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Analytical Method Development and Validation for the Test Related Substances of Pomalidomide in Pomalidomide Capsules

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Abstract:- The percent impurities of pomalidomide in capsules of 1mg, 2mg, 3mg, and 4mg were determined using an HPLC technique. The current study provides the pomalidomide method validation for related chemicals test's procedural design and acceptance criteria. On a 1 mg capsule, the following tests were performed: force degradation, LOD, and LOQ, linearity and range, accuracy, solution stability, filter study, specificity, and robustness. On the pomalidomide capsules, precision and intermediate precision were also conducted. The study's output cleared that method is easy, quick, specific & accurate, making it suitable for regular pomalidomide determination in bulk and pharmaceutical dose forms.

Keywords:- *Pomalidomide; HPLC; Method validation; Capsules.*

I. INTRODUCTION

Chemically pomalidomide is 1,3-dioxo-2-(2,6-dioxopiperidin-3-yl)-4-aminoisoindoline. The formula is $C_{13}H_{11}N_3O_4$ and molecular weight 273.24 g/ mol [1-3]. Pomalidomide is an immunomodulatory medication that is a derivative of thalidomide [4-5]. Pomalidomide as a 5000-fold anti-TNF impact compared to thalidomide and a 10-fold anti-TNF effect compared to lenalidomide [6-8]. In pomalidomide clinical studies, there is a lot of variety in dose, technique, and administration schedules [9-11]. Only a few studies have published quantitative pomalidomide tests in rat plasma, such as HPLC with UV absorbance or fluorescence technique detection, LC-MS or UPLC-MS/MS assays. These investigations, on the other hand, do not give enough verified information for human clinical trials, such as sensitivity, stability, or simplicity [12-16]. As a result, a sensitive, verified, and high-throughput pomalidomide determination technique is required. In this study we developed analytical method validation for the test related substances of pomalidomide in pomalidomide capsules 1mg, 2mg, 3mg, and 4mg capsules.

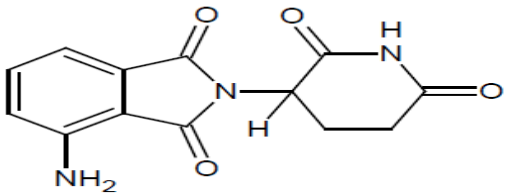

Fig. 1: Structure of pomalidomide

II. MATERIALS & METHODS

Water, Potassium dihydrogen phosphate, Orthophosphoric acid, Acetonitrile, Methanol , 0.45µ Nylon Membrane Disc filter, 0.45µ PTFE Membrane Disc filter, 0.45µ PVDF Membrane Disc filter, 0.45µ Nylon Membrane Syringe filter, Commercial available, pomalidomide 1mg, 2 mg 3mg,and 4mg capsules were procured from local market.

Instrumentation: High performance liquid chromatography was used, Kinetex Phenyl Hexyl, 4.6 x 250 mm, 5 µ or equivalent column with injection volume 10 µL, flow rate 0.7 mL/min, wave length 210 nm (PDA Detector). The Column oven temperature 30°C, auto sampler temperature 25°C, running time 10 minutes, retention about 15 minutes for pomalidomide peak, Mobile phase A: Use Buffer pH 3.0., Mobile phase B: use of Methanol 100%., Solvent A and solvent B in the ratio of 40:60. Mix and degas. Diluent 1- 0.1 % of Orthophosphoric Acid, Diluent 2- Mixture of 0.1 % Orthophosphoric acid (Diluent 1) and Acetonitrile in the ratio of 30:70. Needle wash Methanol: Water (90:10), Seal wash Methanol: Water (10:90)

A. Gradient Programme:

Table 1: Relative Retention Time (RRT)

Time (min)	Mobile phase A (%)	Mobile phase B (%)
0	65	35
25	65	35
30	20	80
40	20	80
50	65	35
55	65	35

- **Preparation of diluent:** Prepared a mixture of Buffer pH 3.0 and Acetonitrile in the ratio of 30:70 v/v and mix well.
- **Preparation of blank solution:**
- **Des-amino pomalidomide impurity stock solution preparation:** Weighed 2 mg of Des-amino Pomalidomide impurity standard into a 20 mL volumetric flask containing 10 mL diluent, sonicated to dissolve, cooled, and diluted to volume with diluent and mixed well. (Concentration of Des-amino Pomalidomide: 100 ppm)

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- **Preparation of pomalidomide nitrodine impurity stock solution:** Weighed correctly and transferred about 2 mg of Pomalidomide Nitrodine impurity standard to a 20 mL volumetric flask containing 10 mL diluent, sonicated to dissolve, diluted to volume with diluent, and mixed. (Concentration of Pomalidomide Nitrodine: 100 ppm)
- **Preparation of system suitability solution:** Further transferred 2.0 mL of Des-amino Pomalidomide impurity stock solution and 2.0 mL of Pomalidomide Nitrodine impurity solution into 20 mL flask, diluted to volume with diluent and mixed well. (Concentration of Des-amino Pomalidomide and Pomalidomide Nitrodine impurity: 10 ppm)
- **Preparation of standard stock solution:** Weighed accurately about 25 mg of pomalidomide standard and transferred into 200 mL volumetric flask, Added roughly 100 mL of diluent, sonicated for 10 minutes, cooled, and made volume up to the mark with diluent and mixed. Filter through a syringe filter with a 0.45 m Nylon membrane. (Concentration: Pomalidomide: About 125 ppm)
- **Preparation of standard solution:** Transfer 4.0 mL of standard stock solution to a 100 mL volumetric flask, dilute to the desired volume with diluent, and mix. (Concentration: Pomalidomide Standard solution: About 5 ppm)
- **Preparation of sample solution:** The average filled weight of 20 capsules was determined. Weighed and transferred inner content equivalent to 20 mg Pomalidomide into a 20 mL volumetric flask with 10 mL diluent, sonicated for 20 minutes with intermittent shaking, cooled and made up to the mark with diluent, centrifuged the solution, and filtered through a 0.45 m Nylon membrane syringe filter. (Concentration: Pomalidomide Standard solution: About 1000 ppm)
- **Preparation of placebo solution:** Weighed accurately and transferred placebo into a 20 mL volumetric flask with a capsule shell equivalent to 20 mg of Pomalidomide content, added about 10 mL of diluent and sonicated for about 20 minutes with intermediate shaking, cooled and made up to the mark with diluent, centrifuged the solution, and filtered through a 0.45 m membrane nylon syringe filter.

Table 2: HPLC Injections

Sr. No.	Description	No. of Injection
1	Blank	1
2	Placebo solution	1
3	System suitability solution	1
4	Standard solution	6
5	Sample solution	1
6	Bracketing standard solution	1

Table 3: Relative Retention Time (RRT)

Impurity Name	RRT
Pomalidomide	1.00
5- Amino Pomalidomide	0.65
Des-amino Pomalidomide	1.20
Pomalidomide Nitrodine	1.29
Pomalidomide Benzyldione	1.59

- **System suitability:** Blank (diluent), Placebo solution, System suitability solution, and Standard solution (six replicate) were all injected into the HPLC in equal amounts. For peak areas of Pomalidomide, the relative standard deviation of six replicate injections should not exceed 5.0 percent. In a system suitability solution, the resolution between Des-amino Pomalidomide impurity and Pomalidomide Nitrodine impurity should not be less than 1.5. Pomalidomide peak should have a tailing factor of no greater than 2.0. Pomalidomide Standard Solution Theoretical Plates should not be less than 2000. Pomalidomide peak area responses obtained from six replicate injections of standard solution and bracketing standard should have a relative standard deviation of less than 5.0 percent.
- **Peak purity:** Injected Blank, Standard solution, placebo solution, System suitability solution, Individual impurity solution, Sample solution (all strength), Spike solution onto the HPLC.
- **Forced degradation:** Prepared and treated the blank and placebo solution same as sample solution.
- **Photolytic degradation:** Weighed and transferred 20 capsules in a two separate Petri Plates (One open Petri plate and another wrapped with aluminium foil); The sample solutions were then subjected to UV and white light for 1.2 million hours with a combined near ultraviolet intensity of not less than 200 watts per square metre. After exposure, weigh and transfer the inner content equivalent to 20 mg Pomalidomide into a 20 mL volumetric flask, add 10 mL diluent, sonicate for 20 minutes with intermittent shaking, cool and make up to the mark with diluent, centrifuge the solution, and filter through a 0.45 m Nylon membrane syringe filter.
- **Thermal degradation:** Determined the average weight of 20 Capsules; taken 20 capsules in a Petri Plate and Heat in oven for 100 ºC for 5 days. After exposure, weigh and transfer inner content equivalent to 20 mg Pomalidomide into a 20 mL volumetric flask, add 10 mL diluent, sonicate for 20 minutes with intermittent shaking, cool and make up to the mark with diluent, centrifuge the solution, and filter through a 0.45 m Nylon membrane syringe filter.

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- **Acid degradation:** The average filled weight of 20 capsules was determined. Weighed and transferred inner content equating to 20 mg Pomalidomide into a 20 mL volumetric flask, along with 10 mL diluents and kept for required time at bench top or heat the solution on the water bath at required temperature for required period of time, in order to achieve 5 to 15 % degradation w. r. t. control sample, sonicated for 20 minutes with occasional shaking, cooled and made up to the mark with diluent and centrifuged the solution; filtered through 0.45 m Nylon membrane syringe filter
- **Base degradation:** Weighed and placed inner content equivalent to 20 mg Pomalidomide into a 20 mL volumetric flask after determining the average filled weight of 20 capsules. 10 mL diluent was added and require volume and concentration of Sodium Hydroxide solution and keep for require time at bench top or heat the solution on the water bath at required temperature for required period of time, in order to achieve 5 to 15 % degradation w. r. t. control sample, neutralized this solution with same volume and same strength of acid., sonicated for 20 minutes with intermittent shaking, cooled and diluted to the desired concentration using diluent, centrifuged, then filtered through a 0.45 m Nylon membrane syringe filter
- **Peroxide degradation:** The average filled weight of 20 capsules was determined. Weighed and placed 20 mg Pomalidomide-equivalent inner content into a 20 mL volumetric flask. 10 mL diluent was added and required volume and concentration of Hydrogen peroxide solution, heated the solution on the water bath for required time if necessary, in order to achieve 5 to 15 % degradation w. r. t. control sample, Allowed it to cool to room temperature and sonicated for 20 minutes with intermittent shaking, cooled and made up to the mark with diluent and centrifuged the solution; filtered through 0.45 µm Nylon membrane syringe filter.
- **Humidity degradation:** Determined the average weight of 20 Capsules, Taken 20 Capsules in Petri plate and kept in desiccator at 90% RH at room temperature for 5 days. After exposure, weigh and transfer inner content equivalent to 20 mg Pomalidomide into a 20 mL volumetric flask, add 10 mL diluent, sonicate for 20 minutes with intermittent shaking, cool and make up to the mark with diluent, centrifuge the solution, and filter through a 0.45 m Nylon membrane syringe filter.
- **Acceptance criteria:** System suitability criteria should be fulfilled. The peak due to active peak and known impurities is pure as shown on the PDA (Photo diode array). The peaks due to Pomalidomide should be pure as shown on the PDA. The angle of purity should be less than the purity threshold. Mass- Balance should be within 95%-105%.
- **Limit of detection and Limit of quantitation:** Determination of LOD/LOQ based on visual evaluation and S/N ratio method. Prepared different concentrations in decreasing order of active to estimate the LOQ and LOD and inject onto HPLC. The predicted LOD level to be injected in triplicate and LOQ solution in six replicate injections and the % RSD was calculated to check the precision onto HPLC.
- **Precision at LOQ:** Injected six replicates of sample solution at LOQ level & determined the % RSD.
- **Acceptance criteria:** System suitability criteria should be fulfilled. LOD and LOQ should be reported. S/N ratio for LOD solution is NLT 3. % RSD for LOQ: NMT 10.0%. S/N ratio for LOQ solution is NLT 10.
- **Linearity and Range:** The Linearity of response will be determined by preparing different concentration for unknown impurity (0.5%) from LOQ to 150 % of the working concentration as given in the table below.
- **Linearity stock solution (For unknown impurities):** Pomalidomide standard was carefully weighed and transferred into a 200 mL volumetric flask; 100 mL diluent was added, sonicated for 10 minutes, chilled, and volume was brought up to the mark with diluent and mixed; Filtered through a syringe filter using a 0.45 m Nylon membrane. (Concentration: Pomalidomide: About 125 ppm)

Table 4: Concentration series

Linearity Level%	Volume taken from Linearity stock solution(mL)	Total volume (mL)	Pomalidomide Concentration(ppm)
LOQ	As predicted in LOD LOQ		
50	2.0	100	2.50
75	3.0	100	3.75
100	4.0	100	5.00
125	5.0	100	6.25
150	6.0	100	7.50

- **Procedure:** Performed system suitability as directed and recorded the results; injected all levels in duplicate except lowest and highest concentration levels (injected in six replicates). Considered average area of each level for determining linearity and plotted a graph between concentration (ppm) v/s area counts and determine linearity, Square of Correlation coefficient (R^2), slope of the regression line and Y-intercept; determined % limit of Y-intercept and residual sum of square.
- **Acceptance criteria:** System suitability criteria should be fulfilled. Response should be linear. Correlation coefficient should not be less than 0.98. Slope of regression line, % Limit of Y-intercept and residual sum of squares, Relative response factor (RRF) shall be reported % Limit of Y- Intercept should be within ± 15.0%
- **Accuracy (Recovery):** Evaluated accuracy at four levels LOQ, 50%, 100% and 150% of the working concentration of Pomalidomide. Each level prepared in triplicates;

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prepared 12 sample preparations. From the amount added and the amount found, calculated % recovery. Calculated mean recovery and % RSD.

- **Accuracy stock solution (For unknown Impurity):** Weighed about 25 mg of Pomalidomide standard and transferred to a 200 mL volumetric flask; added about 100 mL of diluent, sonicated for 10 minutes, cooled, and made volume up to the mark with diluent and mix; filtered through 0.45 m Nylon membrane syringe filter and transferred 8.0 mL of above solution to a 50 mL volumetric flask. With diluent and mixing, I was able to get the volume up to the desired level. (Concentration: Pomalidomide: About 20 ppm)

Table 5 : Recovery level from Pomalidomide standard or API

Recovery Level %	Weight of Placebo (mg)	Amount of Accuracy Stock added (mL)	Total volume (mL)	Concentration(ppm)
				Pomalidomide
LOQ	3780	As predicted in LOD LOQ		
LOQ	3780			
LOQ	3780			
50-1	3780	2.5	20	2.5
50-2	3780	2.5	20	2.5
50-3	3780	2.5	20	2.5
100-1	3780	5.0	20	5.0
100-2	3780	5.0	20	5.0
100-3	3780	5.0	20	5.0
150-1	3780	7.5	20	7.5
150-2	3780	7.5	20	7.5
150-3	3780	7.5	20	7.5

- **Acceptance criteria:** System suitability criteria should be fulfilled. At LOQ Level mean recovery should be in the range 75% to 125%. Mean recovery at each level for 50% to 150% should be in the range of 80.0%- 120.0%.

B. Precision:

Table 6: Acceptance criteria

Impurity level	Acceptance criteria(%RSD)
≤ 0.10	≤ 25%
> 0.10 to 0.25	≤ 20%
> 0.25 to 0.50	≤ 15%
> 0.50 to 1.0	≤ 10%
>1.0	≤ 5 %

- **Ruggedness:** Evaluated intermediate precision by comparing the findings obtained from Ruggedness with those obtained from Method precision; evaluated reproducibility by comparing the results produced from Ruggedness with those obtained from Method precision.
- **Solution Stability:** The Standard solution and Sample solution should be kept at least for 48 hours at sample temperature condition (25ºC) and also Mobile Phase should be kept for at least 3 days, should be injected time to time continuously to check the solution stability.
- **Mobile phase Stability:** The mobile phase should not contain any precipitation or contamination. Cumulative % RSD for Standard and Bracketing Standard Retention time should not be more than 2.0
- **Standard solution Stability:** Cumulative % RSD for peak area of standard should not be more than 5.0. The % relative difference of initial and stability sample should meet acceptance criteria.
- **Robustness:** Under each of the selected experimental conditions, standard and sample allowed to run as per system suitability.
- **Change in chromatographic conditions:**
  - Change in wavelength (± 2 nm)
  - Change in column oven temperature (± 2°C).
  - Change in Buffer pH of mobile phase A (± 0.1 unit)
- **Acceptance criteria:** The system's appropriateness criteria must be met. The RSD for replicate responses of standard solutions should not exceed 5.0 percent; reported RT, RRT, and interference of Blank, placebo, and known impurity at the retention time of Pomalidomide of each altered condition; and reported RT, RRT, and interference of Blank, placebo, and known impurity at the retention time of Pomalidomide of each altered condition.

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III. RESULTS AND DISCUSSION

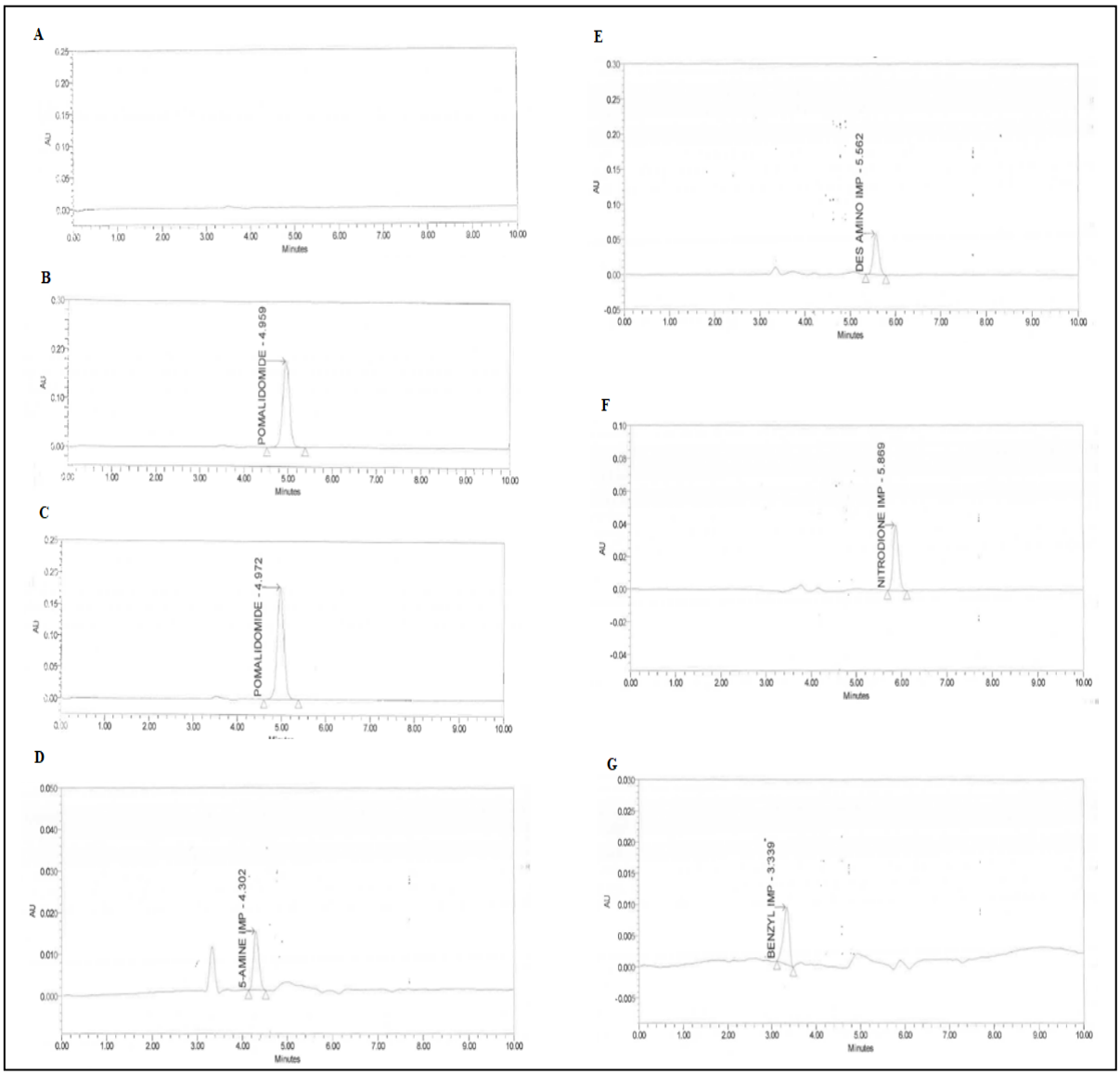

Fig. 2 : Chromatogram of A) Blank, B)Sample, C)Standard, D) 5- Amino Pomalidomide, E) Des-amino Pomalidomide, F) Pomalidomide Nitrodine, G) Pomalidomide Benzyldione

A. Linearity :

Table 7 : Linearity Levels

Sample	Concentration (ppm)	Area
Sample 1	6.0760	531890
Sample 2	12.1520	1119843
Sample 3	18.2280	1705296
Sample 4	24.3040	2269730
Sample 5	36.4560	3383804
Sample 6	48.6080	4447183
Correlation coefficient		0.999
Intercept		5817.902
Slope		92048.2128
% Y-intercept		0.26

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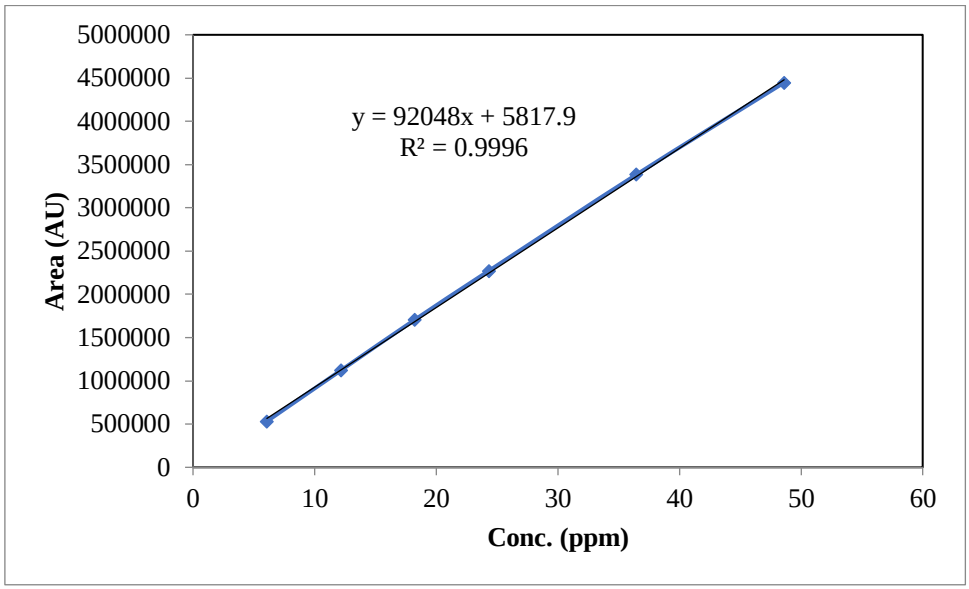

Fig. 3: Linearity Levels

A graph of peak area versus concentration (in ppm) was plotted for pomalidomide at a concentration range between 6 to 48.6 ppm. The linear regression equation and correlation coefficient (R^2) were y = 92048x + 5817 and 0.999 respectively.

B. Accuracy:

Table 8: Accuracy Levels

Level	Conc added (µg/mL)	Peak Area	Conc recovered (µg/mL)	% Recovery
50%	12.917	1175630	13.011	100.7
	12.959	1188158	13.149	101.5
100%	25.320	2316147	25.633	101.2
	24.948	2294390	25.392	101.8
150%	37.104	3417686	37.824	101.9
	37.056	3415336	37.798	102.0
Mean Recovery				101.5
% RSD				0.49

The accuracy of the developed method was evaluated by conducting recovery studies. The accuracy was calculated by assay method at concentrations of 50%, 100%, and 150%. The calculated amount of pomalidomide stock solutions was spiked to get the concentrations. The % mean recovery of spiked samples was found to be 101.5 %.

C. Precision:

Table 9: Test for Precision

Sr. No.	Sample Area
1	2280560
2	2283960
3	2300199
4	2300185
5	2288343
6	2290397
Mean	2290607.333
% RSD	0.34

The intra-day precision studies were performed using pomalidomide reference standard solution. The solution was injected at various time intervals, and the percent related standard deviation (% RSD) was determined. The inter-day precision was studied by injecting the same concentration of the standard solution on consecutive days, and the % RSD was calculated. The relative standard deviation obtained from 6 results was found to be 0.34%.

Robustness Studies: The changes were applied, and system suitability parameters were checked, found to be within the acceptable limits. It was noted that trivial changes in temperature and flow rate does not affect the method and produces results, which passes system suitability. Hence, the method was robust.

IV. CONCLUSION

Pomalidomide in pharmaceutical matrices may be quantified using a simple HPLC approach established in current work. The lack of extra peaks in the chromatogram suggests that the common excipients utilised in the tablets are not interfering. The validation test findings, taken together, indicated a technique with a relatively large linear range, adequate precision and accuracy, and practically dependable sensitivity. The approach allows for easy, selective, sensitive, and specific drug analysis and may be utilised for regular pharmaceutical quality control analysis in a short amount of time. For the analysis of Pomalidomide API, a sensitive and selective RP-HPLC technique has been developed and validated. The result suggests that the developed approach is an incredibly, another acceptable strategy for investigation, immaculateness, and soundness, which may aid in the investigation of Pomalidomide.

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