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Research Article

DEVELOPMENT AND VALIDATION OF STABILITY INDICATING RP-HPLC METHOD FOR QUANTITATIVE ESTIMATION OF LENALIDOMIDE AND ITS IMPURITIES IN SOLID DOSAGE FORM

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ABSTRACT
Highly sensitive RP-HPLC method has been developed for the determination and quantification of Lenalidomide impurities in Lenalidomide liquid dosage formulations. Samples are analysed by means of 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). The flow rate was 1.0 mL/min. The column temperature was maintained at 40°C and sample cooler temperature was maintained at 5°C, injection volume 20 µL and wavelength 210 nm. The developed HPLC method was validated with respect to specificity, precision, linearity, accuracy, solution stability and filter study. Validation study was compared as per ICH guideline.

Keywords: Lenalidomide, Determination of related substances, Forced degradation, Liquid chromatography.

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 shows 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]. Its chemical structure is shown in Fig. 1.

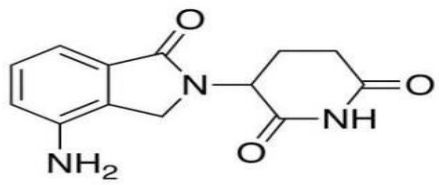

Fig. 1: Chemical structure of Lenalidomide

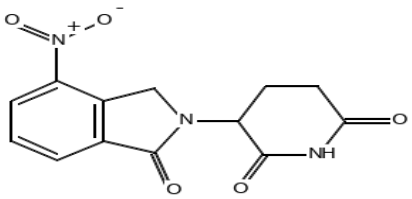

Fig. 2: Lenalidomide impurity-A

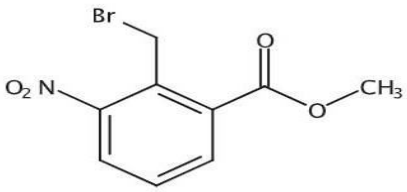

Fig. 3: Lenalidomide impurity-B

Impurity-A: 3-(4-Nitro-1-oxoisoindolin-2-yl) piperidine-2,6-dione.
Impurity-B: 2-(Bromomethyl-3-nitro benzoic acid methyl ester.
The literature survey reveals that there are no HPLC methods reported in major pharmacopoeias like USP, EP, JP and BP. Only few analytical methods [8] were reported till date for the estimation of Lenalidomide by using spectrophotometric [9], RP-HPLC methods [10-11] and LC-MS methods [12-13].

Hence, we tried to develop stability indicating HPLC method the estimation of Lenalidomide and its impurities in liquid dosage form. The present work describes a simple, stability indicating HPLC method for the determination of related substances in Lenalidomide in liquid dosage form according to ICH guidelines [14-15].

2. EXPERIMENTAL

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 were procured from Merck, India.

2.2. Preparation of solutions

2.2.1. Preparation of pH 3.0 phosphate buffer

Weighed accurately 1.3654 g of potassium dihydrogen orthophosphate and transferred into a 1000mL of water and mixed well. The pH was adjusted to 3.0 with diluted orthophosphoric acid solution. Filtered through 0.45 membrane filter and sonicated to degas.

2.2.2. Preparation of mobile phase-A

A phosphate buffer solution (pH 3.0) was used as mobile phase-A

2.2.3. Preparation of mobile phase-B

A mixture of 100mL of acetonitrile and 900 mL of water in the ratio of 100:900 (%v/v) was prepared.

2.2.4. Preparation of diluents

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

2.2.5. Preparation of standard solution

Lenalidomide standard (10.418 mg of into a 100 mL volumetric flask) was prepared, and about 70 mL of diluent was added followed by sonication to dissolve. Diluted to volume with diluent and mixed well. 1 mL of this solution was transferred into 200 mL volumetric flask, diluted to volume with diluent and mixed well.

2.2.6. Preparation of sensitivity solution

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

2.2.7. Preparation of impurity-A stock solution

Accurately weighed and transferred 1.0 mg of impurity-A into 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.

2.2.8. Preparation of impurity-A solution

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

2.2.9. 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.

2.2.10. Preparation of impurity-B solution

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

2.2.11. Preparation of placebo solution

Accurately weighed and transferred placebo powder equivalent to about 25 mg of Lenalidomide into a 100 mL volumetric flask and added about 70 mL of diluent and sonicated 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 0.45µm PVDF syringe filter and discarded first 3 mL of the filtrate.

2.2.12. Preparation of sample solution

Accurately weighed 20 capsules (W1), opened and transferred the powder into a mortar and pestle without loss of 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 sonicated 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 0.45µm PVDF syringe filter and discarded first 3 mL of the filtrate.

2.2.13. Preparation of spiked sample solution

Accurately weighed 20 capsules (W1), opened and transferred the powder into a mortar and pestle without loss of 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 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 the solution through 0.45 µm PVDF filter and discarding the first 3 mL of the filtrate.

2.3. Chromatographic conditions

Analysis was carried out on Waters 2489 U.V-Visible detector/2695 Separation Module, equipped with Empower[3] software. Inertsil ODS-3V (150x4.6mm, 3µm) column was used as stationary phase. The mobile phase consisted 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 HPLC gradient program was set as (time/% mobile phase- B) 0.0/15, 10/15, 15/50, 30/50, 31/15, 40/15. The flow rate was 1.0 mL/min. The column temperature was maintained at 40°C and sample cooler temperature was maintained at 5°C, injection volume was 20 µL and wavelength was 210nm UV detection respectively.

2.4. Method Development

2.4.1. 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 a robust, simple and time efficient method that is capable of being utilized in manufacturing setting.

2.4.2. Selection of wavelength

The sensitivity of the HPLC method depends upon the selection of detection wavelength. An ideal wavelength is one that gives good response for related substances and the drugs to be detected. The wavelength for measurement was selected as 210 nm from the absorption spectrum.

2.4.3. Selection of stationary phase

Proper selection of the stationary phase depends on the nature of the sample and chemical profile. The drug selected for the present study was polar compound and could be separated either by normal phase chromatography or reverse phase chromatography. From literature survey, it was found that different C18 columns could be appropriately used for the separation of related substances for Lenalidomide.

2.4.4. Selection of mobile phase

Different mobile phase and stationary phases were employed to develop a suitable LC method for the quantitative determination of 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 was observed when Hypersil BDS C18 (150mm x 4.6mm, 3µ) and gradient mobile 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 was also not achieved and peak interferences were present.

The second attempt was made using Inertsil ODS-3V, 150 x 4.6 mm, 3µm column, and gradient mobile 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.

The next attempt was made using Inertsil ODS-3V, 150 x 4.6 mm, 3µm column, and gradient mobile 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. RESULTS

3.1. Method Validation

3.1.1. Specificity

Specificity was demonstrated by injected blank solution, placebo solution, standard solution, sample solution, spiked sample and individual impurities and analyzed as per the test method.

It was observed that known impurities are not co eluting with each other and main analyte peak.

3.1.2. Interference from degradation products

A study was conducted to reveal the effective separation of degradants/impurities from Lenalidomide. Sample solutions and placebo solutions were exposed to the following stress conditions to degradation. Stressed and unstressed samples were injected into the HPLC system with photo diode array detector by following test

method conditions. All degrading peaks were resolved from Lenalidomide peak in the chromatograms of all samples and placebo did not show any interference at the retention time of Lenalidomide and impurities under the above conditions.

Degradation study results showed significant degradation 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.

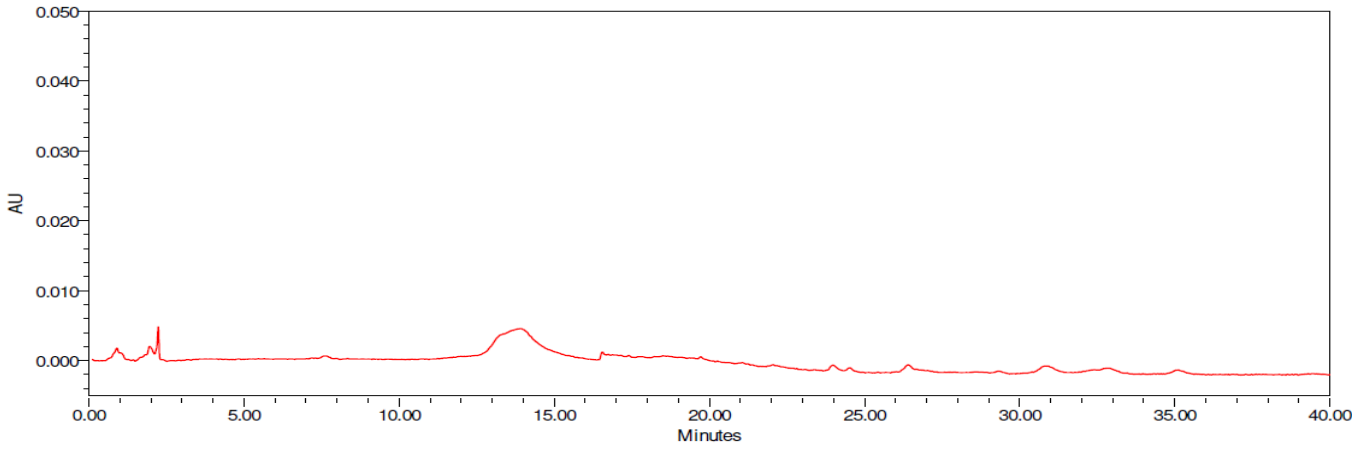

Fig. 4: Typical chromatogram of blank

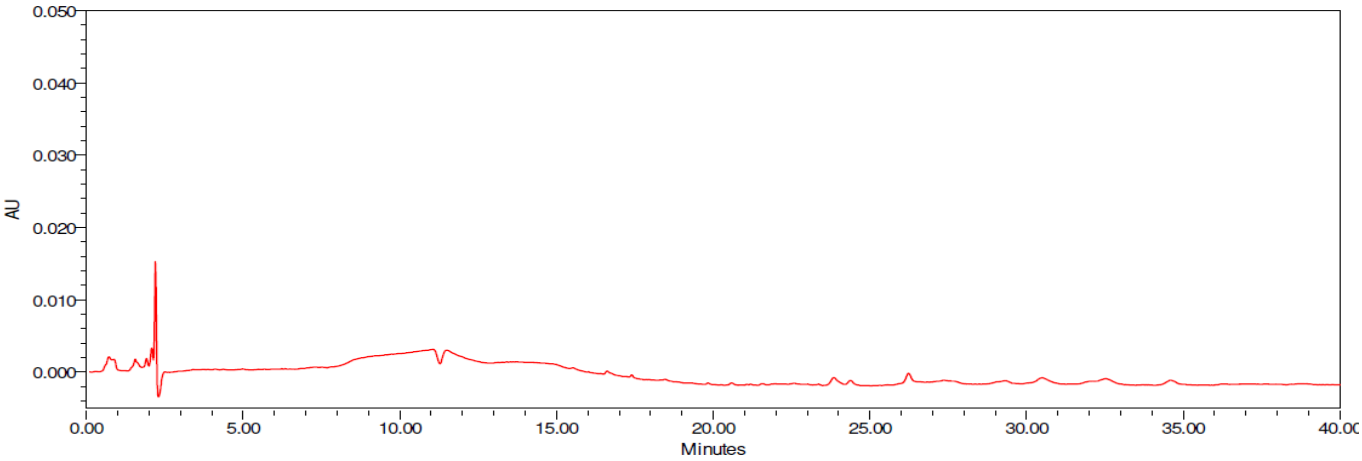

Fig. 5: Typical chromatogram of placebo

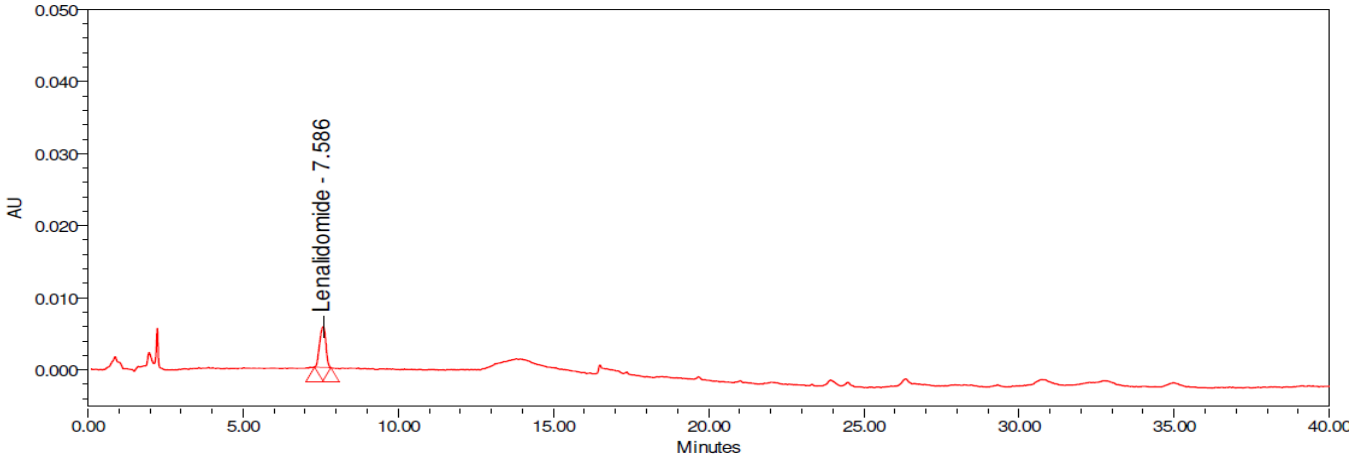

Fig. 6: Typical chromatogram standard

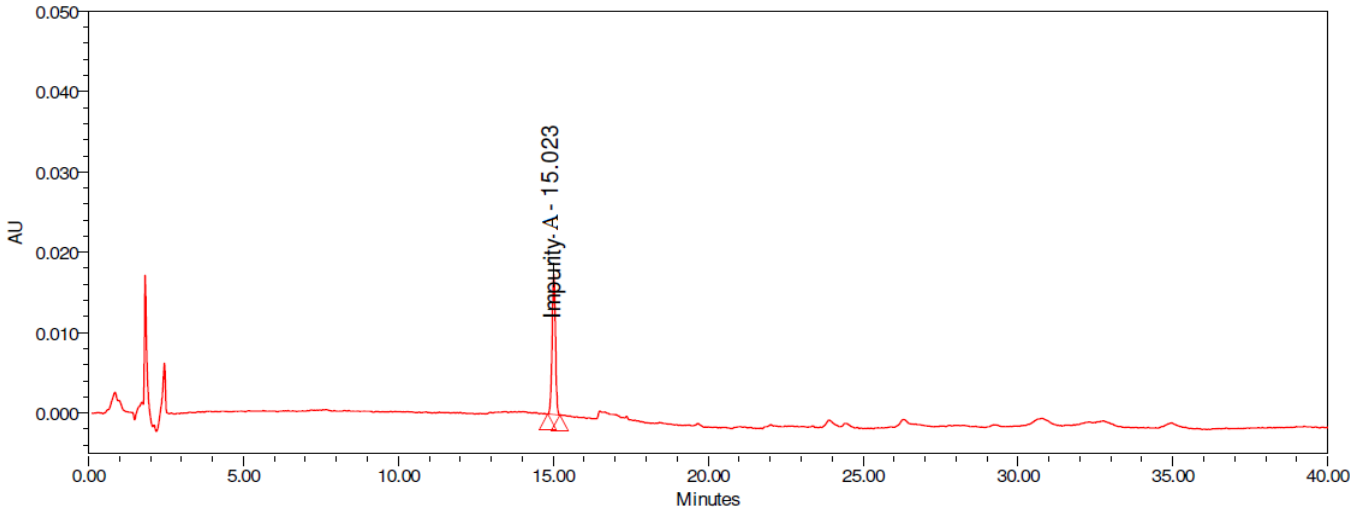

Fig. 7: Typical chromatogram impurity-A

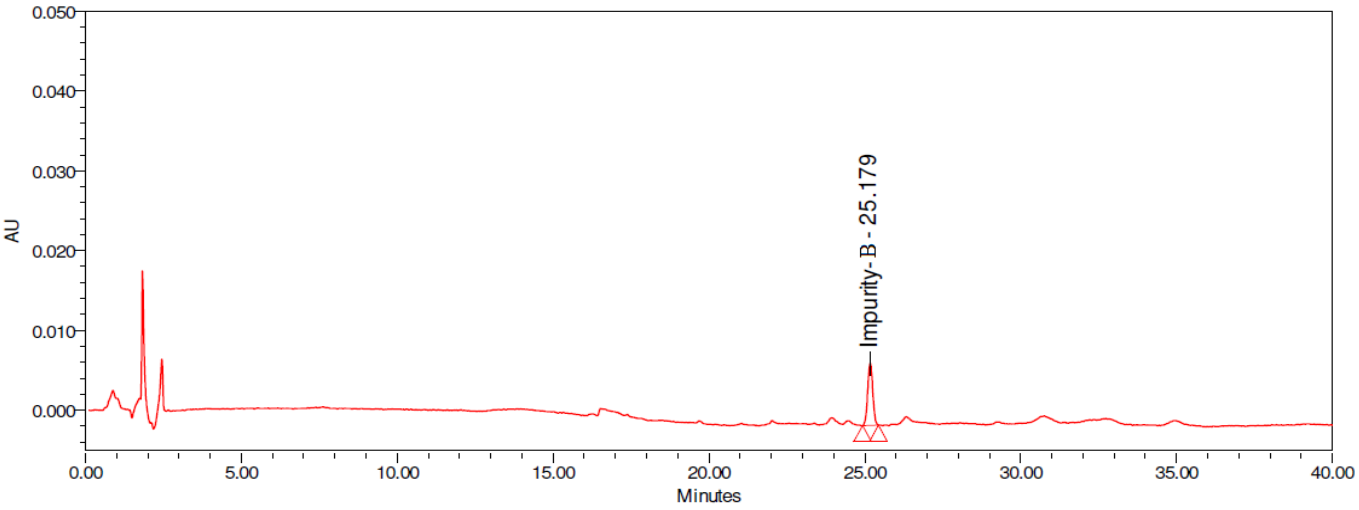

Fig. 8: Typical chromatogram impurity-B

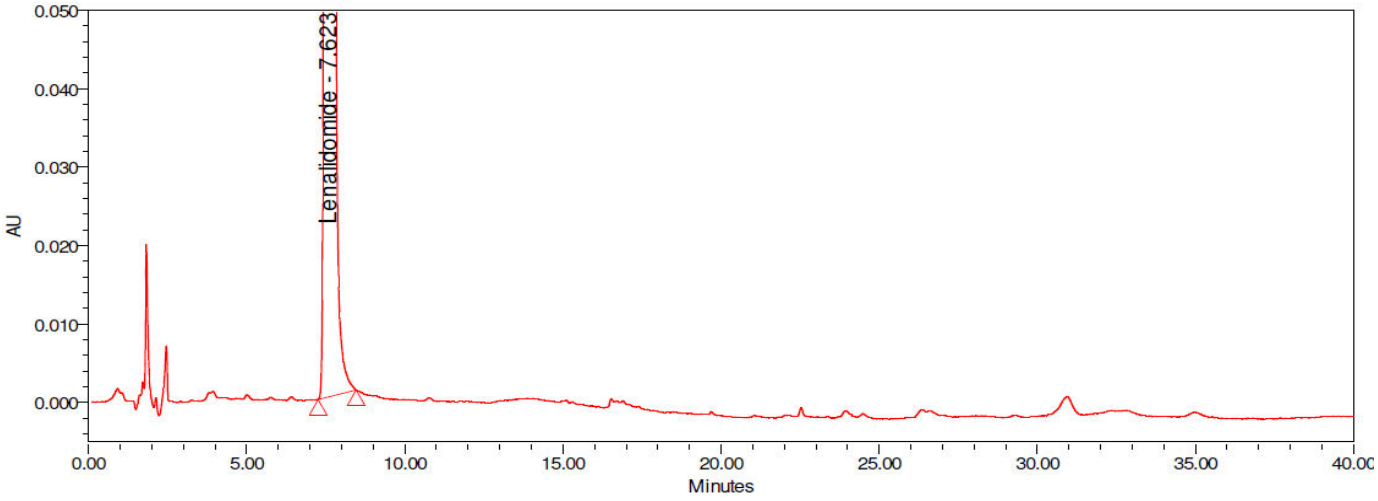

Fig. 9: Typical chromatogram control sample

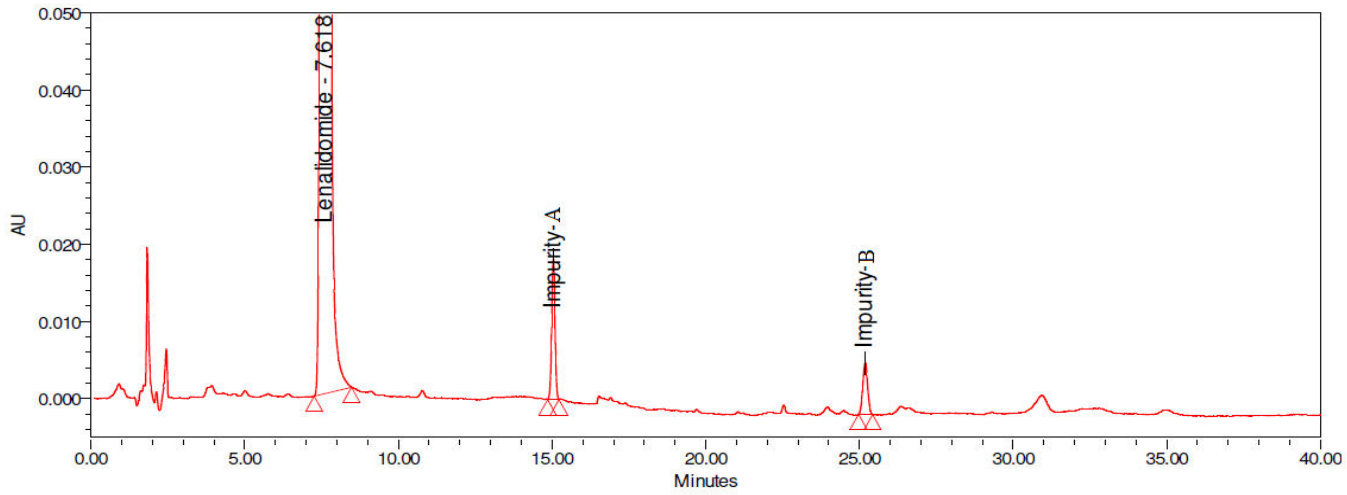

Fig. 10: Typical chromatogram spiked sample

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 2: Forced Degradation results

Degradation	Imp-A	Imp-B	Total impurities (%)	% Assay	Mass Balance (%)
Control sample	0.0125	ND	0.013	100.8	NA
Acid degradation (0.5N HCl/5mL/60°C/2hrs)	0.0142	ND	0.014	100.3	99.5
Base Degradation (0.5N NaOH/5mL/60°C/2hrs)	ND	ND	0.011	100.7	99.9
Peroxide Degradation (30% H2O2/5 mL/BT/24hrs)	6.053	ND	6.128	100.9	94.0
Thermal Degradation (60°C/Thermal oven/48hrs)	ND	ND	0.012	100.7	99.9
Water degradation (Water/5mL/60°C/4hrs)	0.0121	ND	0.012	100.5	99.7

3.2. Precision

3.2.1. System precision

System precision was demonstrated by prepared standard solution as per the test method and injected for six times into HPLC system. The retention time and area response of analyte peak were recorded.

The %RSD of peak area for Lenalidomide was found to be 0.53% which is below 5.0% indicates that the system gives precise result.

3.2.2. Method precision

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

The results were well within the limits. From the above results, it is concluded that method is precise.

Table 3: System precision 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 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	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

3.3. Limit of detection (LOD) & Limit of Quantitation (LOQ)

3.3.1. 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 the three injections.

3.3.2. 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 the six injections.

The limit of quantitation and limit of detection values obtained for each impurity and Lenalidomide are within the acceptance criteria.

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

3.4. Linearity

The linearity of detector response for analytes was demonstrated by preparing solutions over the range of LOQ to 150% level with respect to sample concentration. These solutions were injected into the HPLC system and the responses of the same were recorded. The observations are tabulated below. 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 linear standard curve that was calculated and given in Figs. 11-13 to demonstrate the linearity of the proposed method. From the data obtained which is given in Table 8 to Table 10.

The linearity results for Lenalidomide and all the impurities in the specified concentration range are found satisfactory, with a correlation coefficient greater than 0.99.

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

3.5. 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 as per the method and the results are tabulated in Table 11.

Accuracy at LOQ level to 150% level for impurity-A and impurity-B is meeting the acceptance criteria. From the above results, it is concluded that method is accurate.

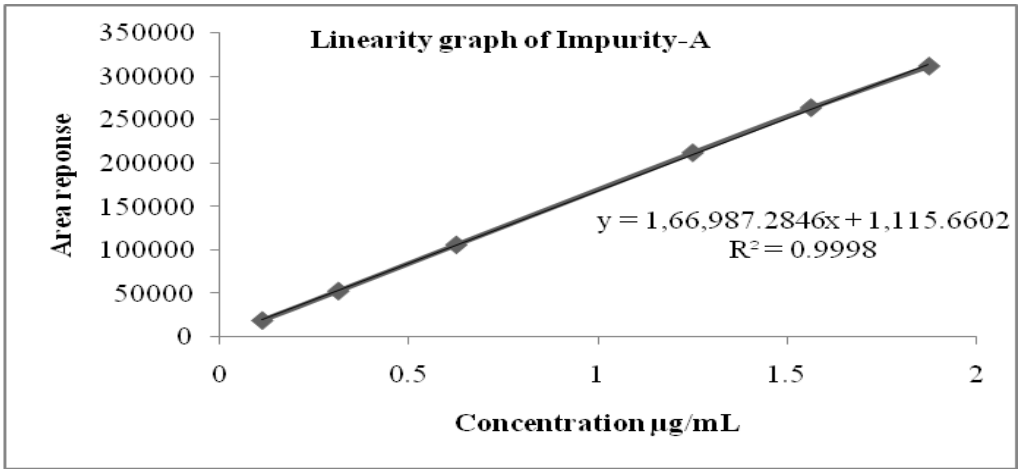

Fig. 11: Linearity graph of Impurity-A

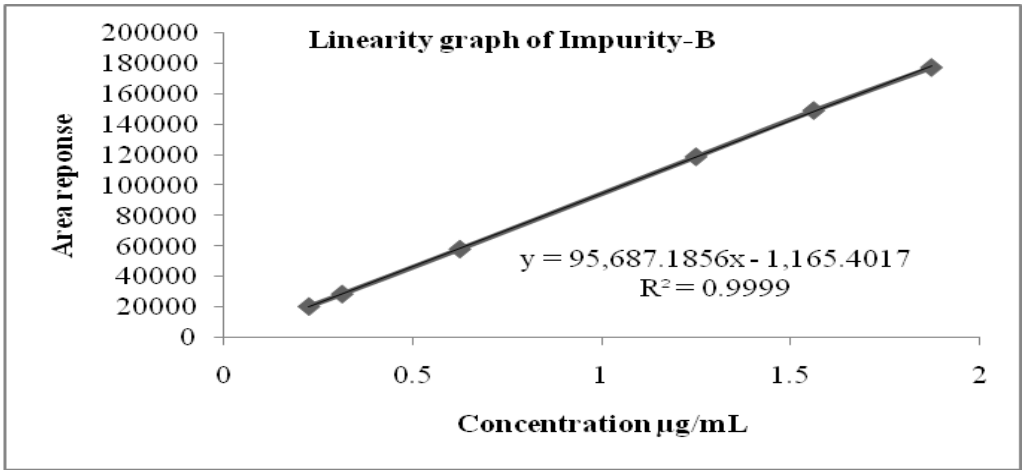

Fig. 12: Linearity graph of Impurity-B

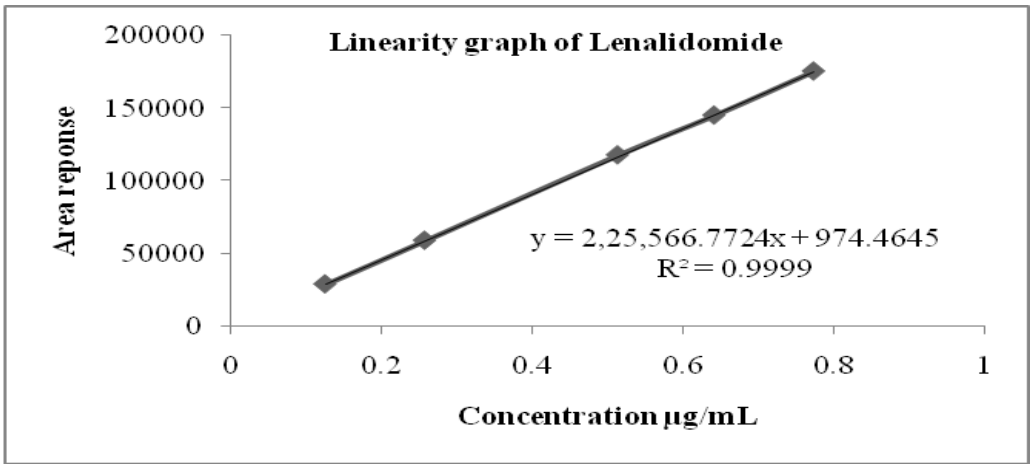

Fig. 13: Linearity graph of Lenalidomide

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

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

3.6. Solution stability of analytical solutions

Standard and sample and spiked sample solutions were kept for 48 hrs at room temperature in transparent bottles in auto sampler and in refrigerator 2-8°C. The stability of standard and sample and spiked sample solutions was determined by comparison of "old" prepared standard solutions with freshly prepared standard solutions.

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).

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 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

3.7. Filter validation

Performed the filter validation for spiked sample solution, one portion of the solution was centrifuged and the other portion of the solution was filtered through 0.45 µm PVDF and 0.45 µm Nylon filters.

Filter validation parameter was established and the filtered samples solutions are compatible for both 0.45 µm PVDF & 0.45 µm Nylon filters.

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 at 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 17: Results for 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

4. DISCUSSION

A simple, economic, accurate and precise HPLC method was successfully developed. The method was carried out by using Inertsil ODS-3V, 150 x 4.6 mm, 3µm column and the mobile phase consisted 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 was 1.0 mL/min. The column temperature was maintained at 40°C and sample temperature was maintained at 5°C, injection volume 20 µL and wavelength was fixed at 210 nm. The results obtained were accurate and reproducible. The method developed was statistically validated in terms of selectivity, accuracy, linearity, precision, and stability of solution.

For selectivity, 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 the determination of related substances in Lenalidomide. There was 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 standard preparations and mixed standard preparations are comparable.

For system precision studies, six replicate injections were performed. %RSD was determined from the peak areas of Lenalidomide and its impurities. The acceptance limit should be not more than 5.0%, and the results were found to be within the acceptance limits.

The limit of detection (LOD) and limit of quantitation (LOQ) for impurity-A were 0.1124 µg/mL and 0.0371 µg/mL, impurity-B were 0.2247 µg/mL and 0.0742 µg/mL respectively.

The linearity results for Lenalidomide and all the impurities in the specified concentration range are found satisfactory, with a correlation coefficient greater than 0.99.Calibration curve was plotted and correlation co-efficient for Lenalidomide and its impurities found to be 0.9998 and 0.9999 respectively.

The accuracy studies were shown as % recovery for Lenalidomide and its impurities at specification level. The limit of % recovered shown is in the range of LOQ and 150% and the results obtained were found to be within the limits. Hence the method was found to be accurate.

Solution stability parameter was established; standard, sample and spiked sample solutions are stable upto 48 hrs on bench top in refrigerator. Filter validation parameter was established and the filtered spiked sample solutions are compatible for both 0.45 µm PVDF & 0.45 µm Nylon filters.

5. CONCLUSION

The developed method was validated for various parameters as 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, it can

be concluded that the developed method is simple, precise, cost-effective, eco-friendly, and safe and can be successfully employed for the routine analysis of Lenalidomide and its impurities in Lenalidomide liquid dosage forms.

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Conflict of interests

The authors claim that there is no conflict of interest.

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