

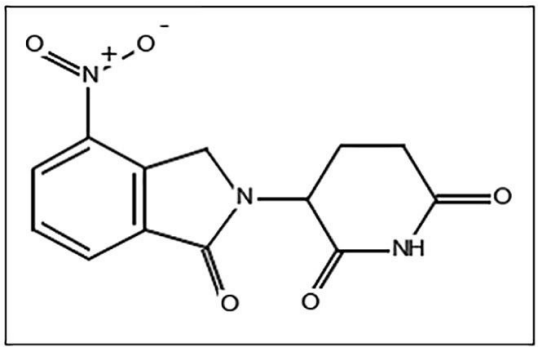

Fig. 2: Lenalidomide Impurity-A

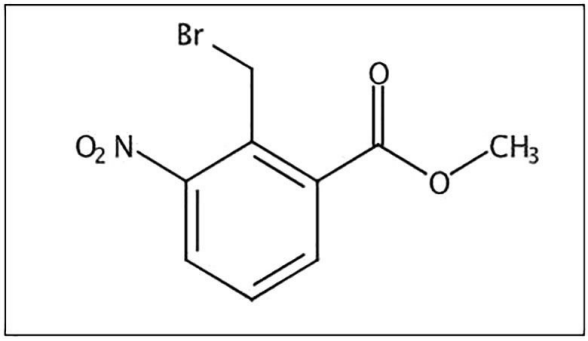

Fig. 3: Lenalidomide impurity-B

Impurity-A: 3-(4-Nitro-1-oxoisoindolin-2-yl)piperidine-2,6-dione.
Impurity-B: 2-(Bromomethyl-3-nitro benzoic acid methyl ester.

2. MATERIALS AND METHODS

2.1. Chemicals and reagents

Potassium dihydrogen orthophosphate, orthophosphoric acid, Hydrochloric acid, Sodium hydroxide, and Hydrogen peroxide were purchased from Merck, Mumbai, India. Acetonitrile, Methanol, and Milli-Q, water HPLC grade, procured from Merck, India.

2.2. Preparation of pH 3.0 phosphate buffer

We accurately weighed 1.3654 g of potassium dihydrogen orthophosphate, transferred it into 1000 mL of water, and mixed it well. And we adjusted the pH to 3.0 with a diluted orthophosphoric acid solution. Finally, filtered through 0.45 membrane filtered and sonicated to degas.

2.3. Preparation of mobile phase-A

Use pH 3.0 phosphate buffer solution as mobile phase-A

2.4. Preparation of mobile phase-B

Prepared a mixture of 900 mL of acetonitrile and 100 mL of water in the ratio of 900:100 (%v/v).

2.5. Preparation of diluent

Mix 450 mL of methanol, 50 mL of acetonitrile, and 500 mL buffer solution in the ratio of (45:5:50) v/v/ Sonicate to degas for 10 minutes and mix well.

2.6. Preparation of standard solution

Weighed and transferred 10.418 mg of Lenalidomide standard into a 100 mL volumetric flask and added about 70 mL of diluent and sonicate to dissolve. It was diluted to volume with diluent and mixed well. Next, transfered 1 mL of this solution into a 200 mL volumetric flask, diluted to volume with diluent, and mix well.

2.7. Preparation of sensitivity solution

Transferred 5 mL of standard solution into 20 mL volumetric flask, diluted to volume with diluent and mixed well.

2.8. Preparation of impurity-A stock solution

Accurately weighed and transferred 1.0 mg of impurity-A into a 20 mL volumetric flask, added 10 mL of acetonitrile, shaken for 5 minutes to dissolve, and diluted to the volume with diluent and mixed well.

2.9. Preparation of impurity-A solution

Transferred 0.3 mL of the above impurity-A stock solution into a 25 mL volumetric flask and diluted to volume with diluent and mixed well

2.10. Preparation of Impurity-B stock solution

Accurately weighed and transferred 1.0 mg of impurity-B into 20 mL volumetric flask, added 10 mL of methanol, shaken for 5 minutes to dissolve, and diluted to the volume with diluent and mixed well.

2.11. Preparation of impurity-B solution

Transferred 0.3 mL of above impurity-B impurity stock solution into a 25 mL volumetric flask, diluted to volume with diluent, and mixed well.

2.12. Preparation of placebo solution

Accurately weighed and transferred placebo powder equivalent to about 25 mg of Lenalidomide into a 100 mL volumetric flask added about 70 mL of diluent and sonicate for 30 minutes with intermediate shaking (maintained the sonicator bath temperature between 20- 25°C), then diluted to volume with diluent and mixed well. Filtered the solution through a 0.45µm PVDF syringe filter and discarded the first 3 mL of the filtrate.

2.13. Preparation of sample solution

Accurately weighed the 20 capsules (W1), opened and transferred the powder into a mortar and pestle without losing any weighed portion. Weighed the empty capsule shells (W2). Weighed and transferred capsule powder equivalent to about 25 mg of Lenalidomide into a 100 mL volumetric flask, added about 70 mL of diluent and sonicate for not less than 30 minutes with intermediate shaking (maintained the sonicator bath temperature between 20-25°C), then diluted to volume with diluent and mixed well. Filtered the solution through a 0.45µm PVDF syringe filter and discarded the first 3 mL of the filtrate.

2.14. Preparation of spiked sample solution

Accurately weighed the 20 capsules (W1), opened and transferred the powder into a mortar and pestle without

losing any weighed portion. Next, weighed the empty capsule shells (W2). Weighed and transferred capsule powder equivalent to about 25 mg of Lenalidomide into a 100 mL volumetric flask, added about 70 mL of diluent and sonicated for 20 minutes with intermediate shaking (maintain the sonicator bath temperature between 20-25°C), and added 0.6 mL of impurity-A solution and 0.6 mL of Impurity-B solution, diluted to volume with diluent and mixed well, filtered this solution through 0.45 µm PVDF filter and discarding the first 3 mL of the filtrate.

3. METHOD DEVELOPMENT

Optimization of chromatographic conditions

3.1. Method optimization parameters

An understanding of the nature of API (functionality, acidity, or basicity), the synthetic process, related impurities, the possible degradation pathways, and their degradation products are needed for successful method development in reverse-phase HPLC. In addition, successful method development should result in a robust, simple, and time-efficient method capable of being utilized in manufacturing settings.

3.2. Selection of wavelength

The sensitivity of the HPLC method depends upon the selection of detection wavelength. An ideal wavelength responds well to related substances and the drugs to be detected. Therefore, the wavelength for measurement was selected as 210 nm from the absorption spectrum.

3.3. Selection of stationary phase

Properly selecting the stationary phase depends on the nature of the sample and chemical profile. The drug selected for the present study was a polar compound that could be separated by normal or reverse-phase chromatography. A literature survey found that different C18 columns could be appropriately used to separate related substances for Lenalidomide.

3.4. Selection of mobile phase

Different mobile and stationary phases were employed to develop a suitable LC method for quantitatively determining impurities in Lenalidomide. Different mobile phase compositions were tried to get good peak shapes and selectivity for the impurities present in Lenalidomide. Poor peak shape and resolution were observed when Hypersil BDS C18 (150mm x 4.6mm, 3µ) and mobile gradient phase programmed of mobile phase: A pH 3.0 phosphate buffer and mobile phase: B acetonitrile. There was no proper resolution of impurities, and analyte peak and efficiency of the peak were also not achieved, and peak interferences were present. In the second attempt made using Inertsil ODS-3V, 150 x 4.6 mm, 3µm column, and mobile gradient phase programmed of mobile Phase: A pH 3.0 phosphate buffer and mobile Phase: B acetonitrile. There was no proper resolution of impurities and analyte peaks. The next attempt was made using Inertsil ODS-3V, 150 x 4.6 mm, 3µm column, and mobile gradient phase programmed of mobile phase: A pH 3.0 phosphate buffer and mobile phase: B acetonitrile: water. The resolution of both drug and impurities was achieved. These chromatographic conditions were selected for validation studies.

3.5. Optimized Chromatographic conditions

The Waters 2489 U.V-Visible detector/2695 Separation Module was analyzed and equipped with Empower[3] software. The Inertsil ODS-3V (150 x 4.6mm, 3µm) column was used as a stationary phase. The mobile phase consists of channels A and B. channel-A: pH 3.0 phosphate buffer and Channel-B: acetonitrile: water (90:10 v/v) in the proportion of gradient elution. The HPLC gradient program was set as (time/% mobile phase- B) 0.0/15, 10/15, 15/50, 30/50, 31/15, and 40/15. The flow rate is 1.0 mL/min. The column temperature was maintained at 40°C and the sample cooler temperature at 5°C, with injection volume 20 µL and wavelength 210 nm UV detection, respectively.

4. METHOD VALIDATION RESULTS

4.1. *Specificity*

Specificity was demonstrated by injecting the blank solution, placebo solution, standard solution, sample solution, spiked sample, and individual impurities and analyzing as per the test method. The observations are tabulated below in Table 1 and Fig. 4-10.

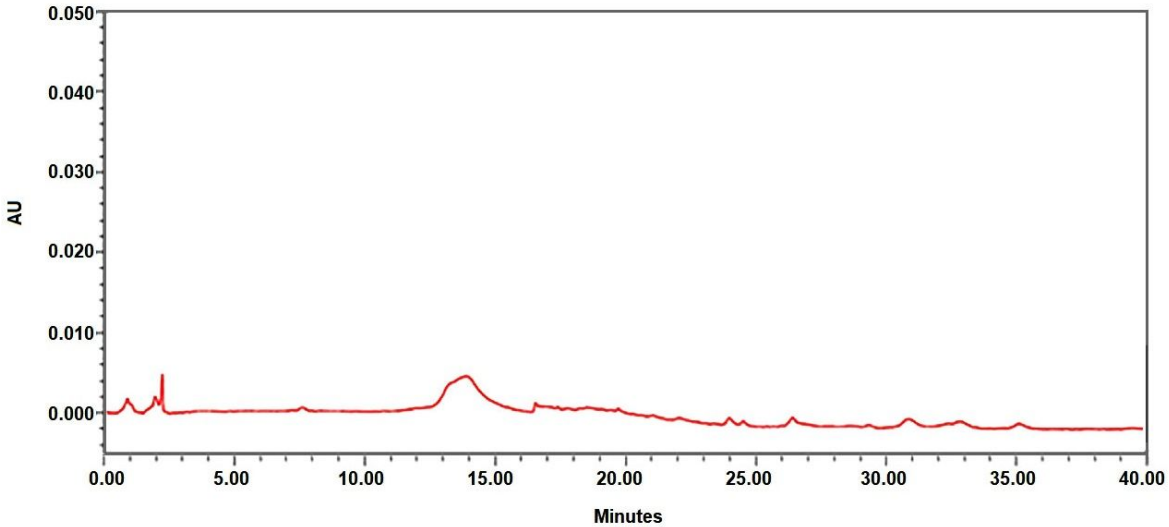

Fig. 4: Typical chromatogram of blank

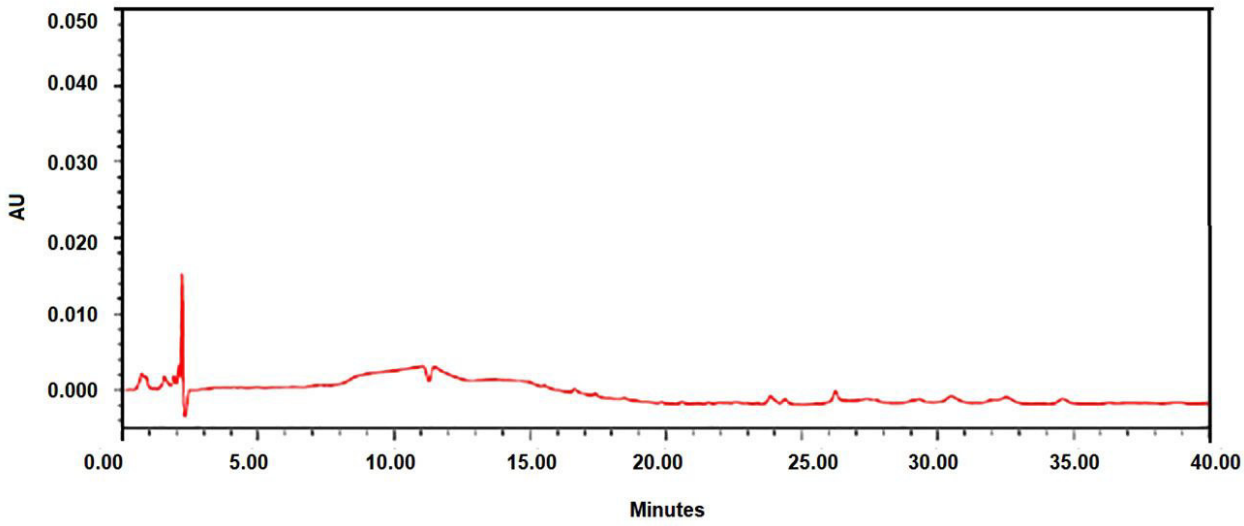

Fig. 5: Typical chromatogram of placebo

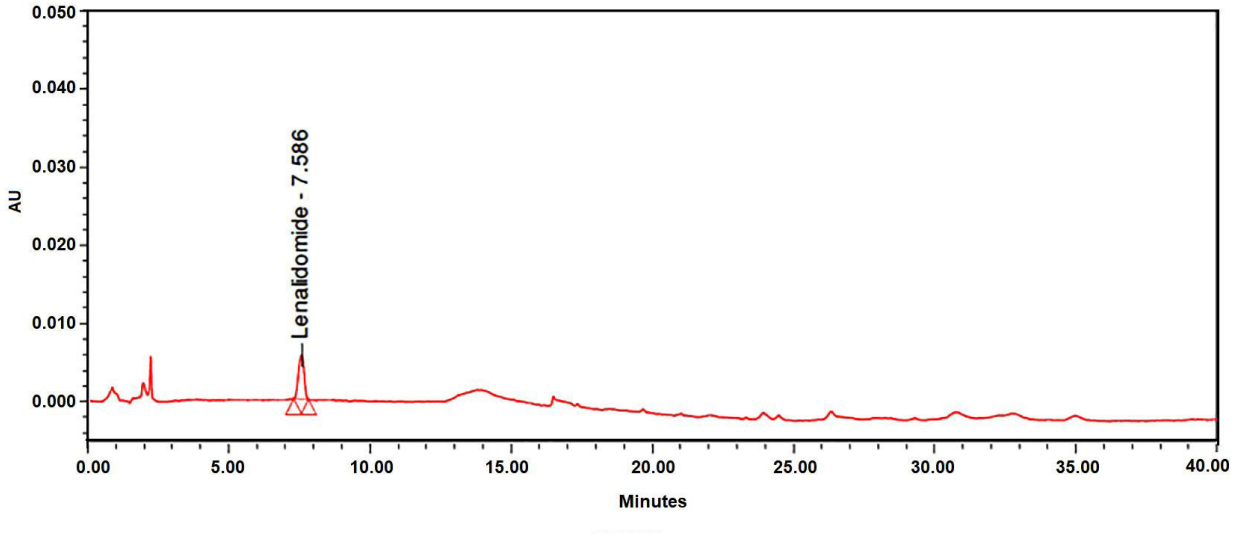

Fig. 6: Typical chromatogram standard

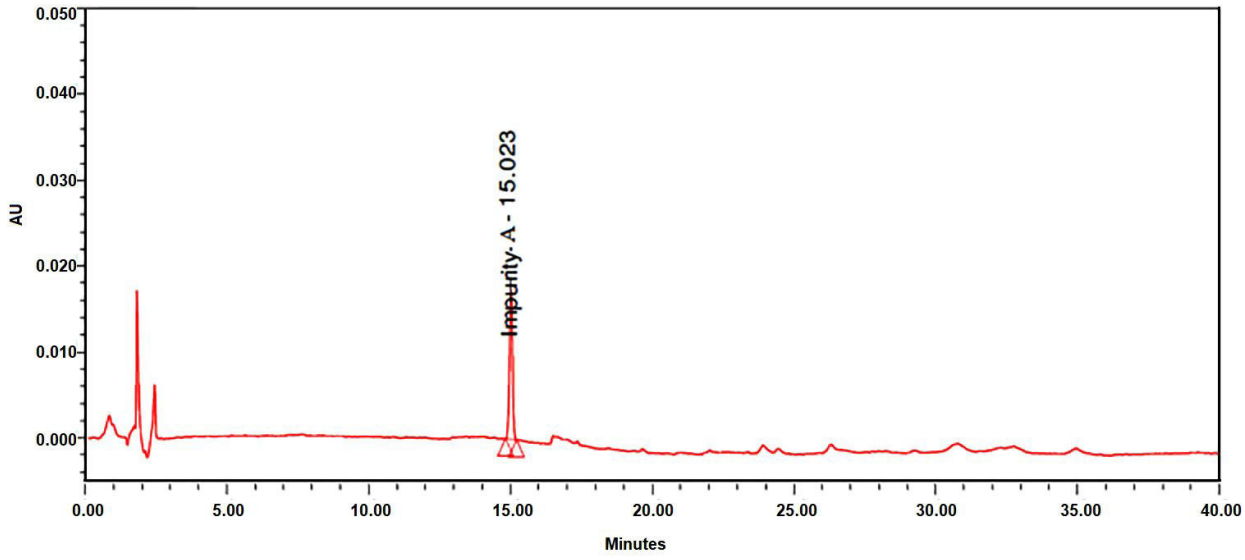

Fig. 7: Typical chromatogram impurity-A

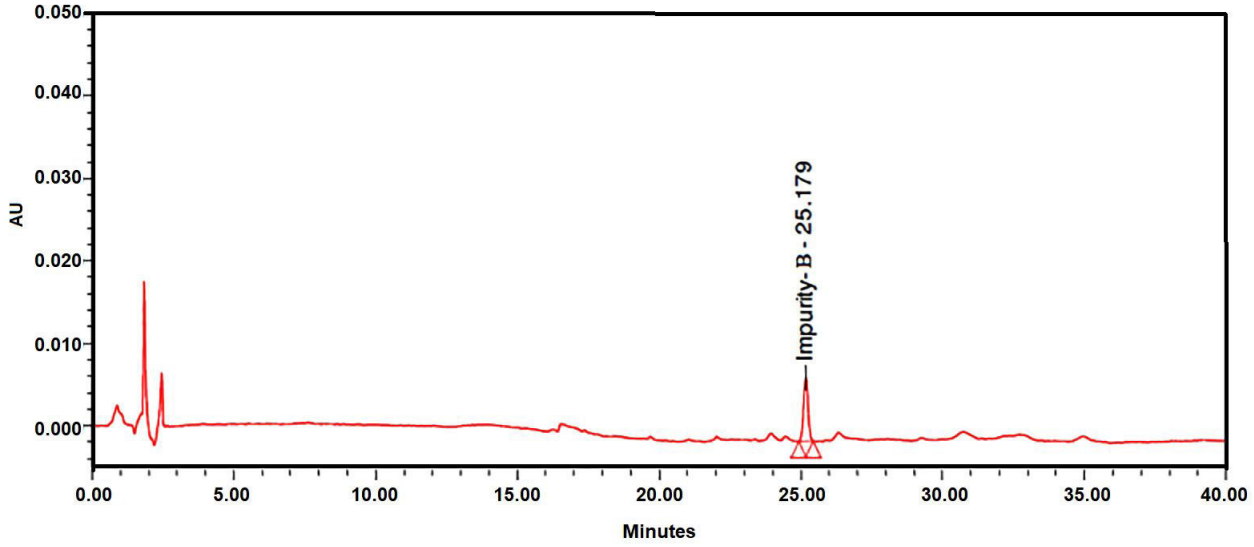

Fig. 8: Typical chromatogram impurity-B

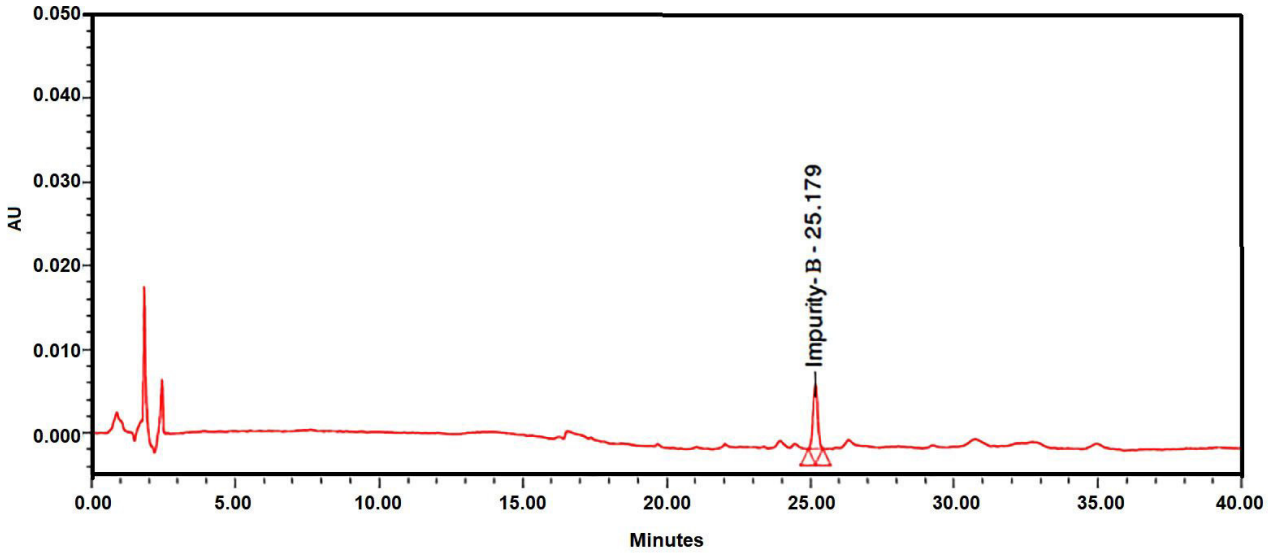

Fig. 9: Typical chromatogram control sample

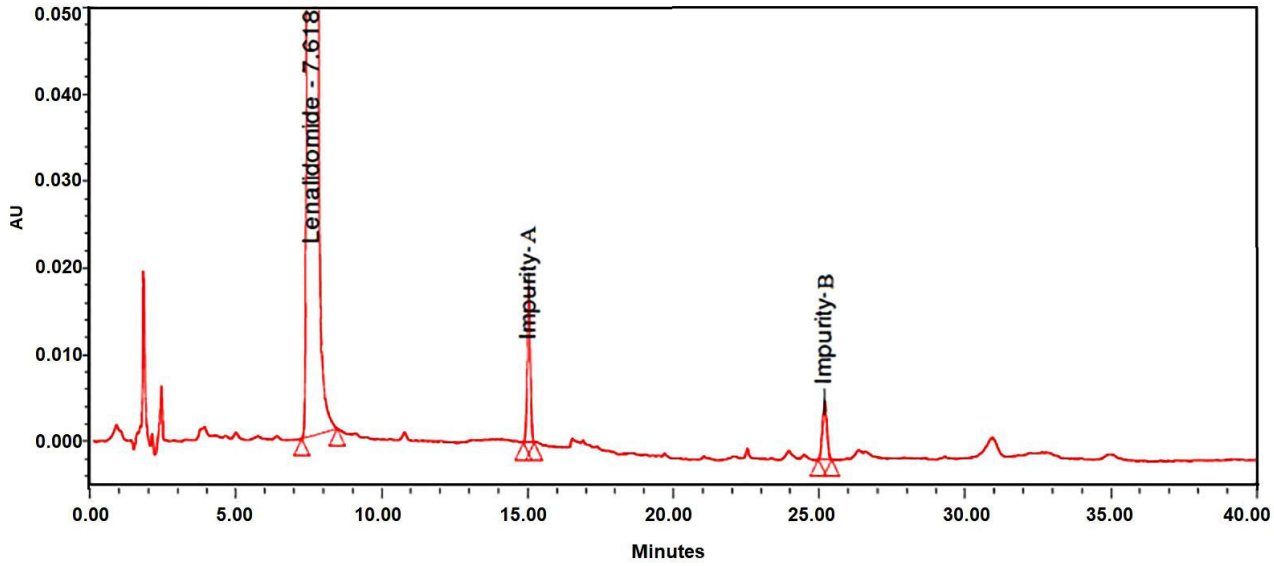

Fig. 10: Typical chromatogram spiked sample

Table 1: Impurity interference data (Specificity results)			
Peak Name	Retention Time	Blank	Placebo
Blank	ND	NA	NA
Placebo	ND	NA	NA
Impurity-A	15.023	No	No
Impurity-B	25.179	No	No
Lenalidomide	7.586	No	No

Table 1 and Figures 4 to 10 illustrate that the specificity of the chromatograms was recorded for blank, placebo, sample, sample, and spiked sample solutions of Lenalidomide and its related substances. Specificity studies reveal that the peaks are well separated from each other. Therefore, the method is selective for determining related substances in Lenalidomide. It was observed that known impurities are not coeluting with each other and the main analyte peak. There is no interference between diluent and placebo at Lenalidomide and impurities peaks.

4.2. *Interference from degradation products*

A study was conducted to reveal the effective separation of degradants/impurities from Lenalidomide. Sample and placebo solutions were exposed to the following stress conditions for degradation. Stressed and unstressed samples were injected into the HPLC system with a photodiode array detector by following test method conditions. All degrading peaks were resolved from the Lenalidomide peak in the chromatograms of all samples. The placebo did not interfere with the retention time of Lenalidomide and impurities under the above conditions. The observations are tabulated below in Table 2.

Table 2: Forced Degradation results						
S.No.	Degradation	Imp-A	Imp-B	Total impurities (%)	% Assay	Mass Balance (%)
1	Control sample	0.0125	ND	0.013	100.8	NA
2	Acid degradation (0.5N HCl/5mL/60°C/2hrs)	0.0142	ND	0.014	100.3	99.5
3	Base Degradation (0.5N NaOH/5mL/60°C/2hrs)	ND	ND	0.011	100.7	99.9
4	Peroxide Degradation (30% H2O2/5 mL/BT/24hrs)	6.053	ND	6.128	100.9	94.0
5	Thermal Degradation (60°C/Thermal oven/48hrs)	ND	ND	0.012	100.7	99.9
6	Water degradation (Water/5mL/60°C/4hrs)	0.0121	ND	0.012	100.5	99.7

Table 2 illustrates that the degradation study results were shown significant degradation was observed in oxidation (peroxide) stress conditions. Hence, Lenalidomide is sensitive to oxidation. Furthermore, the results proved the developed method has good selectivity and specificity.

4.3. *System suitability or System precision*

System suitability or precision was demonstrated by a prepared standard solution per the test method and injected six times into the HPLC system. In addition, the retention time and area response of the analyte peak were recorded. The observations are tabulated below in Table 3.

Table 3: System suitability results	
Injection No.	Area response
1	117633
2	116736
3	117275
4	118191
5	118460
6	117420
Average	117619
SD	627.9794
% RSD	0.53

Table 3 illustrates that the %RSD of the peak area for Lenalidomide was found to be 0.53%, below 5.0%, indicating that the results are satisfactory.

4.4. *Method precision*

Method precision was demonstrated by preparing six control and six samples by spiking impurities at the specification level and analyzing them as per the test method. The samples were prepared as per the method, and the result of the precision study is tabulated in Table 4. and Table 5.

Table 4: Results of method precision (Control samples)

S.No.	Sample Details	Impurity-A (%)	Impurity-B (%)
1	Prep-1	ND	ND
2	Prep-2	ND	ND
3	Prep-3	ND	ND
4	Prep-4	ND	ND
5	Prep-5	ND	ND
6	Prep-6	ND	ND
Average		NA	NA
Std. Dev		NA	NA
% RSD		NA	NA

Table 5: Results of method precision (Spiked samples)

S.No.	Sample Details	Impurity-A (% recovery)	Impurity-B (% recovery)
1	Prep-1	100.3	98.9
2	Prep-2	100.2	99.1
3	Prep-3	100.0	100.3
4	Prep-4	99.6	98.5
5	Prep-5	101.0	99.8
6	Prep-6	100.2	100.9
Average		100.2	99.6
Std. Dev		0.3817	0.9131
% RSD		0.38	0.92

Table 4 to Table 5 illustrates that the method precision was demonstrated by preparing six control samples and six samples by spiking impurities at the specification level and analyzing them as per the method. The results control samples and six samples were well within limits. From the above results, it is concluded that the method is precise.

4.5. *Limit of detection (LOD) & Limit of Quantitation (LOQ)*

- ***Limit of detection***

The worst found signal-to-noise ratio for each peak was greater than 3 in each injection. All the peaks were detected in all three injections.

- ***Limit of Quantitation***

The worst found signal-to-noise ratio for each peak was greater than 10 in each injection. All the peaks were detected in all six injections. The observations are tabulated below in Table 6 and Table 7.

Table 6: LOD and LOQ concentrations and S/N values

Name of the impurity	Concentration in (ppm)		Signal-to-noise ratio value	
	LOD	LOQ	LOD	LOQ
Impurity-A	0.0371	0.1124	4	18
Impurity-B	0.0742	0.2247	5	15

Table 7: LOQ precision results

S.No.	Name of the solution	Impurity-A	Impurity-B
1	LOQ precision-1	17054	21069
2	LOQ precision-2	16955	20607
3	LOQ precision-3	17526	20285
4	LOQ precision-4	16625	19728
5	LOQ precision-5	17747	20963
6	LOQ precision-6	16865	20369
Avg.		17128	20503
SD		424.1563	491.6847
%RSD		2.48	2.40

Table 6 to Table 7 illustrates that the limit of detection (LOD) and limit of quantitation (LOQ) for impurity-A 0.1124 µg/mL and 0.0371 µg/mL, Impurity-B 0.2247 µg/mL and 0.0742 µg/mL respectively. The limit of quantitation and detection values obtained for each impurity and Lenalidomide are within the acceptance criteria.

4.6. *Linearity*

The linearity of detector response for analytes was demonstrated by preparing solutions over the range of LOQ to 150% level concerning sample concentration. These solutions were injected into the HPLC system, and the responses were recorded. The observations are tabulated below. In addition, the calibration curve of the analytical method was assessed by plotting concentration versus peak area and represented graphically. Therefore, the HPLC method was found to be a standard linear curve calculated and given in Fig. 11 to Fig. 13. to demonstrate the linearity of the proposed method from the data obtained, which is given in Table 8 to Table 10.

Table 8: Linearity for Impurity-A

S.No	Levels	Concentration in ppm	Area response
1	LOQ	0.112	18985
2	25	0.313	52823
3	50	0.625	105945
4	100	1.25	211882
5	125	1.563	263704
6	150	1.875	311528
Correlation coefficient (r^2)			0.9998
Slope			166987.2846
Intercept			1115.6602
% Y-Intercept			0.53

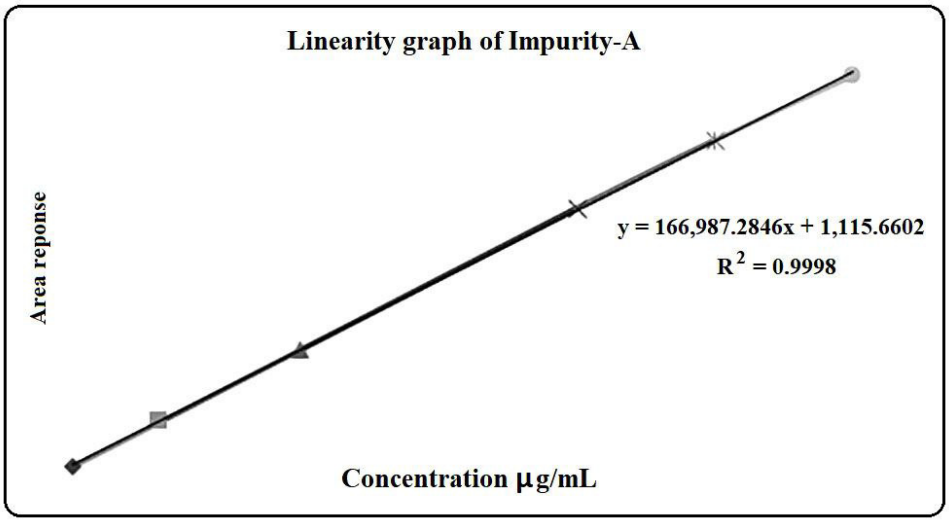

Fig. 11: Linearity graph of Impurity-A

Table 9: Linearity for Impurity-B

S.No	Levels	Concentration in ppm	Area response
1	LOQ	0.225	20515
2	25	0.313	28662
3	50	0.625	58228
4	100	1.251	118855
5	125	1.563	149226
6	150	1.875	177483
Correlation coefficient (r^2)			0.9999
Slope			95687.1856
Intercept			-1165.4017
% Y-Intercept			-0.98

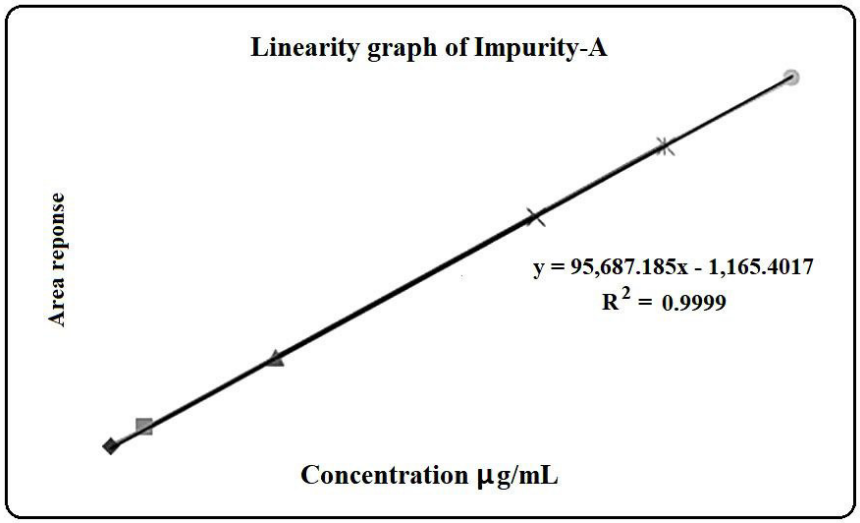

Fig. 12: Linearity graph of Impurity-B

Table 10: Linearity for Lenalidomide

S.No.	Levels	Concentration in ppm	Area response
1	LOQ	0.125	28926
2	50	0.257	58929
3	100	0.513	117628
4	125	0.641	144978
5	150	0.773	175245
Correlation coefficient (r^2)			0.9999
Slope			225566.7724
Intercept			974.4645
% Y-Intercept			0.83

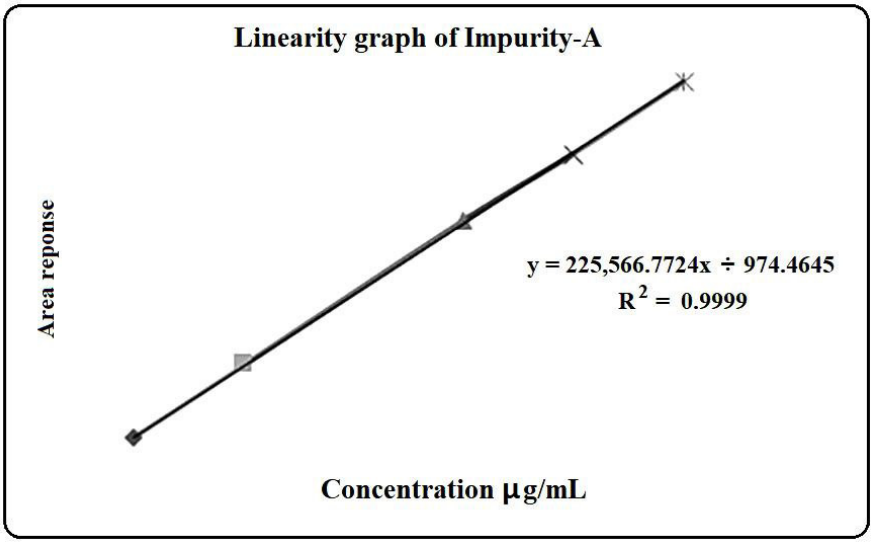

Fig. 13: Linearity graph of Lenalidomide

Table 8 to Table 10 and Figures 11 to 13 illustrate that the linearity results for Lenalidomide and all the impurities in the specified concentration range are satisfactory, with a correlation coefficient greater than 0.99. Therefore, the calibration curve and correlation coefficient for Lenalidomide and its impurities were plotted to be 0.9998, 0.9999, and 0.9999, respectively.

4.7. *Accuracy*

Recovery of Lenalidomide impurities in Lenalidomide was performed. The sample was taken, and varying amounts of Lenalidomide impurities representing LOQ to 150 % of specification level were added to the flasks. The spiked samples were prepared per the method, and the results are tabulated in Table 11.

Table 11: Accuracy results of Lenalidomide impurities

S.No.	Theoretical (%)	% Mean Recovery	
		Impurity-A	Impurity-B
1	LOQ	109.6	104.9
2	50	100.9	98.3
3	100	101.2	98.7
4	150	98.5	99.4

Table 11 illustrates that the accuracy at the LOQ level, 50% level, 100% level, and 150% level for impurity-A and Impurity-B meets the acceptance criteria. Therefore, from the above results, it is concluded that the method is accurate.

4.8. Solution stability of analytical solutions

Standard and sample and spiked sample solutions were kept for 48 hrs at bench top (room temperature) and refrigerator 2-8°C. The solution stability of standard and sample and spiked sample solutions was determined by comparison of old prepared standard solutions with freshly prepared standard solutions. The observations are tabulated below in Table 12 to Table 16.

Table 12: Results for solution stability of standard

Time Interval	%Recovery	
	Room temperature	Refrigerator
Initial	NA	NA
24hrs	99.3	100.4
48hrs	100.3	100.6

Table 13: Results for solution stability of test solution at room temperature

Component	Initial	After 24Hrs	% Difference	After 48Hrs	% Difference
Impurity-A	ND	ND	NA	ND	NA
Impurity-B	ND	ND	NA	ND	NA
Maximum unknown impurity	ND	ND	NA	ND	NA
Total impurities	NA	NA	NA	NA	NA

Table 14: Results for solution stability of test solution in the refrigerator

Component	Initial	After 24Hrs	% Difference	After 48Hrs	% Difference
Impurity-A	ND	ND	NA	ND	NA
Impurity-B	ND	ND	NA	ND	NA
Maximum unknown impurity	ND	ND	NA	ND	NA
Total impurities	NA	NA	NA	NA	NA

Table 15: Results for solution stability of spiked sample at room temperature

Component	Initial	After 24Hrs	% Difference	After 48Hrs	% Difference
Impurity-A	0.503	0.499	0.004	0.494	0.009
Impurity-B	0.492	0.489	0.003	0.485	0.007

Table 16: Results for solution stability of spiked sample at refrigerator

Component	Initial	After 24Hrs	% Difference	After 48Hrs	% Difference
Impurity-A	0.503	0.501	0.002	0.498	0.005
Impurity-B	0.492	0.490	0.002	0.487	0.005

Table 12 to Table 16 illustrates the solution stability of standard, sample, and spiked samples at different time intervals studied; from the above results, it is concluded that standard, sample, and spiked sample solutions are stable up to 48 hours in both the conditions (bench top and refrigerator).

4.9. Filter validation

Performed the filter validation for spiked sample solution, one portion of the solution was centrifuged, and the other portion was filtered through 0.45 µm PVDF and 0.45 µm Nylon filters. The observations are tabulated below in Table 17.

Table 17: Results of Filter validation

Component	Filter Type	Area Response	Difference
Impurity-A	Centrifuged sample	206945	NA
	0.45 µm PVDF Filtered Sample	207626	0.3
	0.45 µm Nylon Filtered Sample	211268	2.1
Impurity-B	Centrifuged sample	110571	NA
	0.45 µm PVDF Filtered Sample	110673	0.1
	0.45 µm Nylon Filtered Sample	108351	-2.0

Table 17 illustrates the filter validation study for spiked sample solution with different filters (0.45 µm PVDF filters and 0.45 µm Nylon filters) compared with unfiltered sample solution (centrifuged). Based on the above results, the filtered sample solutions are compatible with both 0.45 µm PVDF & 0.45 µm Nylon filters.

5. DISCUSSION

A simple, economical, accurate, and precise HPLC method was successfully developed. This method was carried out using Inertsil ODS-3V, 150 x 4.6 mm, 3µm column and the mobile phase consists of two channels, A and B. Channels A and B. channel-A: pH 3.0 phosphate buffer and Channel-B: acetonitrile: water (900:100 v/v). The flow rate is 1.0 mL/min. The column temperature was maintained at 40°C, sample temperature was maintained at 5°C, injection volume was 20 µL, and wavelength was fixed at 210 nm. The results obtained were accurate and reproducible. The method developed was statistically validated regarding selectivity, accuracy, linearity, precision, stability of the solution, and filter study. For selectivity [24-25], the chromatograms were recorded for standard and sample solutions of Lenalidomide and its related substances. Selectivity studies reveal that the peaks are well separated from each other. Therefore, the method is selective for determining related substances in Lenalidomide. There is no interference of diluent and placebo at Lenalidomide and impurities peaks. The elution order and the retention times of impurities and Lenalidomide obtained from individual and mixed standard preparations are comparable. For system precision[26-27] studies, six replicate injections were performed. %RSD was determined from the peak areas of Lenalidomide and its impurities. The acceptance limit should be less than 5.0%, and the results should be within acceptable limits. The linearity[28-33] results for Lenalidomide and all the impurities in the specified concentration range are satisfactory, with a correlation coefficient greater than 0.99. The calibration curve was plotted, and the correlation coefficient for Lenalidomide and its impurities was found to be 0.9998, 0.9999, and 0.9999, respectively. The accuracy[34] studies were shown as % recovery for Lenalidomide and its impurities at the specification level. The limit of % recovered is shown in the range of LOQ and 150%, and the results obtained were found to be within limits. Hence the method was found to be accurate. The limit of detection (LOD) and limit of quantitation (LOQ)[35-39] for impurity-A 0.1124 µg/mL and 0.0371 µg/mL, Impurity-B 0.2247 µg/mL and 0.0742 µg/mL respectively. Solution stability[40] parameter was established; standard, sample, and spiked sample solutions are stable up to 48 hrs on a benchtop in a refrigerator. Filter validation[41] parameter was established, and the filtered spiked sample solutions are compatible with both 0.45 µm PVDF & 0.45 µm Nylon filters. Degradation study[42-44] results were shown significant degradation was observed in oxidation (peroxide) stress conditions. Hence it can be concluded that Lenalidomide is sensitive to oxidation. The results proved that the developed method has good selectivity and specificity.

6. CONCLUSION

The developed method was validated for various parameters per ICH guidelines, like accuracy, precision, linearity, specificity, LOD and LOQ, solution stability, and filter validation. The results obtained were within the acceptance criteria. So, the developed method is simple, precise, cost-effective, eco-friendly, and safe. Therefore, it can be successfully employed for the routine analysis of Lenalidomide and its impurities in Lenalidomide capsule dosage forms.

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8. AUTHORS CONTRIBUTION STATEMENT

We have assured that "all authors have read and approved the manuscript." Furthermore, all the authors have equal contributions and participation in this research work. Sitamahalakshmi has analyzed all samples on HPLC instruments and completed the experimental work, and was a major contributor to writing the manuscript. He completed his work under the supervision of Ramachandran, who helped him elaborate on the methodology and theoretical approach.

9. CONFLICT OF INTEREST

Conflict of interest declared none.

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